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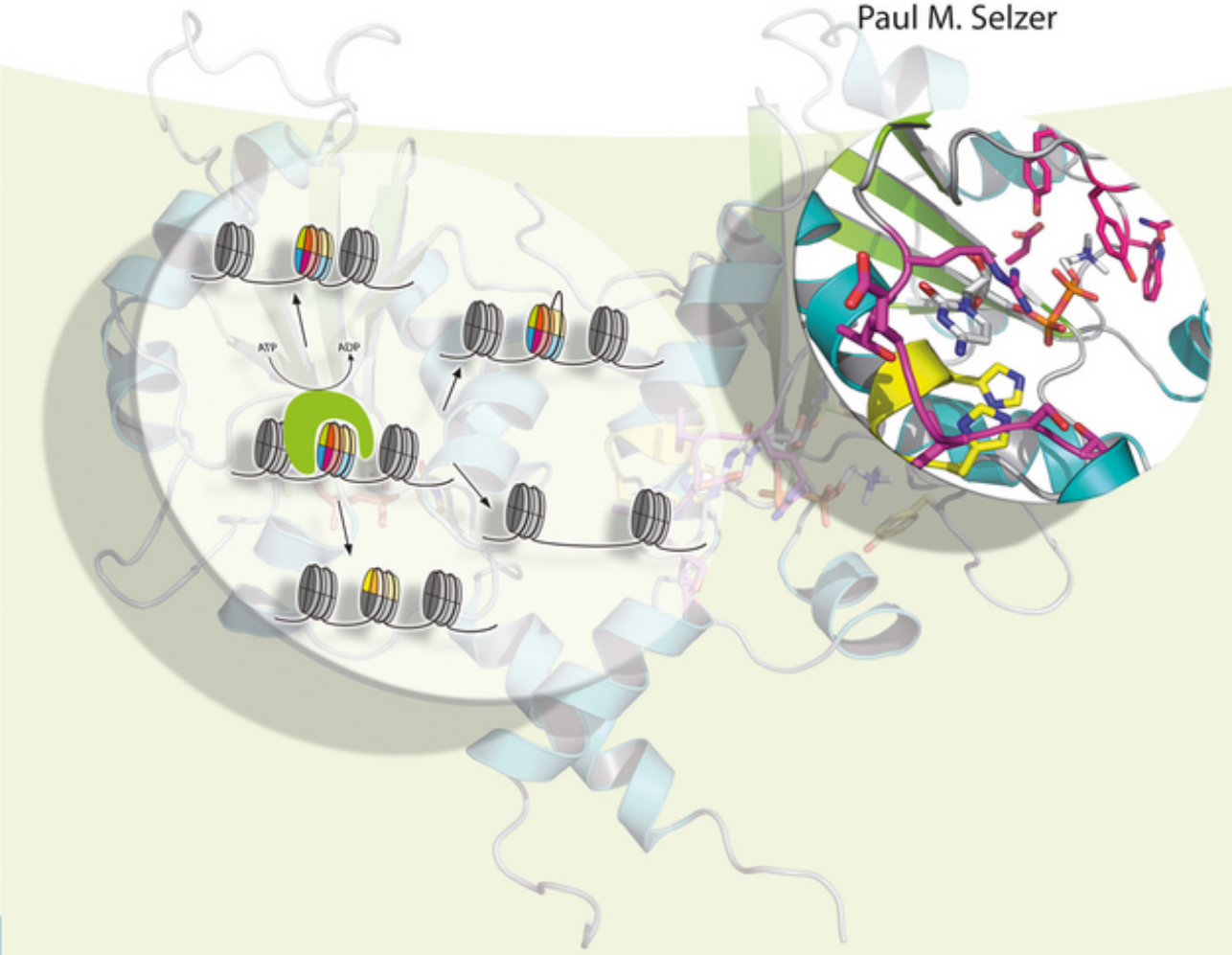
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Comprehensive Analysis of Parasite Biology

From Metabolism to Drug Discovery

Volume 7

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Comprehensive Analysis of Parasite Biology

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Editors

Prof. Sylke Müller
University of Glasgow
Medical, Veterinary & Life Sciences
120 University Place
G12 8TA Glasgow
United Kingdom
sylkemuller@hotmail.co.uk

Prof. Rachel Cerdan
University Montpellier
DIMNP, UMR5235 CNRS
Place Eugène Bataillon
34095 Montpellier Cedex 5
France
rachel.cerdan@univ-montp2.fr

Prof. Ovidiu Radulescu
University Montpellier
DIMNP, UMR5235 CNRS
Place Eugène Bataillon
34095 Montpellier Cedex 5
France
ovidiu.radulescu@univ-montp2.fr

Series Editor
Prof. Dr. Paul M. Selzer
Head of Antiparasitics R&D
Boehringer Ingelheim Animal Health GmbH
Binger Strasse 173
55216 Ingelheim am Rhein
Germany
paul.selzer@boehringer-ingelheim.com

Cover

Three-dimensional model of the catalytic domain of *Plasmodium falciparum* CTP:phosphocholine cytidylyltransferase - the rate-limiting enzyme of the phosphatidylcholine biosynthesis pathway - with the bound product CDP-choline. The protein is shown in ribbon representation. CDP-choline is depicted in stick representation. The inset shows a close-up view of the active site with residues coordinating CDP-choline depicted in stick representation. The structure visualization was prepared on the basis of a structural model provided by E. Guca *et al.*, Chapter 7.

The positioning of nucleosomes along eukaryotic genomes is organized by ATP-dependent chromatin remodeling complexes that can promote various changes to the nucleosome landscape, including nucleosome sliding, unwrapping, eviction, and histone exchange. These changes result in altered DNA accessibility and can affect transcriptional activity, E.M. Bunnik & G. LeRoch, chapter 18.

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List of Contributors

Frederick Annang
Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía
Fundación MEDINA
Screening and Target Validation
Parque Tecnológico de Ciencias de la Salud
Avenida del Conocimiento 34
E-18016 Granada
Spain

Barbara M. Bakker
Vrije Universiteit Amsterdam
Department of Molecular Cell Physiology
De Boelelaan 1085
1081 HV Amsterdam
The Netherlands
and
University of Groningen
University Medical Center Groningen
Center for Liver Digestive and Metabolic Diseases Systems Biology
Centre for Energy Metabolism and Ageing
Department of Pediatrics
Antonius Deusinglaan 1
9713AV Groningen
The Netherlands

Richárd Bártfai*
Radboud University
Department of Molecular Biology
Radboud Institute for Molecular Life Sciences
Geert Grooteplein 28
6525GA Nijmegen
The Netherlands
r.bartfai@ncmls.ru.nl

Daniela Begolo*
Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH)
DKFZ-ZMBH Alliance
Im Neuenheimer Feld 282
69120 Heidelberg
Germany
begolo.daniela@gmail.com

Angus Bell
Moyne Institute
Trinity College Dublin
School of Genetics and Microbiology
Department of Microbiology
Dublin 2
Ireland

* Corresponding author.

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Florian Bellvert
Université de Toulouse
Institut National des Sciences Appliquées
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

and

Université de Toulouse
Centre national de la recherche scientifique, UMR5504
Institut national de la recherche agronomique, UMR792é
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

Alessandra Bianchin*
University College Dublin
Conway Institute of Biomolecular and Biomedical Science
Belfield, Dublin 4
Ireland
Alessandra.Bianchin@ucdconnect.ie

Marco Biddau
University of Glasgow
Institute of Infection, Immunity and Inflammation
College of Medical, Veterinary and Life Sciences
120 University Place
G12 8TA Glasgow
UK

Jürgen Bosch*
Johns Hopkins University
Johns Hopkins Malaria Research Institute
Johns Hopkins Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology
615 North Wolfe Street
W8708 Baltimore, MD 21205
USA
jbosch2@jhu.edu

Lauren E. Boucher
Johns Hopkins University
Johns Hopkins Malaria Research Institute
Johns Hopkins Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology
615 North Wolfe Street
W8708 Baltimore, MD 21205
USA

James A. Brannigan
University of York
Department of Chemistry
Structural Biology Laboratory
Wentworth Way
Heslington
YO10 5DD York
UK

Ana Brennand
Rayne Institute
King's College London
Faculty of Life Sciences and Medicine
Division of Diabetes and Nutritional Sciences
Denmark Hill Campus
123 Coldharbour Lane
SE5 9NU London
UK

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Frédéric Bringaud
Université de Bordeaux
Microbiologie Fondamentale et Pathogénicité
Centre national de la recherche scientifique, UMR 5234
146, rue Léo Saignat
33076 Bordeaux
France

Evelien M. Bunnik
University of California Riverside
Center for Disease Vector Research
Department of Cell Biology and Neuroscience
Institute for Integrative Genome Biology,
900 University Avenue
Riverside, CA 92521
USA

Edern Cahoreau
Université de Toulouse
Institut National des Sciences Appliquées
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

and

Université de Toulouse
Centre national de la recherche scientifique, UMR5504
Institut national de la recherche agronomique, UMR792
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

Rachel Cerdan*
University Montpellier
Laboratory of Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France
rachel.cerdan@univ-montp2.fr

Anmol Chandele*
International Center for Genetic Engineering and Biotechnology
Aruna Asaf Ali Marg
New Delhi 110 067
India
chandeleanmol@gmail.com

and

ICGEB-Emory Vaccine Center
Molecular Medicine Group
ICGEB
Aruna Asaf Ali Marg
New Delhi 110 067
India

Anthony J. Chubb
Royal College of Surgeons in Ireland
Department of Molecular and Cellular Therapeutics
123 St Stephen's Green
Dublin 2
Ireland

Christine Clayton
Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH)
DKFZ-ZMBH Alliance
Im Neuenheimer Feld 282
69120 Heidelberg
Germany

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Alicia Contet
University Montpellier
Laboratory of Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France

Darren J. Creek*
Monash University
Monash Institute of Pharmaceutical Sciences
Drug Delivery Disposition and Dynamics
381 Royal Parade
Parkville, VIC 3052
Australia
darren.creek@monash.edu

Elany Barbosa da Silva
Universidade Federal de Minas Gerais
Departamento de Bioquímica e Imunologia
Instituto de Ciências Biológicas
Av. Antônio Carlos 6627
Belo Horizonte
MG 31270-901
Brazil

Glaécia Aparecida do Nascimento Pereira
Universidade Federal de Minas Gerais
Departamento de Bioquímica e Imunologia
Instituto de Ciências Biológicas
Av. Antônio Carlos 6627
Belo Horizonte
MG 31270-901
Brazil

François du Toit
Stellenbosch University
Department of Biochemistry
Matieland
South Africa

Katherine M. Ellis
Monash University
Monash Institute of Pharmaceutical Sciences
Drug Delivery Disposition and Dynamics
381 Royal Parade
Parkville, VIC 3052
Australia

Maria Fatarova
Université de Toulouse
Institut National des Sciences Appliquées
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

and

Université de Toulouse
Centre national de la recherche scientifique, UMR5504
Institut national de la recherche agronomique, UMR792
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés
135 Avenue de Rangueil
31077 Toulouse
France

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Rafaela Salgado Ferreira*
Universidade Federal de Minas Gerais
Departamento de Bioquímica e Imunologia
Instituto de Ciências Biológicas
Av. Antônio Carlos 6627
Belo Horizonte
MG 31270-901
Brazil
rafaelasf@gmail.com

Sabine Anne-Kristin Fraschka
Radboud University
Department of Molecular Biology
Radboud Institute for Molecular Life Sciences
Geert Grooteplein 28
6525GA Nijmegen
The Netherlands

Olga Genilloud*
Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía
Fundación MEDINA
Screening and Target Validation
Parque Tecnológico de Ciencias de la Salud
Avenida del Conocimiento 34
E-18016 Granada
Spain
olga.genilloud@medinaandalucia.es

Carlo R. Giannangelo
Monash University
Monash Institute of Pharmaceutical Sciences
Drug Delivery Disposition and Dynamics
381 Royal Parade
Parkville, VIC 3052
Australia

Dolores González Pacanowska*
Departmento de Bioquímica y Farmacología Molecular
Instituto de Parasitología y Biomedicina "López-Neyra"
Consejo Superior de Investigaciones Científicas
Avda del Conocimiento s/n
18016 Granada
Spain
dgonzalez@ipb.csic.es

Melisa Gualdrón-López
Federal University of Minas Gerais
Institute for Biological Sciences
Laboratory of Immunoregulation of Infectious Diseases
Department of Biochemistry and Immunology
Avenida Antonio Carlos 6627
Pampulha
Belo Horizonte
MG 31270-901
Brazil

Ewelina Guca
University Montpellier
Laboratory of Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France

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Jurgen R. Haanstra*
Vrije Universiteit Amsterdam
Department of Molecular Cell Physiology
De Boelelaan 1085
1081 HV Amsterdam
The Netherlands

and

Vrije Universiteit Amsterdam
Department of Systems Bioinformatics
De Boelelaan 1085
1081 HV Amsterdam
The Netherlands
j.r.haanstra@vu.nl

Fabien Jourdan
Université de Toulouse
TOXALIM (Research Centre in Food Toxicology)
Institut National de la Recherche Agronomique (INRA)
UMR1331 Toulouse
France

Barbara Kappes*
Friedrich-Alexander University Erlangen-Nürnberg
Department of Chemical and Bioengineering
Institute of Medical Biotechnology
Paul-Gordon Street 3
91052 Erlangen
Germany
baerbel.kappes@mbt.uni-erlangen.de

Karine G. Le Roch*
University of California Riverside
Center for Disease Vector Research
Department of Cell Biology and Neuroscience
Institute for Integrative Genome Biology
900 University Avenue
Riverside, CA 92521
USA
karine.leroch@ucr.edu

Glenn A. McConkey*
University of Leeds
School of Biology
Faculty of Biological Sciences
Clarendon Road
LS2 9JT Leeds
UK
G.A.McConkey@leeds.ac.uk

Paul A.M. Michels*
University of Edinburgh
Centre for Translational and Chemical Biology
Institute of Structural and Molecular Biology
School of Biological Sciences
King's Buildings
Max Born Crescent
EH9 3BF Edinburgh
UK
paul.michels@ed.ac.uk

Alexia S. Miller
Johns Hopkins School of Medicine
Department of Biophysics and Biophysical Chemistry
725 N Wolfe St
608D WBSB
Baltimore, MD 21205
USA

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Sylke Müller*
University of Glasgow
Institute of Infection
Immunity and Inflammation
College of Medical
Veterinary and Life Sciences
120 University Place
G12 8TA Glasgow
UK
sylkemuller@hotmail.co.uk

Gerald Penkler
Stellenbosch University
Department of Biochemistry
Private Bag X1
Matieland 7602
South Africa

and

Vrije Universiteit Amsterdam
Department of Molecular Cell
Physiology
De Boelelaan 1085
1081 HV Amsterdam
The Netherlands

Jean-Charles Portais*
Université de Toulouse
Institut National des Sciences
Appliquées
Laboratoire d'Ingénierie des
Systèmes Biologiques et des
Procédés
135 Avenue de Rangueil
31077 Toulouse
France
portais@insa-toulouse.fr

and

Université de Toulouse
Centre national de la recherche
scientifique, UMR5504
Laboratoire d'Ingénierie des
Systèmes Biologiques et des
Procédés
Institut national de la recherche
agronomique, UMR792
135 Avenue de Rangueil
31077 Toulouse
France

Ovidiu Radulescu*
University Montpellier
Dynamique des Interactions
Membranaires Normales et
Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France
ovidiu.radulescu@univ-
montp2.fr

Eva Rico
University of Edinburgh
Centre for Immunity
Institute of Immunology and
Infection Research
School of Biological Sciences
Infection and Evolution
King's Buildings
Charlotte Auerbach Road
EH9 3FL Edinburgh
UK

Stefan Ringgeler
Friedrich-Alexander University
Erlangen-Nürnberg
Department of Chemical and
Bioengineering
Institute of Bioprocess
Engineering
Paul-Gordan Street 3
91052 Erlangen
Germany

Downloaded From Vet-eBooks.com

Matthew D. Roberts
University of St Andrews
Biomedical Sciences Research Complex
North Haugh
St Andrews
KY16 9ST Fife
UK

Paul M. Selzer*
Boehringer Ingelheim Animal Health GmbH
Binger Straße 173
55216 Ingelheim am Rhein
Germany

and

Universität Tübingen
Interfakultäres Institut für Biochemie
Hoppe-Seyler-Str. 4
72076 Tübingen
Germany

and

University of Glasgow
Wellcome Trust Centre for Molecular Parasitology
Institute of Infection Immunity and Inflammation
Faculty of Biomedical and Life Sciences
120 University Place
G12 8TA Glasgow
Scotland
UK
paul.selzer@boehringer-ingelheim.com

Partho Sen
University Montpellier
Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France

Anna E. Sexton
Monash University
Monash Institute of Pharmaceutical Sciences
Drug Delivery Disposition and Dynamics
381 Royal Parade
Parkville, VIC 3052
Australia

Sanu Shameer
Université de Toulouse
TOXALIM (Research Centre in Food Toxicology)
Institut National de la Recherche Agronomique (INRA)
UMR 1331
180 chemin de Tournefeuille – BP93173
31027 Toulouse Cedex 3
France

Amit Sharma
International Center for Genetic Engineering and Biotechnology
Molecular Medicine Group
Aruna Asaf Ali Marg
New Delhi 110 067
India

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Terry K. Smith*
University of St Andrews
Biomedical Sciences Research Complex
North Haugh
St Andrews
KY16 9ST Fife
UK
tks1@st-andrews.ac.uk

Jacky L. Snoep
Stellenbosch University
Department of Biochemistry
Private Bag X1
Matieland 7602
South Africa

and

Vrije Universiteit Amsterdam
Department of Molecular Cell Physiology
De Boelelaan 1085
1081 HV Amsterdam
The Netherlands

Daniel Stoessel
Monash University
Monash Institute of Pharmaceutical Sciences
Drug Delivery Disposition and Dynamics
381 Royal Parade
Parkville, VIC 3052
Australia

Francis Isidore Totanes
University of Leeds
School of Molecular and Cell Biology
Faculty of Biological Sciences
Clarendon Road
LS2 9JT Leeds
UK

María Valente
Departmento de Bioquímica y Farmacología Molecular
Instituto de Parasitología y Biomedicina "López-Neyra"
Consejo Superior de Investigaciones Científicas
Avda del Conocimiento s/n
18016 Granada
Spain

David D. van Niekerk
Stellenbosch University
Department of Biochemistry
Private Bag X1
Matieland 7602
South Africa

Henri J. Vial
University Montpellier
Laboratory of Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France

Francisca Vicente
Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía
Fundación MEDINA
Screening and Target Validation
Parque Tecnológico de Ciencias de la Salud
Avenida del Conocimiento 34
E-18016 Granada
Spain

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Antonio E. Vidal
Departmento de Bioquímica y Farmacología Molecular
Instituto de Parasitología y Biomedicina "López-Neyra"
Consejo Superior de Investigaciones Científicas
Avda del Conocimiento s/n
18016 Granada
Spain

Tina Weber
University of Glasgow
Institute of Infection
Immunity and Inflammation
Marie Curie Initial Training Network "ParaMet"
120 University Place
G12 8TA Glasgow
Scotland
UK

Kai Wengelnik
University Montpellier
Laboratory of Dynamique des Interactions Membranaires Normales et Pathologiques
UMR 5235 CNRS, UM
Place Eugène Bataillon
34095 Montpellier Cedex 5
France

David R. Westhead
University of Leeds
School of Molecular and Cell Biology
Faculty of Biological Sciences
Clarendon Road
LS2 9JT Leeds
UK

Anthony J. Wilkinson*
University of York
Department of Chemistry
Structural Biology Laboratory
Wentworth Way
Heslington
YO10 5DD York
UK
tony.wilkinson@york.ac.uk

Simon A. Young
University of St Andrews
Biomedical Sciences Research Complex
North Haugh
KY16 9ST St Andrews, Fife
UK

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Foreword

I was delighted to write this Foreword for the book for two main reasons. Firstly, the subject area fits very well with my personal scientific biases (e.g., multiple protozoan parasites rather than simply one, multidisciplinary approaches, multinational), and secondly, I think that one can express one's opinions freely in such an article in the sure knowledge that no one will be offended as few apart from the Editors are likely to read it. I shall attempt do a little bit to rectify the demise of Prefaces/Forewords later in this piece. Unfortunately, books themselves are now a much less important means of communication in science than they were; many are not easily available online and are expensive, in contrast to the enormous amount of literature available free on the Internet. Thus, Introductions to scientific papers can be easily filled with grand-sounding reviews and papers without the need to pay money (or, seemingly on occasions, even to read the articles except for the Abstract). Their lack of easy availability means that articles in books do not get cited (or found by search engines such as Pubmed) and hence cannot become high profile, even when they are excellent. Thus, in some countries, the United Kingdom being one example, they are perceived as valueless except for youngsters trying to make their way into a scientific career. This is a great sorrow and, perhaps, can be changed – as I suggest as follows.

It has not always been like this, and one message I shall try to convey is that much is being lost in the current approach to focus only on readily available and very recent articles when investigating a topic. It would indeed make life easier if this was adequate, but, unfortunately, in my opinion, one usually loses out greatly if one simply relies upon the interpretation of others of the appropriate literature; they too may not have read all the key articles. When I entered parasitology, books were a vital resource. I recall fondly scanning the pages of Biochemistry of Parasites (second edition, edited by Von Brand, 1974); it contained so much detailed facts as well as appropriate references (it saved many hours scanning through Current Contents). Many of these data are indeed still highly relevant and have not been superseded. I strongly encourage those with interests in the areas to consult such tomes (I am sure that good University libraries still have a copy of this and other similar volumes). Interestingly, some good books can now be obtained freely and in full on the Internet, such as Biochemistry and Molecular

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Biology of Parasites (edited by JJ Marr and Miklos Muller in 1995; Chapter 3 appeals particularly to me), Biochemical Protozoology, and Molecular Basis of Drug Design and Resistance, a short list, which, admittedly, reflects my biases, but nevertheless containing excellent and still relevant reviews with data that sometimes have not been published elsewhere. These are readily available now, but do they get read? It would be interesting to know, but I suspect that the answer is "rarely," primarily as current young researchers probably do not know of their existence. Perhaps, the more experienced scientists should be encouraging those in the early stages of their career to remember appropriate past work rather than dismiss it as "ancient" and so irrelevant. I am sure that the Prefaces/Forewords do often not get read; hence, I (modestly) recommend them here as a good read.

Thus, it will be apparent that I believe that books should continue to have a place in research in biological science; good ones should provide the foundations upon which to build one's knowledge of a subject area. The aim of this book is to do that and to provide stimulus to those venturing into drug discovery against parasitic diseases. I hope that it succeeds and that it, too, is made available on the Internet and soon; certainly, this would facilitate its availability enormously. Drugs against protozoan parasites were the start of chemotherapy *per se* and successes were numerous. Indeed, new antiprotozoal drugs were the highlights of the early days of chemotherapy. Situations change and new antiprotozoal drugs have been very scarce in recent times, whereas the need has not decreased and, in some cases, the advent of drug resistance has increased the problems. The development of new technologies always brings with it the hope for massive improvements in health. Usually, the technologies yield large amounts of data (and many scientists keen to apply the methods, Review Boards of grant-awarding bodies being keen to support novel approaches), but translating that into useful and practical products has proved to be hard in very many cases. In some instances, I believe that the basic biology gets overlooked through ignorance or expediency in the rush to apply the new technologies. Thus, I am pleased to see that this volume focuses not only on the new but also takes into account the need to address important biological questions using biologically relevant materials. This is, in my opinion, an essential ingredient of all biological research. I recall learning early in my career the importance to use good and thorough scientific approaches and having important aims (such as providing steps along the road to new and needed medicines such as antiprotozoals). I also learnt the benefits of multidisciplinary approaches, and harnessing multiple sets of skills in one program can be so beneficial. Learning to collaborate with others is crucial, and that takes skill as well as patience. Aiding such interactions is an important role for funding bodies, especially such as the EC. I experienced first-hand how networks of scientists can work well, for example, COST Actions. This facilitates the building of collaborations as well as understanding of different approaches and attitudes; in my opinion, money well

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spent even if the apparent bureaucracy of European networks and the associated administration can have its torments. This book is the result of one such network, and I hope that the contents of the book are useful not only to the current network but also to many more extensive networks in the future.

December 2015

Graham H. Coombs
Emeritus Professor of Biochemical Parasitology
University of Strathclyde
Glasgow, UK

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Preface

Infectious diseases caused by parasites are widespread in humans and their domestic animals. The human diseases strike hardest in the poorest nations and not only cause severe pain and distress but also impair child development, educational progress, and adult productivity, thus contributing significantly to poverty. Treatments currently available are limited by severe side effects, development of drug resistance, and/or high-cost and inadequate means of administration. We believe that there is a moral duty for researchers from the richest nations to work on control methods of diseases that afflict the poorest nations. There is, however, also self-interest for the developed nations. In direct terms, infection of travelers (and peacekeeping forces) is a constant threat. Moreover, there is a risk that parasites that had previously been eliminated from Europe may return from their reservoirs in the developing world, or – given global climate changes – novel parasites may be introduced into newly amenable habitats. Furthermore, alleviating parasitic diseases contributes to significant economic improvements, and this enhanced prosperity would contribute to future economic growth across the world.

Unfortunately, the lack of financial resources in the poorest countries in the world that are most affected by parasitic diseases has for a long time limited the interest of pharmaceutical industry in finding cures for the diseases. In recent years, the pharmaceutical industry has committed itself more to investing into neglected diseases caused by parasites, primarily because a genuine feeling of mission among influential decision-makers has emerged; however, such altruistic actions clearly have to be limited for a commercial enterprise. There has remained a limited core of academic scientists focusing on the problems caused by parasites, but successes with applied outcomes have been few. Some of the research efforts of academics have perhaps been naïve and divorced from the realities of the pharmaceutical industry, such that the results of their efforts can never be practically exploited.

In an effort to bring together the innovative approaches of academic scientists and the tenets of pharmaceutical industry, we created a doctoral training network that had the goal of enabling doctoral students to gain deep insights into and experience of the different and yet complementary approaches of both sectors in the area of drug discovery against parasitic diseases. The program, called

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ParaMet, was funded by the European Commission through a Marie Curie Action. It provided a unique blend of interdisciplinary research projects covering areas integral to the drug discovery process in academia and industrial settings. This volume is one of the outcomes that this venture has generated.

This volume provides a comprehensive summary of the multidisciplinary approaches currently applied in drug discovery programs aimed against parasitic diseases. The volume is organized into four parts that introduce the various main aspects of the drug discovery pipelines. Its first part details individual protein groups or pathways that are specific to protozoan parasites and thus hold promise for future drug development. Several structure-based approaches that underpin the rational design of drugs are exemplified. Such rational approaches are complemented by use of empirical, phenotypical screening methods. The different screening methods are described with a particular focus on the use of natural compound libraries, showing how these will help to expand chemical diversity to identify unique and novel chemical scaffolds acting against parasites and parasite-specific features.

The second part of the volume outlines metabolomics approaches to identify parasite-specific pathways and metabolic nodes that are exploitable for drug discovery. Description of pathways, mechanisms, and targets is accompanied by presentation of metabolomics and lipidomics technologies needed for metabolic pathway reconstruction.

Regulation of gene expression is vital for parasites with complex life cycles. The third part of the volume describes how advances made in understanding the multitude of epigenetic mechanisms regulating the way parasites read their own genes lead to antiparasite drug discovery. Details of proteins determining nuclear organization and modulating transcription, but also of proteins involved in synthesis of resources for translation, are given.

The outcome of modern biology technologies is the generation of large datasets, which are ideal for analysis by bioinformatics and mathematical modeling procedures. Mathematical models can be used to fill in the gaps in knowledge and offer a better understanding of complex aspects of parasite physiology such as drug resistance. The fourth part of the volume describes such analytical approaches that have been applied in antiparasite drug discovery in particular to provide a platform that can be used to query parasite systems in order to make more informed decisions about potential drug targets and the likely mode of actions of drugs and parasite resistance to them.

The editors wish to thank all the authors for their diligence and insight, and the series editor Paul M. Selzer for his many useful suggestions and for his contribution to this volume.

December 2015

Glasgow and Montpellier
Rachel Cerdan, Sylke Muller, and Ovidiu Radulescu

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Part One
Identification and Validation of New Drugs and Targets

Comprehensive Analysis of Parasite Biology: From Metabolism to Drug Discovery,
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1
Discovery of the Mechanism of Action of Novel Compounds That Target Unicellular Eukaryotic Parasites

Daniela Begolo and Christine Clayton*

Abstract

In recent years, most new candidate antiparasitic drugs have been found by screening huge numbers of compounds for their ability to kill parasites, followed by counterscreening for toxicity to mammalian cells. Several public–private initiatives have supported this, yielding many hits each for Plasmodia and Kinetoplastids. From these, candidates are selected for further investigation. Although knowledge of the precise mode of action is not necessary for successful development, detailed understanding of the drug's uptake, activation, and target can be very useful in guiding medicinal chemistry, toxicology, and pharmacology. Knowledge of the target can also provide information for further drug discovery studies and in choosing partner drugs in combinations. A multiplicity of complementary approaches can be applied to investigate the drug mode of action. Examples include selecting drug-resistant parasites and identifying the resistance-causing mutations, reverse genetics to find genes required for drug susceptibility, metabolomics, and biochemical approaches such as affinity purification. Here, we review the myriad possibilities, including numerous examples.

Introduction

The development of new antiparasitic drugs is a necessary process, because many of the currently used drugs are unacceptably toxic and resistance is emerging [1, 2]. This review focuses in particular on compounds against *Plasmodium* spp., *Leishmania* spp., *Trypanosoma brucei*, and *Trypanosoma cruzi*. Multiple initiatives, for example, the public–private partnerships Drugs for Neglected Diseases initiative (DNDi, www.dndi.org) [3] and Medicines for Malaria Venture (MMV, www.mmv.org) [4] were founded to support discovery and approval of new drugs. There is continued discussion concerning the virtues of different drug discovery

*Corresponding author.

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methods [5–7]. In the phenotypic drug discovery approach, large numbers of compounds are screened for their ability to kill the target pathogen, without regard to possible mechanism of action (MoA) [5, 7, 8]. The target-based approach, in contrast, first focuses on a particular mechanism and second considers the ability to kill the pathogen [5, 9]. A few years ago, an analysis of all approved first-in-class compounds showed that the phenotypic approach has, in practice, been more successful for the development of licensed drugs targeting infectious diseases [5]. As a consequence of these observations, efforts have been redirected toward phenotypic screens [10]. In the last few years, under the auspices of public–private partnerships, millions of chemical compounds have been tested for their abilities to kill protist pathogens without affecting the mammalian cells [11, 12]. As a result, thousands of drug-like molecules are now available for potential development.

Phenotypic screening is an agnostic approach: target, activation pathway (if relevant), and entrance route – which together are summarized as MoA – are initially not known. Indeed, drugs can achieve clinical approval even if their MoA is unknown [6, 7, 13]. Nevertheless, MoA knowledge is extremely helpful for discovery programs [6]. Target identification aids medicinal chemists, since 3D structure determination illuminates structure–activity relationships [14] and thus facilitates lead optimization [15]. MoA knowledge can also help to predict and monitor possible resistance emergence, as well as on-target side effects [16]. Even if a particular drug candidate is unsuccessful, knowledge of its MoA will suggest development of alternative compounds with the same MoA. Finally, compounds with novel MoA deepen our understanding of parasite biology [14, 16].

A multitude of techniques is available for target deconvolution. Usually, it is necessary to integrate several complementary approaches [16, 17]. Once a possible target has been identified, it must be validated by independent methods [18]. Here we give an overview of different method, and present examples – usually for molecules that target parasitic protists, but occasionally also other organisms (Table 1.1).

Principles

A drug that encounters a pathogen must be taken up first, which may involve either passive diffusion or active transport [2]. In some cases, it may need to be transferred to an organelle and/or activated by pathogen enzymes [2]. The activity of the drug in the cells may involve inhibition of a single or multiple enzymes, binding to macromolecules, or less specific toxicity [6]. In the latter case, selective toxicity of the drug for the pathogen, rather than the host, must rely either on accumulation of the drug in the pathogen or a particular compartment or on activation by a pathogen-specific pathway.

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Table 1.1 Overview of drugs often cited in the text.

Compound or class name	Other names – related compound	Target organism	Genes implicated in mode of entrance	Genes implicated in mode of action and resistance
Antimonials	SbV, SbIII	*Leishmania* spp.	Entrance through AQP1 [80]; export through PGPA [98]	SbV is reduced to SbIII [76–78]; nonspecific toxicity
Artemisinin		*Plasmodium* spp.		Targets phosphatidylinositol-3-kinase [105]; resistance can be cause by mutation in Kelch-13 propeller [104]
Atovaquone		*Plasmodium* ssp.		Inhibits cytochrome b/c [106]
Benznidazole		*T. cruzi*	Possibly through TcABCG1 [100]	Activated by type 1 NTR [57]; probably causes oxidative stress [107]
Chloroquine		*P. falciparum*, *Plasmodium malariae*	PfCTR, probably in vacuoles [63, 64]	Probably inhibiting hemozoin formation [37, 38]; PfMDR1 is involved in resistance [64]
Difluoro-methylornithine	DFMO, eflornithine	*T. brucei*	Entrance through AAT6 [55]	It targets and covalently binds ornithine decarboxylase [50]
Melarsoprol		*T. brucei*	Low-affinity transporter AT1 [46], most likely high-affinity AQP2 [108, 109]	Mechanism unclear; cross-resistance with Pentamidine [47]
Miltefosine		*Leishmania* ssp.	Transported through LdMT [71], if in the presence of LdRos3 [72]	Probably several activity, among them increased oxidative stress [110, 111]
Nifurtimox		*T. cruzi*, *T. brucei*		Activated by type 1 NTR [57]; probably causes oxidative stress ; possibly DNA/RNA degradation [112]
Pentamidine	Diamidine	*T. brucei*	Low-affinity transporter AT1 [46], most likely high-affinity AQP2 [108, 109]	Mechanism unclear; cross-resistance with Melarsoprol [47]

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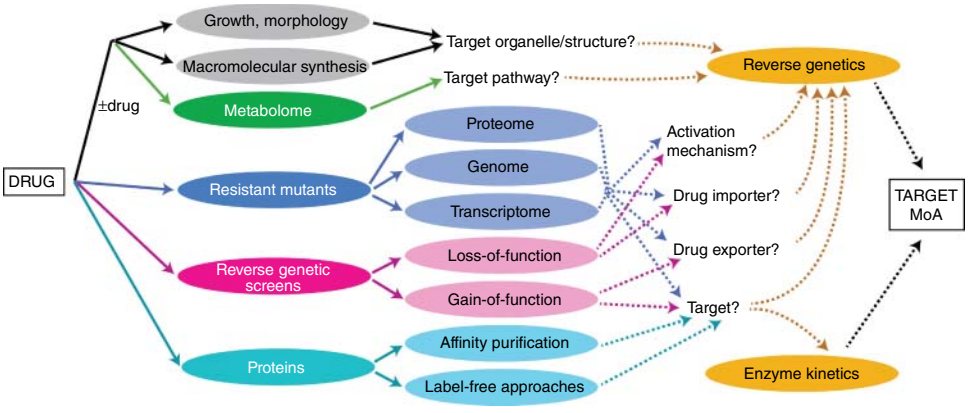


Figure 1.1 Schematic representation of the different possible approaches for target deconvolution described in the text.

Figure 1.1 shows a simplified version of the entire target deconvolution process. First, basic characteristics need to be established:

1) What effect does the drug have on parasite morphology?
2) Is it cytostatic, cytotoxic, or cytolytic?
3) How long does it take to act?
4) What is the effect on cell cycle/at what stage in the parasite cell cycle does it act?
5) How does it affect macromolecular synthesis?
6) Can possible targets be predicted from the chemical structure?

Once these basics are known, further steps can be chosen:

7) What are the effects on metabolites?
8) Does the drug accumulate within the cell or within a particular subcellular compartment?
9) Can resistant lines be generated? If so, what is the mechanism? Which genes are mutated in resistant lines? Do resistant lines show alterations in gene expression?
10) A library of cells with inducible or constitutive knockdown of all genes is made and selected with drug. Which genes are no longer expressed in the resistant cells?
11) A library of cells with inducible or constitutive overexpression of all genes is made and selected with drug. Which genes are overexpressed in the resistant cells?
12) The drug molecule is attached to an affinity matrix and incubated with cell lysate. Which proteins selectively bind to the drug?

Finally, potential mechanisms of action are tested:

13) If a mutant gene has been identified in a resistant line: Does introduction of that mutant version into normal cells render them drug-resistant?

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Reciprocally, in the resistant line, does replacement of the mutant allele with the wild-type render the cells drug-sensitive?

14) If a target enzyme has been identified: Does the drug inhibit that enzyme with a potency that is compatible with the intracellular drug concentration? Do the inhibitory activities of compounds in the relevant series parallel their activities against the target organism?

Initial Investigations

Initial general tests can give substantial information. Molecules that disrupt, or form pores in, the plasma membrane rapidly cause cell lysis, as do molecules that cause irreversible chemical damage. For example, arsenical drugs that are used to treat late-stage African trypanosomiasis kill cells within half an hour even when added at low nanomolar concentrations, most likely via nonspecific covalent binding to thiol groups and resulting oxidative stress [19]. They derive selective action from accumulation within the parasites (described later). Antimalarial endoperoxides such as artemisinin and derivatives, or synthetic alternatives, are also fast-acting and cytotoxic, probably at least partially via oxidative radicals [20]. Compounds that affect biosynthesis of the plasma membrane are expected ultimately to be cytolytic [21]. In contrast, compounds that inhibit DNA synthesis – whether directly, or via nucleotide depletion – act more slowly and are likely to be cytostatic [22].

Morphology and Stage Specificity

Some antiparasitics have very specific effects on morphology, which are observed either with live cells or on fixed ones after specific organellar staining. Kinetoplastids, such as *T. cruzi*, *T. brucei* and *Leishmania* spp., contain a large assemblage of mitochondrial DNA called the *kinetoplast*, which consists of concatenated circles (kDNA). After appropriate staining, the kinetoplast is readily seen by light or fluorescence microscopy [23]. Isometamidium – an ethidium bromide derivative that is used to treat cattle African trypanosomiasis – intercalates selectively in the kDNA, probably affecting the kDNA integrity preventing division of the network [24, 25]. In this case, the drug is itself fluorescent, so its accumulation in the kDNA network can be observed directly [25–27]. The action of drugs that affect the mitochondrial membrane potential can be detected by staining with MitoTracker. Salicylhydroxamic acid is an irreversible inhibitor of the trypanosome alternative oxidase, which is required for efficient glycolysis. The first noticeable effect of the drug is a slowing in parasite motility – presumably as a consequence of ATP depletion [28, 29]. Finally, apolipoprotein L1 – not a drug, but a protein of human serum – kills *T. brucei* via damage to the lysosome; this is visible by staining with lysosome probes or immunofluorescence targeting lysosomal proteins [30].

Apicomplexan parasites, such as *Plasmodium* spp., contain a secondary plastid-derived organelle called the *apicoplast*. This contains various pathways

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and has a genome that encodes the essential proteins [31]; in the blood form, the only apicoplast-specific metabolic pathway that is required for multiplication and survival is isoprenoid biosynthesis [32]. Compounds that inhibit apicoplast replication and protein expression affect the parasites only gradually: defects in apicoplast division and segregation are seen and the cells undergo "delayed death," after several cell divisions [33–35]. Effects at particular stages of the cell cycle are often also manifest morphologically. An inhibitor of DNA or deoxyribonucleotide synthesis will most likely cause parasites to accumulate in G1 or S-phase. Intraerythrocytic *Plasmodium* parasites obtain amino acids partially through digestion of hemoglobin in a lysosome-like organelle called the *food vacuole* [36]. The released heme is highly toxic in the soluble state, but is detoxified by biomineralization into insoluble hemozoin crystals [36]. Drugs that inhibit hemozoin formation, such as chloroquine, are not toxic to the early ring stages, but kill the trophozoites, which have very active food vacuoles [37, 38]. Such drugs also cause clear microscopically visible alterations in hemozoin accumulation [39].

Macromolecular Synthesis

The inhibition of macromolecular biosynthesis is most readily detected by labeling with radioactive precursors followed by trichloroacetic acid (TCA) and/or ethanol precipitation: ^{35}S-methionine for protein, ^{3}H-thymidine for DNA, and ^{3}H-uracil for RNA [40]. To our knowledge, there are currently no examples of drugs that directly target these processes in the nucleus or cytosol of protists: the machineries involved may be too similar to those in the mammalian host. However, effects will be seen from inhibitors that prevent the provision of precursors.

Structure-Based Prediction

In some cases, possible targets can be predicted from chemical structures. For example, huge numbers of inhibitors of mammalian protein kinases are already well characterized and libraries of kinase inhibitors have been published [41]. Molecules that share the same or similar scaffolds may inhibit parasite kinases (e.g., [42]). This can be tested directly in enzyme assays, if pure proteins and appropriate assays are available.

Biochemical Methods and Candidate Genes

Simple biochemical methods can be used to test hypotheses concerning drug action – if predictions are possible. Some examples are described as follows. These methods were a mainstay of drug target identification before it was possible to sequence RNA and DNA at the genome/transcriptome level or to identify many proteins and metabolites in complex mixtures. Now, they are used mainly to confirm hypotheses that are generated based on high-throughput data.

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Melarsoprol and Diamidines

Melarsoprol, which is used against trypanosomiasis, is a highly toxic molecule containing arsenic and exhibits strong reactivity with thiols [19]. An explanation for its (rather poor) selectivity for trypanosomes – such as active accumulation – is therefore needed. To find the mechanism of drug import using biochemical approaches, one would ideally measure the drug directly, for example, using radioactively labeled drug, since the amounts from cell samples are unlikely to be sufficient for bioassay [43]. As a surrogate, however, at least for competition assays, the effect of the drug on the organism can be used. The structures of melarsoprol and the diamidines, both drugs used against African trypanosomes, bear some resemblance to adenosine. This suggested to Carter *et al.* [44, 45] that a purine transporter might be involved in drug accumulation. Indeed, adenine, adenosine, and dipyridamole compete for uptake and reduce the effect of the drugs. The responsible transporter was named the P2 transporter [44]. To find the gene encoding the P2 transporter, Mäser used *Saccharomyces cerevisiae* with a defect in adenine biosynthesis. The strain was complemented with a *T. brucei* cDNA library and selected for the ability to grow with adenine as the sole purine source. This resulted in identification of the adenosine transporter 1 (*AT1*) gene, a member of the equilibrative nucleoside transporter family [46]. TbAT1 was validated afterward: cells lacking *AT1* have reduced sensitivity to melarsoprol and diamines [47].

Meanwhile, others followed up the hypothesis that melarsoprol might act via thiol groups and could perhaps be exported as a thiol conjugate. Trypanosomes have several homologs of the multidrug resistance protein (MRP) family, which were first discovered as exporters of anticancer drugs [48]. The major small thiol in trypanosomes is trypanothione, a conjugate of glutathione with spermidine. Prompted by the previous results with *Leishmania* (see the following section), Shahi *et al.* [49] followed a candidate gene approach. They showed that overexpression of one transporter, multidrug resistance protein A (MRPA), can cause a low degree of melarsoprol resistance, especially when coexpressed with two enzymes of trypanothione biosynthesis, ornithine decarboxylase, and gamma-glutamyl-cysteine synthase [49]. These studies, however, partially illuminated an export mechanism rather than the MoA.

DFMO

Difluoromethylornithine (DFMO or eflornithine) is a suicide inhibitor of ornithine decarboxylase [50]. It inhibits the enzyme in mammalian cells – indeed, it was initially developed for cancer therapy [51], but it has been revived and is currently used to prevent growth of facial hair [52]. The specificity of the drug is due to its irreversible action, combined with much slower turnover of the trypanosome enzyme compared with the human one. The consequent loss of polyamine biosynthesis causes death of the parasites [53]. Although resistance to DFMO can be obtained *in vitro* through overexpression of ornithine

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decarboxylase [54], when trypanosomes resistant to eflornithine were selected, no differences were found in the target enzyme [50] or in the metabolomic profile [55]. Instead, reduced drug accumulation was observed [55]. Since eflornithine is an amino acid analog, loss of an amino acid transporter was hypothesized. Therefore, the members of amino acid permease gene family were studied. One copy of *TbAAT6* was found to be absent from the resistant cells [55]. RNA interference (RNAi) on the transporter mRNA conferred a degree of resistance comparable to the selected cell lines.

Drug Activation

Two drugs that require intracellular activation are currently in clinical use against parasitic protists. In both cases, activation results in a highly reactive and toxic free radical. Metronidazole is used to treat the anaerobes *Trichomonas* and *Giardia* and is activated by reduced ferredoxin [56]. Prior knowledge that nitroheterocyclic compounds are prodrugs, which need to be activated, led to the identification of a candidate enzyme, the mitochondrial type 1 nitroreductase (NTR) in *T. brucei* and *T. cruzi*. Reduced expression of NTR leads to resistance against nifurtimox and benznidazole [57].

Classical Genetics and Genomics

One very successful method to identify the targets of a novel drug is to generate pathogens that are resistant to the drug. The changes responsible for resistance are then identified [2, 16]. They could be, for example, loss of a transporter that takes up the drug (loss of drug import), loss of drug activation, mutation of an enzyme target such that it is no longer affected by the drug, and increased expression of the drug target. Loss of function might be by gene deletion or mutation, or decreased expression, and increases in activities can be via mutation, gene amplification, or activated expression. However, quite often such experiments do not yield any information about the mechanism of drug action. Instead, they can reveal mechanisms for increased drug export or inactivation.

Before the Genomes

Before the era of easy genome sequencing, identification of the causes of drug resistance relied on genetic crosses or the testing of hypotheses. Examples are listed as follows.

Chloroquine and Quinine

Chloroquine is one of the examples for which resistance studies revealed an export mechanism rather than a direct MoA. Genetic studies of *Plasmodium falciparum* are greatly facilitated by the fact that it has an obligatory sexual stage in the mosquito definitive host, but is haploid in the mammalian intermediate

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host [58]. This means that controlled crosses can be done, and the progeny can be studied to identify genomic regions, and even single genes, linked to drug resistance [16, 59]. A landmark study using this approach was a laboratory cross between cloned chloroquine-resistant and chloroquine-sensitive *P. falciparum* field isolates [60]. Using 85 restriction fragment length polymorphisms that distinguished the resistant and sensitive strains, a 400 kb segment of chromosome 7 was linked to the transmission of chloroquine resistance to the progeny [60]. Afterward, the area was restricted to a region of 36 kb [61]. Polymorphisms were then identified and few genes proposed as candidates. To test the validity of the proposed resistance alleles, the relevant DNA segments were sequenced from field isolates of known drug sensitivities, and the authors looked for mutations that correlated with resistance. As the number of sequences increased, two initial candidates [61, 62] were successively eliminated before the real culprit, chloroquine resistance transporter in *P. falciparum* (PfCRT), was identified [63]. PfCRT is a transmembrane protein of the digestive vacuole, probably exporting the protonated version of chloroquine [64] from its site of action. Overexpression of the mutant version increased resistance of the cells to the drug and resistance selection *in vitro* led to mutations [63]. Allelic exchange was performed in sensitive strains using mutated PfCRT of different origins; this showed that mutating this gene was enough to obtain resistant cells [65].

Genetic crosses are extremely laborious and have now been made almost superfluous by the development of high-throughput DNA sequencing [16]. Nevertheless, the progeny lines from the genetic cross have been used extensively to obtain additional insights into drug action. The quantitative trait locus (QTL) approach (a statistical method that identifies stretches of DNA that correlate with a particular measured phenotype [66]) was used to identify parts of the genome apart from PfCRT that contribute to chloroquine resistance [67]. One of the identified genes was *pfmdr1* [64]. PfMDR1 is a protein of the multidrug resistance transporter class, and mutations may modulate chloroquine sensitivity [64]. Resistance to quinine, another antimalarial drug, is linked not only to *pfctr* and *pfmdr1* (multi drug resistance) genes but also to a *pfnhe* (sodium/hydrogen ion exchanger) [68]. Recently, it was shown that not only *pfcrt* but also a HECT ubiquitin protein ligase (*pfut*) was a conserved trait in quinine resistance [69]. Both genes were present in chromosome 7 but in different chromosomal domains of restricted genetic diversity; therefore, they were inherited independently [69]. It was suggested that the ubiquitin action could be part of a multifactorial resistance, modulating the stability of proteins involved in survival under quinine pressure [69].

Miltefosine

Miltefosine is an anticancer drug that is now approved for oral use against visceral leishmaniasis [70]. A candidate drug transporter (Miltefosine transporter in *Leishmania donovani* (LdMT)) was discovered by taking a resistant strain and transfecting it with a cosmid library of genomic DNA [71]. The authors then looked for

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drug sensitivity. Just one cosmid restored sensitivity. By further subcloning, the gene *LdMT* was identified that encodes a protein similar to P-type ATPase APT subfamily. The retransfection of *LdMT* into a resistant strain increased the uptake of miltefosine. The resistant cell line was defective in glycerophospholipid uptake, and this was also reversed by retransfection of *LdMT* [71]. The protein was shown to be active only in the presence of another protein, LdRos3, which is presumably part of the same translocation machinery [72].

Antifolates and Antimonials in *Leishmania*

Leishmania are susceptible to methotrexate, an inhibitor of dihydrofolate reductase. To confirm the mechanism, cells resistant to methotrexate were generated by stepwise selection in increasing concentrations of drug. The resistant cells contained specific amplified episomes. Some of these indeed encoded the target enzyme, dihydrofolate reductase [73]. However, others encoded a protein of the MRP family, which was named MDR1 [74]. *T. brucei* MRPA is the homolog of MDR1.

Pentavalent antimonials have been used against *Leishmania* for more than 65 years [75]. Similar to the arsenicals, these are nonspecifically toxic compounds, so selectivity must lie in drug activation or accumulation. The more active trivalent antimonials are produced through reduction of pentavalent antimonials, in macrophages and within *Leishmania* [76–78]. It was known that trivalent metalloid uptake can occur through aquaglyceroporins [79]. Therefore, different *Leishmania* spp. were investigated to look for homologous genes of human aquaglyceroporin 9 [80]. A gene was found that encoded aquaglyceroporin 1 (AQP1); when overexpressed in *Leishmania* cell lines, AQP1 produced hypersensitivity to antimonials [80].

Post-Genomic Era

The availability of protist parasite genomes, together with steadily improving techniques for reverse genetics, has enormously facilitated target deconvolution [59]. Initially, the genome sequences were invaluable in the identification of candidate genes (see the previous section). More recently, the availability of relatively cheap, high-throughput sequencing has revolutionized the way in which targets can be identified [59]. Nevertheless, screens merely yield candidates whose roles in drug action must be tested individually by more laborious methods [81].

Microarrays for Analysis of Resistant Cell Lines

High-density "tiling" microarrays are available for *P. falciparum.* Although their primary purpose was originally to study gene expression through hybridization with labeled cDNA [82, 83], they can also be hybridized with fragmented genomic DNA and thus used to find single nucleotide polymorphisms (SNPs), which lead to

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decreased signals, and copy number variations (CNVs), where an increased copy number gives an increased signal [84]. The imidazopyrazines are a new class of antimalarial compounds found by phenotypic screening [83]. Resistant *Plasmodium* lines were selected and labeled genomic DNA was hybridized with the array. One gene was found to be modified consistently: it encodes phosphadytilinositol-4-OH kinase [83]. By reverse genetics, it was shown that either the mutations or the overexpression of the wild-type protein conferred resistance to the cells [83].

The same array was used to study spiroindolones [82], which are in clinical trials to cure malaria [85]. Six independent resistant clones were all found to carry mutations in the gene encoding the P-type cation-transporter ATPase4 (PfATP4) [82]. The role of the gene was confirmed by reverse genetics, as described for the imidapyrazoles.

Whole Genome Sequencing of Resistant Lines

Microarrays have limited sensitivity and reproducibility. At least in the case of protists with relatively small genomes, they have now been replaced for most purposes by whole genome sequencing [59]. Aminopyrazoles were found by phenotypic screening against *P. falciparum*, and sequencing of the genomes of laboratory-generated resistant lines revealed, as for spiroindolones, mutations in the gene encoding PfATP4 [59, 85]. PfATP4 maintains the Na^+ imbalance across the plasma membrane and the drug inhibits this [85]. It is interesting to notice how two classes of antimalaria compounds, even though chemically very different, affect the same target; clearly, PfATP4 is not only crucial for the life of *Plasmodium* but also eminently druggable [85]. This is critical information, but it is unlikely to lead to searches for further PfATP4 inhibitors for two reasons. Firstly, PfATP4 is a transmembrane protein, not amenable for recombinant protein production and inhibitor screens; and secondly, in order to avoid cross-resistance between drugs, it is important to limit the number of compounds that attack only one target.

Whole genome sequencing was used to analyze miltefosine-resistant *Leishmania major* [86]. This study showed the necessity of sequencing single clones of resistant cells, since the resistant populations showed great variability in phenotype and genotype [86]. After sequencing single resistant clones, no CNVs were found, but single point mutations in the miltefosine transporter known to cause resistance were identified [86]. Similar experiments with antimony resistant clones failed to reveal any SNPs linked to resistance, but DNA insertions and deletions were identified [87]. In particular, a deletion of the gene encoding AQP1, the protein previously identified to take up the drug [80], was found.

Linkage group selection is a method to analyze uncloned progeny of a genetic cross, comparing progeny selected or not selected with drug; genome wide markers are evaluated in a quantitative way and markers of the sensitive parent are expected to be located close to genes determining the phenotype after

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selection [88]. In *Plasmodium chabaudi* and *Plasmodium vivax*, orthologs of *pfcrt* and *pfmdr1* are not involved in chloroquine resistance [89]. Linkage group selection was initially performed by PCR amplification of known polymorphisms [88, 90] of three independent genetic crosses between artemisinin-sensitive and artemisinin-resistant *P. chabaudi* [90]. To improve the resolution of the analysis, a similar approach was used with crosses of parasites resistant to artemisinin [91] and chloroquine [89], coupled with whole genome resequencing of the initial cell lines [89, 91]. A deubiquitinating enzyme (encoded by *ubp1*) [90, 91] and putative amino acid transporter encoded by *aat1* [89] were thereby implicated in multidrug resistance.

Whole genome sequencing analyses are only useful if several independent drug-resistant lines are available. If these are derived in the laboratory, they are compared with the original line [85]. If, in contrast, field isolates are used, several independent sensitive isolates will be needed as controls. Work on halofuginone, which is used in Chinese medicine against malaria, illustrates some potential pitfalls of taking a simplistic approach [92, 93]. Whole genome sequencing of two independently selected resistant clones leads to the identification of nonsynonymous mutations in *cPRS*, a putative proline aminoacyl-transfer RNA (tRNA) synthetase [93]. *S. cerevisiae* are resistant to halofuginone; to test whether cPRS could be the drug target, the *cPRS* gene was replaced by the wild-type version of the *Plasmodium* gene, and this resulted in sensitivity to halofuginone in *S. cerevisiae* [93]. Halofuginone was shown to affect the response pathway to amino acid deprivation, but the effect was seen only when the wild-type version of the *cPRS* gene was present [93]. Therefore, Herman *et al.* hypothesized that mutations in the gene were the cause of resistance in *Plasmodium.* However, upon closer investigation, it transpired that establishment of resistance was more complex [92]. Initially, proline homeostasis was modified, then mutations in the *cPRS* gene were observed; however, these mutations lead to cells with reduced fitness. The amplification of the *cPRS* gene was actually a late, compensatory event, which increased the fitness of the resistant cells [92].

Recently, the genomes of 45 *P. falciparum* strains from Senegal were sequenced to look for genome-wide association study (GWAS) with drug resistance [94]. Genes previously known to be linked to drug resistance were identified, such as *pfmdr1*, *pfcrt*, and *dhfr.* However, other genes were also found, some of which encode steps in metabolic pathways that might modulate the response to drug stress [94]. A similar approach was applied to a set of nine other *P. falciparum* isolates with different genetic backgrounds; SNPs were documented and then compared with 57 other genome sequences [95]. In addition to the previously known genes, a new locus, PF10_0355, was identified to be connected to halofantrine resistance [95]. Overexpression of the protein encoded by PF10_0355 conferred resistance to halofantrine and other structurally related drugs [95]. The function of this protein is as yet unknown, and we do not know whether it is a halofantrine target or somehow reduces halofantrine efficacy.

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Changes in mRNA Levels

The amounts of proteins in cells often do not correlate directly with the levels of the corresponding mRNAs [96], but nevertheless, changes in mRNA level can provide information regarding changes in the state of cells. Changes in gene expression that are an immediate response to drug treatment can offer clues about drug action if cells have feedback mechanisms that regulate expression to compensate for damage or metabolic disruption. Such adaptations do not involve any changes to the genome. So far, however, most gene expression results relevant to parasite chemotherapy are from comparisons between drug-resistant and drug-sensitive cells: in this case, differences in expression levels or pattern can reflect either gene regulation or changes in gene copy number.

Candidate Genes

Before high-throughput methods were available, a "candidate gene" approach was adopted. For example, the expression of genes previously known to be involved in drug resistance could be checked in drug-resistant lines [97]. The expression 44 candidate genes from arsenite- and antimony-resistant *Leishmania* was measured in a "mini-array" format and overexpression of mRNAs encoding the ATP-binding cassette (ABC) transporter *Leishmania* ABC transporter (PGPA) (the homolog of *T. brucei* MRPA), γ-glutamylcysteine synthetase, and glutathione synthetase was detected [98]. Glutathione synthetase and γ-glutamylcysteine synthetase are enzymes on the trypanothione synthesis pathway, and MRPA/PGPA exports antimonials and arsenicals as conjugates with trypanothione. Using the same gene array with RNA from methotrexate-resistant cells, increased expressions of the target (dihydrofolate reductase) and pteridine reductase and S-adenosylmethionine synthase were found [98]. Using a different mini-array containing all *Leishmania* genes encoding ABC proteins, three were found to be overexpressed in antimony-resistant cells [99].

Microarrays and Sequencing

Candidate approaches are, of course, not useful for the discovery of novel modes of drug action or resistance. Genome-wide microarrays are therefore a considerable improvement – although the genes discovered have not always been novel. For example, they were used to identify overexpression of the ABC transporter TcABCG1 in benznidazole-resistant *T. cruzi* [100]; as confirmation, overexpression using a *T. cruzi* expression vector resulted in resistance to the drug [100]. In contrast, SNPs in the gene did not correlate with resistance [101]. A whole-genome microarray was also used to analyze the artemisinin tolerance of some Cambodian *P. falciparum* isolates [102]. The comparison of three resistant

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and three sensitive strains revealed numerous differences. This, combined with an observed slowing of metabolism and growth but increased protein turnover, suggested wide-ranging regulatory alterations [102]. More recently, 1043 clinical *P. falciparum* isolates from 13 different endemic regions, and with different resistance levels, were analyzed by RNASeq [103]. Again, numerous changes were detected in resistant isolates, affecting 10% of the 5000 detected transcripts [103]. Most of the upregulated genes had functions in protein metabolism and quality control [103]. Downregulation of genes involved in DNA replication was also seen, which might imply developmental stalling [103]. The changes in protein quality control are consistent with genomic analyses, which found that mutations in the *P. falciparum* Kelch-13 propeller correlated with artemisinin resistance [104]. A search for inhibitors of *Plasmodium* phosphatidylinositol-3-kinase revealed that artemisinin is a potent inhibitor of enzyme activity, preventing formation of phosphatidylinositol-3-phophate [105]. In ring stages, a range of secreted proteins is modified by phosphatidylinositol-3-phophate, resulting in their retention in the endoplasmic reticulum; polyubiquitination and Kelch-13 binding result in degradation of phosphatidylinositol-3-kinase [105]. The Kelch-13 mutations in resistant cells result in an increased level of phosphatidylinositol-3-kinase, thus combating the action of artemisinin in rings [105]. Other MoA are, however, likely to be more important in trophozoites – which explains the complexity of resistance phenotypes.

Reverse Genetic Screens

Gain-of-Function Screens

One subset of reverse genetic approaches exploits the fact that overexpression of either the drug target or the proteins involved in drug metabolism or export can cause drug resistance [95, 113–115].

In *L. major*, cosmid vectors can be maintained as episomes. If the cosmid encodes a protein that can confer drug resistance, its copy number will increase upon stepwise selection [115]. After transfection of a library covering the whole genome, drug treatment will select for those cosmids that cause resistance [115]. For example, two sterol synthesis inhibitors selected for nine resistance genes. One of them was squalene synthase, which acts in the sterol biosynthesis pathway, but upstream of the previously known drug targets. It was suggested that squalene synthase overexpression increases the levels of intermediates upstream of the target enzymatic reactions, compensating by mass action for the inhibition of enzymes further along the pathway [115]. In another case, the library was transfected into antimony-resistant *Leishmania mexicana* strains, and the authors looked for parasites that had become antimony-sensitive [116]. The identified cosmid encoded AQP1, a previously known transporter of antimony [116]. It turned out that the only difference between the resistant strain and the wild-type strain was a reduced level of *AQP1* mRNA [116].

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In each of these *Leishmania* experiments, the selected cosmids were recovered individually, which meant that only the cosmids that conferred the highest resistance would be recovered. High-throughput sequencing has obviated the need to do this. Recently, a protein overexpression library in *T. brucei* was created [117]. A plasmid library for tetracycline-inducible expression of mRNAs from random genomic fragments was transfected with high efficiency using a meganuclease-based system [118]. Although the library had an average of 10-fold coverage of each gene, it was limited by the fact that the inserts had an average length of about 1.2 kb [54, 117]. The trypanosome library was selected with DFMO and DDD85646 [119, 120]. After selection, DNA was prepared, the library inserts were PCR-amplified, and the resulting mix was sequenced. Comparison with the original library showed which overexpressed genes were conferring a growth advantage [54]. The only functional genes recovered were ornithine decarboxylase, the target of DFMO [50], and *N*-myristoyltransferase, which is the target of DDD85646 [119, 120]. This shows that the approach can not only identify targets but also be used to confirm drug specificity [54].

Loss-of Function Screens

High-throughput loss-of-function screens are possible in parasites for which dominant-negative mechanisms of gene downregulation exist. So far, the best examples are for *T. brucei*, which has the RNAi pathway [121, 122]. The plasmids comprising the libraries are designed to integrate into the parasite genome. In each plasmid, a random fragment of DNA is flanked by opposing tetracycline-inducible promoters. As described earlier for the *T. brucei* overexpression library, the plasmid library is transfected into *T. brucei* and a library of parasites – each containing a different inducible RNAi fragment – was created [118]. Upon tetracycline addition, the respective inserts are transcribed into both directions, creating a double-stranded RNAs. In the absence of drug, there is selection against essential genes [122]. As described for the overexpression library earlier, plasmid inserts that were selected or lost are identified by PCR followed by deep sequencing. In the presence of drugs, it is possible to select cells with RNAi targeting any gene that is required for drug action. These could be nonessential genes, but it is also possible to select for trypanosomes that have moderate downregulation of an essential gene. In this case, the growth advantage from a decreased gene expression is overridden by the advantage that is obtained in surviving the drug pressure. Selection of a direct drug target is in contrast most improbable, since RNAi is expected to synergize with the drug. For studies of drugs against kinetoplastids, at least, such analyses seem likely to become routine.

The negative selection approach has so far proved most useful in elucidating the MoA of drugs that have relatively nonspecific action, but are selective by virtue of specific accumulation or activation. Selection of a trypanosome RNAi library with DFMO retrieved the amino acid transporter AAT6 [55] and selection with melarsoprol identified the transporter AT1 [121]; choline analogs selected for loss

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of a mitochondrial carrier protein 14 (MCP14) [123]. None of these proteins is essential for cell viability, but an *MCP14* knockout led to a reduced growth rate. Downregulation of MCP14 conferred resistance to choline analogs and ectopic copy expression increased susceptibility, confirming MCP14 involvement in drug action [123].

The RNAi library approach has greatly increased our understanding of the MoA of four trypanocidal drugs that are in clinical use: suramin, pentamidine, arsenicals, and nifurtimox [108, 122, 124]. For nifurtimox, which probably acts as a free radical, loss of an activating enzyme, the NTR type 1, was detected. The MoA of suramin is unclear; one possibility is inhibition of glycolysis. Quite a large number of RNAi inserts was selected by suramin; notably, they included a selection of genes involved in endocytosis and lysosomal function [108]. One of the selected RNAi targets, invariant surface glycoprotein of molecular weight 75 kDa (IGS75), is thought to be on the surface and in lysosomes; it was demonstrated to bind suramin. This was consistent with the previously known accumulation of suramin within lysosomes. It was therefore suggested that suramin enters by receptor-mediated endocytosis, with ISG75 as the receptor [108]. It was already known that AT1 is not the only transporter for melarsoprol and pentamidine, and the RNAi library identified the culprits: two aquaporins, aquaglyceroporin 2 (AQP2) and aquaglyceroporin 3 (AQP3) [108, 109]. Treatment-refractory isolates often show loss of the AQP2 gene, or rearrangements involving AQP2 and AQP3 [125, 126], although the roles of the AQPs in resistance have been challenged and the link could be more complex [127]. When the RNAi library was used with CpdA, an inhibitor of cyclic AMP (cAMP) phosphodiesterases, reduced expression of cAMP response proteins (CARPs) was found to confer resistance [128]. cAMP response protein 1 (CARP1) is unique to kinetoplastid parasites, and CARP3 is unique to *T. brucei*; they are all believed to be part of a novel cAMP signaling pathway [128].

Proteins and Proteomes

In the field of drug target deconvolution, high-sensitivity mass spectrometry is finding increased use in two areas: identification of proteins that bind to affinity columns and comparison of drug sensitive and resistant lines [18].

Proteomes

CNVs can suggest that a protein might be overexpressed in a line with additional copies, but changes in expression due to changes in regulatory sequences cannot be predicted. It is therefore extremely useful to be able to compare expression directly [18, 129]. Until 10 years ago, mass spectrometry applications were characterized by limited analytical depth and quantification capability [18]. Experiments

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to compare two protein preparations therefore involved initial separation of the proteins on gels, visualization by staining, followed by selection of small gel segments, which appeared to show differences. Because one-dimensional gel electrophoresis is relatively insensitive, proteins were usually separated on two-dimensional gels [130]. Since the latter is poorly reproducible, the preparations to compare were ideally prestained with two different fluorophores, then mixed before the separation was performed [131, 132].

Using 2-D electrophoresis, methionine adenosyltransferase (MAT) was found to be overexpressed in *L. major* cells with methotrexate as well as a resistant mutant [130]. Overexpression of *MAT* gene in a sensitive strain did not confer resistance, but it was demonstrated that it modified the level of resistance to methotrexate [130]. The method was also used to study the effect of doxycycline in *P. falciparum*, suggesting the mitochondrion and apicoplast as targets [131]. However, by this time, an additional method was also available to confirm the results. Isobaric tag for relative and absolute quantitation (iTRAQ). iTRAQ involves the use of different chemical labels for each polypeptide sample. Up to three samples can be pooled and then analyzed by liquid chromatography and mass spectrometry (LC-MS) [131].

Another common approach for protein labeling is stable isotope labeling by amino acids in cell culture (SILAC). In this case, one culture includes heavy nonradioactive isotopes – which are incorporated into the proteins – whereas the other does not [133–136]. Again, the samples are mixed prior to protein identification. This approach was used in *P. falciparum*, to compare the effects of chloroquine and artemisinin [133]. In this case, gel electrophoresis was avoided entirely. Instead, multidimensional protein identification technology (MudPIT) was used: an unfractionated digested protein mixture was separated by biphasic liquid chromatography and analyzed in tandem mass spectrometry. 1253 *P. falciparum* proteins were identified. In total, 22 proteins were changed after chloroquine and 23 after artemisinin treatment [133]. In both cases, just under half of the identified proteins have no known function. SILAC was also used to study the effects of amphotericin B and antimony on *Leishmania infantum* [135, 136]. No significant new information was obtained, but the antimony study at least identified the ABC transporter MRPA (the same as the *L. major* MDR1), which validates the technique [135].

Affinity Chromatography

Chemical proteomics or chemoproteomics are terms used to define a field of research in which a small molecule is chemically modified and attached to a solid support such as beads. Protein extracts are incubated with the beads, which are then extensively washed. Afterward, the attached proteins are eluted and identified by mass spectrometry [15, 81, 107, 137–140]. The method is simple in

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principle but can be limited in practice [81, 141]. First of all, the compound has to be chemically derivatized; this means using one of the chemical groups in the molecule to add a linker and bind it to a support. Adding groups to a molecule can be difficult when no suitable reactive groups are present [15, 81]. Moreover, changes in the molecule structure may affect the pharmacophore, influencing the specificity or even abolishing drug–protein interactions [142].

This approach was used to look for targets of the antimalarial quinoline drugs primaquine and hydroxychloroquine; ATP served as a control [143]. After incubation of cell extracts with the drugs, bound to sepharose, the matrix was washed. Then, the bound proteins were eluted using the relevant drug; in addition, elution with other quinolines, such as chloroquine, mefloquine, quinacrine, and quinine, were performed. Two erythrocyte proteins were found in the eluates: quinone reductase 2 (QR2) and aldehyde dehydrogenase 1 [143]. Interestingly, QR2 was potently inhibited *in vitro* by quinoline and inhibitors targeting QR2 specifically were lethal to the parasites, probably because of oxidative stress generation [143].

Derivatives of the natural compound naphthoquinone, active against *Trypanosoma brucei rhodesiense*, *L. major*, and *T. cruzi* [144], were also used as affinity probes. This led to the identification of two proteins, glycosomal glycerol kinase and glycosomal glyceraldehyde-3-phosphate dehydrogenase; both proteins were inhibited by the compound and especially the second one seems to be part of the multitarget effect characteristic of these molecules [140].

To avoid the pitfalls associated with derivatization, different derivatized molecules can be used in parallel and the results are compared [142]. When a trypanocidal compound (4-[5-(4-phenoxyphenyl)-2*H*-pyrazol-3-yl]morpholine) was used in this way, only one of the derivatized versions identified *T. brucei* adenosine kinase as a potential target. The authors validated the interaction of this compound with adenosine kinase using circular dichroism spectroscopy and isothermal titration calorimetry, based on the principle that a protein binding to its ligand will have higher thermal stability [142]. The substrate transformation kinetics indicated that the compound was limiting the substrate inactivation pathway. The authors speculated that the parasites died because deregulation of the enzyme activity disrupted purine interconversion [142].

Another possible problem is steric hindrance – beads preventing interactions or protein complexes blocking the pores [145]. To circumvent this, it is possible to label the compound with biotin, incubate the biotinylated drug with protein extracts, and then trap the complexes on streptavidin matrices [146]. Elution can be done with an excess of biotin; alternatively, the tag can be designed to be cleavable; in this way, mild conditions can be used to elute the proteins [146].

A further advancement is the application of click chemistry; small reactive groups are added to the chemical structure and the beads are linked through this reactive group only after the drug is already bound to the proteins [147–149]. This limits the influence of bulky chemical groups and beads. Click chemistry was used with an azide group linked to compounds that inhibit a major cysteine protease active in *T. cruzi* called *cruzain* – although they may well inhibit other

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cysteine proteases as well. Rather unsurprisingly, mammalian cathepsin B was identified as off-target [147].

The major issue in these approaches is the presence of a significant amount of proteins that are very abundant, prone to aggregation or stick to any matrix. This means that many of the proteins detected do not actually bind to the drug. Although this can be alleviated to some extent by changing linkers and matrices, and by including suitable negative controls, a core of contamination always persists [15, 145]. This can be defined through several independent and unrelated experiments, and lists of frequent contaminants can be generated and thus excluded from further considerations [81]. An excellent negative control is a matrix bearing an inactive version of the test compound; this can be processed in parallel with the active version [14]. However, the possibility that the inactive version can still bind to the target – even if it cannot inhibit it – cannot be ruled out [81]. A competition approach can also be used: a protein extract is first incubated with the drug in solution, to saturate the target-binding sites; afterward, the preabsorbed material is normally processed for target identification [18, 81, 141, 145]. The identified proteins are compared with those seen without preabsorption; the proteins present in a usual affinity purification experiment but missing from the competition experiment are more likely to be binding directly to the compound and not the matrix; therefore, they are candidate targets [147, 148, 150, 151].

Several additional factors can influence the likelihood of success of these approaches. The ability of the compound to select a protein target is optimal only if binding is stable and occurs rapidly with high affinity [14]. Some proteins may be inactivated by proteolysis during cell lysis, detergents may change protein conformation, membrane proteins may not be extracted at all [14, 81]. One option here is to use cross-linkers that bind the drug to the target before lysis [152]. Covalent attachment of drug to target has the additional virtue of allowing more stringent, and even denaturating, wash conditions [14, 15, 149].

A compound, ACT-213615, with low nanomolar activity against *P. falciparum*, was developed from a screen of molecules resembling aspartic protease inhibitors, but the MoA is unknown. A derivative that retains activity was made with a photosensitive group and biotin label [14]. The compound was added to infected erythrocytes, activated, and then pulled down from cell lysates using streptavidin beads. Proteins identified using liquid chromatography coupled with tandem mass spectrometry included the well-known multidrug resistance protein 1 (PfMDR1) [153]. The derivatized molecule was also used for cell imaging, since the biotin label could be detected using fluorescent probes [14].

Activity-Based Probes

Activity-based probes are drugs that are modified to react, not only with the solid support but also with an amino acid in the active site of the target protein [137,

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141, 154]. Such probes can only be made if the target is already thought to belong to a specific protein class, such as protein kinases [148, 151] or proteases [150].

Hypothemycin is a fungal natural product that inhibits some human kinases through covalent binding to an active site thiol [155, 156]. It can also kill trypanosomes [148]. Coupling this compound to a matrix permitted purification of a subset of *T. brucei* kinases [148]; it is not yet known which of these are responsible for the *in vivo* effect. A similar approach was used to identify unknown proteins, mostly kinases, using staurosporine as an ATP-like competitive inhibitor; addition of a photoreactive group and biotin tag allowed purification of 70 *L. donovani* ATP-binding proteins [151]. K11777 is a cysteine protease inhibitor [157]; an activity-based probe based on it was used in *T. brucei*, and it identified previously expected targets, such as rodhesain and cathepsin B, but also other interesting candidates such as proteasome components [150]. Visualization of the K11777 probe was shown to be possible: interestingly, in procyclic forms, the probe was equally distributed throughout the parasite, with no specific localization, whereas in bloodstream forms, it was mainly found in lysosomes [150].

Label-Free Approaches

Recently, new approaches have been developed using the original compound, without derivatization [18, 137]. It is hoped that in this way, artifacts will be reduced.

One of these techniques is the target identification using drug affinity responsive target stability (DARTS) [158]. This approach is based on the concept that binding of a small molecule to a protein will make the protein less flexible and therefore more resistant to proteolysis [137]. Cell extracts with and without drug are treated with limited proteolysis and then subjected to liquid chromatography coupled with mass spectrometry. For example, α-ketoglutarate extends the lifespan of *Caenorhabditis elegans*; ATP synthase subunit β was shown to be more protease resistant in the presence of α-ketoglutarate [159]. The method was recently used to study a novel anticancer compound, called RX-5902, and as a result, p68 RNA helicase was found to be the target, inhibiting the ATPase activity depending on β-catenin [160].

Another recently developed approach focuses on changes in stability under oxidative stress. It is called stability of proteins from rates oxidation (SPROX) [161]. Proteins or mixture of proteins are placed in the presence of a fixed amount of oxidant and increasing amounts of chemical denaturant. The more denatured the protein becomes, the more amino acids become accessible to oxidation. The oxidized residues are then detected by mass spectrometry [161]. In the presence of a ligand that stabilizes the protein folding, less oxidation occurs [161]. In a proof of concept study, yeast extracts were incubated with cyclosporin A and hydrogen peroxide, to identify proteins whose oxidation was affected by the drug [162]. Ten proteins were found to be changed and two known targets of cyclosporin A, cyclophilin A and UDP-glucose-4-epimerase, were among them [162].

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These techniques are relatively new, and to our knowledge, they have not been applied yet to the study of drugs against parasites, but we think they could be useful as complementary approaches.

Proteome Display

Protein collections of various sorts can be assayed directly for drug binding. This can be especially useful if the target of interest is of low abundance or is unstable *in vivo* [137]. Although cDNAs can be used for this, the proteins produced may be truncated or contained undesired sequences at their N-termini [163]. The ideal way to generate proteome arrays is to clone intact open reading frames. Various methods are available for protein library display. In one, using DNA templates, the mRNAs are transcribed and translated *in vitro*; they can be assayed either on microtiter plates or on solid supports [117]. Phage display technology is another possibility; in this case, the open reading frames are fused to gene encoding a phage coat protein. Each phage displays one unique protein. After affinity purification using the compound of interest, the phage can be amplified and rescreened to enhance the enrichment [164].

Metabolomics

To determine the effect of drug treatment on metabolism, presence and concentration of many metabolites (<1400 Da) can be measured by mass spectrometry or nuclear magnetic resonance (NMR) [16, 165–167]. This provides more immediate insights into cellular homeostasis than analyses of DNA, RNA, and proteins [168–172]. The technology has yet not been used extensively in parasite drug target identification [112], although it has been used to define metabolic pathways [168, 173]. NMR detects molecules unequivocally and quantitatively, but has low sensitivity [166]. On the other hand, mass spectrometry has very high sensitivity and can identify more than a thousand different compounds in one sample, but it is less quantitative [16, 166]. It has been suggested that a combination of MS and NMR is likely to be most powerful [166, 169]. Normally, cell extracts are fractionated by liquid or gas chromatography prior to the MS analyses in order to reduce complexity. Normal-phase liquid chromatography is used to resolve polar compounds, reverse-phase columns are used for nonpolar molecules [169], gas chromatography is used for volatile metabolites [166], and capillary electrophoresis is the method used for the analyses of polar or volatile compounds [170, 174].

Targeted metabolomic experiments are hypothesis-driven, aiming to quantify a defined subset of metabolites [167]. Atovaquone inhibits the cytochrome b/c complex of *P. falciparum*, and mutations in cytochrome b result in resistance [106]. Inhibition of cytochrome b/c causes loss of membrane potential and organelle dysfunction; but the lethal result is the lack of production of intermediates for pyrimidine synthesis [175]. Quinolone lead compounds with antimalarial

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activity targeting the bacterial-like type II NADH:ubiquinone oxidoreductase were also found to affect cytochrome b/c [175]. At various times after drug addition, 35 key metabolites were monitored by LC-MS and the disruption of pyrimidine metabolism was confirmed: accumulation of dihydroorotate and a reduction of downstream intermediates [175]. Similarly, fosmidomycin was thought to target *Plasmodium* deoxyxylulose 5-phosphate reductoisomerase, an enzyme involved in the production of isoprenoids [176, 177]. Five metabolites in this pathway were quantified, and it was found that in fact another enzyme, methylerythritol phosphate cytidylyltransferase, was inhibited [177]. This result prompted further investigation, and indeed, overexpression of methylerythritol phosphate cytidylyltransferase conferred resistance to fosmidomycin [177].

If drug targets are not known, untargeted metabolomics can be used to identify candidate target pathways [167]. Taking samples at different times is important to characterize the drug effect and use of different drug combinations can also be informative. The effects of nifurtimox and DFMO on trypanosomes were studied as a proof of concept [112]. Since DFMO blocks polyamine biosynthesis, and therefore also affects trypanothione production, it was expected to cause oxidative stress. Nifurtimox, meanwhile, was believed to generate oxidative stress. Unexpectedly, the two drugs were found to be antagonistic. The authors suggested that this was because DFMO is cytostatic, while nifurtimox is active only on proliferating cells [112]. As expected, DFMO affected mainly the polyamine biosynthetic pathway [112], although a decrease in phospholipids was also detected [112]. Nifurtimox, on the other hand, caused an increase in nucleotides and nucleobases: it was speculated that this was a consequence of DNA and RNA degradation [112]. The drug combination did not result in any additional changes [112].

Both benznidazole and nifurtimox are used to treat *T. cruzi* and are thought to have similar modes of action: activation by type 1 NTR and subsequent oxidative stress [107, 178]. Metabolomic analysis of *T. cruzi* resulted in identification of about 1000 metabolites [107]. Benznidazole caused small differences, mostly in trypanothione metabolism, supporting the oxidative stress MoA; as a bonus, some benznidazole metabolites were detected [107]. An NMR metabolomic study of *T. cruzi* treated with plant extracts focused on the culture medium: a mitochondrial reductase was proposed as a target [179]. In contrast, an untargeted study of *L. infantum* promastigotes treated with miltefosine yielded so many differences in the metabolome that no obvious primary targets could be postulated; there were alterations in internal lipid metabolism and increases in alkanes, sugars, and nucleotides. The results suggested that miltefosine is characterized by multiple effects, which might include an increase in oxidative stress [110]. To increase sensitivity, it is also possible to combine different chromatographic methods [170]. This was employed to further study the MoA of miltefosine; it was showed that metabolites of the arginine and polyamine pathways in *Leishmania* were affected. The authors suggested that this supported the idea that the MoA

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relates to an increase in oxidative stress [111]. The effect of miltefosine on *L. donovani* was the subject of a study that focused specifically on phospholipids, sterols, and fatty acids [180]. The authors observed a significant decrease in the amount of phosphatidylcholine and increases in lysophosphatidylcholine and phosphatidylethanolamine [180]. Lysophosphatidylcholine is thought to arise by degradation of phosphatidylcholine.

Sitamaquine is a possible drug against visceral leishmaniasis [181]. Sitamaquine is positively charged and therefore cannot cross membranes, but it was found to interact with negatively charged phospholipids. It was suggested that after this interaction, an aromatic moiety of sitamaquine can insert into the membrane [182]. Resistant cells did not show a decrease in positively charged phospholipids, which meant that reduced uptake via the proposed mechanism could not explain the resistance [183]. Nevertheless, a strong impact of the drug on lipid metabolism was seen, supporting the idea that the drug acts on such pathways: decreased sterols, reduced phosphatidylcholine, and accumulation of phosphatidylethanolamine [183].

Metabolomics is relatively fast [111] and can be used to compare many different strains and conditions. It is, however, often very difficult to distinguish specific alterations from the background of nonspecific effects that reflect growth inhibition or general metabolic disruption. Changes in oxidative stress-related molecules and lipids are a recurring theme. For example, the metabolomes of three genetically rather similar *L. donovani* strains with different levels of antimonial resistance [184] were studied in different growth and drug pressure conditions [171]. The results were rather inconclusive, although resistance appeared to be related to better protection against oxidative stress and higher membrane fluidity [171]. Another study of *L. donovani* resistant to various drugs revealed several common adaptations, such as increased resistance to oxidative stress and changes in the saturation levels of glycerophospholipids [172].

Validation

All these approaches can result in the identification of different genes possibly involved in drug action. As we saw in many examples, hypotheses generated by one method need to be validated using others. Overexpression of a putative target protein or export transporter should increase the EC_{50} of the drugs [71, 100, 123]. If mutations are thought to influence drug action, the mutated gene can be expressed in the cells – or the wild-type gene replaced – to assess the effect on drug sensitivity [63, 65, 101]. Drug targets are expected to be essential, so downregulation should inhibit cell growth and increase drug sensitivity, and gene knockout should either cause severe growth inhibition or be impossible [123].

When an enzyme is suspected to be involved in drug action, effects of the drug on kinetics and binding to the enzyme can be tested [142]. If a transporter is

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suspected to be the route of entrance or to be blocked by a compound, channel inhibitors can be used to test this hypothesis, evaluating the influence on drug action [44].

Conclusions

Phenotypic drug discovery is now revealing new promising compounds to fight diseases caused by protists. Very often, nothing is known about their MoA. We surveyed different methods that have been used more or less successfully for drug target deconvolution. In some cases, one method was enough to identify specific targets or causes of resistance. However, in many cases, the MoA is complex and several approaches are needed. There is no golden rule about which is the best method to be used, every drug is different and has different effects on the cells. Therefore, the strategy chosen to assess the MoA needs careful consideration and fine-tuning.

Abbreviations

AAT1	Amino acid transporter 1
AAT6	See TbAAT6
ABC	ATP-binding cassette
ABCG1	ATP-binding cassette subfamily G member 1
AQP1	Aquaglyceroporin 1
AQP2	Aquaglyceroporin 2
AQP3	Aquaglyceroporin 3
AT1	See AT1
cAMP	Cyclic AMP
CARP	cAMP response proteins
CARP1	cAMP response protein 1
cDNA	Complementary DNA
CNVs	Copy number variations
cPRS	Cytosolic proline aminoacyl-tRNA synthetase
DARTS	Drug affinity responsive target stability
DFMO	Difluoromethylornithine or eflornithine
DHFR	Dihydrofolate reductase
DNDi	Drugs for neglected diseases initiative
EC_{50}	Half maximal effective concentration
GWAS	Genome-wide association study
ISG75	Invariant surface glycoprotein of molecular weight 75 kDa
iTRAQ	Isobaric Tag for relative and absolute quantitation
kDNA	Kinetoplast DNA
LC	Liquid chromatography mass spectrometry
LdMT	Miltefosine transporter in *L. donovani*

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MCP14	Mitochondrial carrier protein 14
MDR1	Multidrug resistance protein 1
MMV	Medicines for Malaria Venture
MoA	Mechanism of action
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MRPA	Multidrug resistance protein A
MS	Mass spectrometry
MT	See LdMT
MudPIT	Multidimensional protein identification technology
NMR	Nuclear magnetic resonance
NTR	Nitroreductase
PfATP4	P-type cation-transporter ATPase4 in *P. falciparum*
PfCRT	Chloroquine resistance transporter in *P. falciparum*
PfMDR1	Multidrug resistance protein 1 in *P. falciparum*
PfNHE	Sodium/hydrogen ion exchanger in *P. falciparum*
PfUT	HECT ubiquitin protein ligase in *P. falciparum*
PGPA	*Leishmania* ABC transporter
PROSC	Proline synthetase co-transcribed homolog
QR2	Quinone reductase 2
QTL	Quantitative trait locus
RNAi	RNA interference
RNAseq	RNA high-throughput sequencing
SILAC	Stable isotope labeling by amino acids in cell culture
SNPs	Single nucleotide polymorphisms
SPROX	Stability of proteins from rates of oxidation
TbAAT6	Amino acid transporter gene in *T. brucei*
TbAT1	Adenosine transporter 1 in *T. brucei*
TCA	Trichloroacetic acid
TcABCG1	ABCG1-like transporter in *T. cruzi*
TRiC	TCP-1 Ring Complex
tRNA	Transfer RNA
UBP1	Ubiquitin-specific protease 1

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2
Antiparasitics from Algae

*Stefan Ringgeler and Barbara Kappes**

Abstract

In the past years, the drug discovery programs of the scientific community have shifted from screening of purely synthetic drugs enabled by combinatory chemistry to natural compounds. The latter regained the center stage for drug discovery strategies because of their chirality, which renders them most versatile to interact with biological targets. In line with this paradigm shift, the efforts to identify antiparasitics from marine sources have increased tremendously during the last years and resulted in the discovery of some promising candidates. In this chapter, we summarize the current data on antiparasitic activities of extracts and compounds from macro- and microalgal species and discuss genus- and species-specific effects on particular parasites. Parasitic species being addressed are (i) *Plasmodium falciparum*, the causative of malaria tropica, (ii) the kinetoplastidal parasites, *Trypanosoma brucei rhodesiense*, *T. brucei* gambiense, *T. cruzi* and *Leishmania* sp. causing African sleeping sickness, Chagas disease, and leishmaniasis, respectively, (iii) the anaerobic protozoan parasites *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis*, the pathogens of the enteric infections amoebiasis and giardiasis and of the sexually transmitted trichomoniasis, and (iv) parasitic worms such as *Brugia malayi*, a causative of lymphatic filariasis.

Introduction

Recent trends in drug discovery research from natural sources have spotted the marine environment as the largest source of biodiversity and, as a consequence, as one of the most promising sources of novel bioactive compounds with a broad spectrum of biological activities such as antibiotic, antifungal, antiviral, antiparasitic, anthelmintic, anticoagulant, antidiabetic, antimitotic, antineoplastic, and anti-inflammatory [1, 2]. A comparative analysis by Kong *et al*. emphasizes the superiority of marine natural products in terms of chemical novelty when

*Corresponding author.

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compared to terrestrial natural products [3]. The exploration of marine organisms for pharmaceutical and medical applications has procured important chemical candidates for the discovery of new compounds against major tropical diseases, such as malaria, neglected tropical diseases such as the African sleeping sickness, Chagas disease, and leishmaniasis, enteric infections such as amoebiasis and the neglected giardiasis, stimulating further investigations into this topic.

This book chapter describes the main substances and extracts with antiparasitic activities biosynthesized from macro- and microalgae and compiles the relevant information. Algae survive in a highly competitive environment [4]. This setting led to the development of a high level of diversity in chemical structures of numerous metabolic pathways as defense strategies not being found in terrestrial environments. This book chapter is structured in a way that active substances or algae extracts from macroalgae are discussed in the context of the major parasite and/or parasite groups. *Plasmodium falciparum* as the causative of malaria is dealt with in a separate section. Information on the kinetoplastidal parasites *Trypanosoma brucei* sp. to which the causatives of African sleeping sickness belong, on *Leishmania* sp., the causatives of leishmaniasis, and on *Trypanosoma cruzi*, the pathogen responsible for Chagas disease, is reviewed in the next section. This part is followed by a section on the anaerobic parasites discussing available data for *Entamoeba histolytica*, the causative of amoebic dysentery, *Giardia lamblia*, the most prevalent diarrheal pathogen worldwide, and the sexually transmitted parasite *Trichomonas vaginalis*, the pathogenic agent of trichomoniasis. The limited information on helminths is compiled in the final section. Microalgae are addressed separately in the final paragraph.

Plasmodium falciparum

Malaria is one of the most important infectious diseases worldwide. Globally, about 3.3 billion people are at risk of being infected with malaria and developing disease, and 1.2 billion are at high risk, meaning a >1 in 1000 chance of getting malaria in a year [5]. According to the latest WHO estimates, 198 million cases of malaria occurred globally in 2013, resulting in 584 000 deaths. Sub-Saharan Africa carries the heaviest burden with an estimated 90% of all malaria deaths, affecting children under the age of 5. Although there exist five *Plasmodium* species that cause malaria in humans, *P. falciparum* is the causative of malaria tropica, the most severe form of malaria and, further, the malaria parasite with the highest prevalence worldwide. Therefore, it is not surprising that all data on antiplasmodial activity of algae extracts or compounds in the literature deal with this parasite species.

Several compounds with an antiplasmodial activity have been isolated from diverse algae species [6]. An aromatic compound (*p*-hydroxybenzaldehyde) with a half maximal inhibitory concentration (IC_{50}) in the low micrograms per milliliter range (IC_{50}'s of 7.3 and 5.6 μg/ml against the *P. falciparum* strains D6 and W2) has been isolated from the red alga *Laurencia papillosa* (Table 2.1)

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Table 2.1 Algae-derived products evaluated for antiparasitic activity.

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Macroalgae (phylum/class)						
Red algae (Rhodophyta/Florideophyceae)						
Amphiroa crassa	Corallinales	δ-Tocopheryl-hydroquinone	*P. falciparum*	220 µM	—	[7]
		δ-Tocopheryl-quinone		10 µM	—	
Asparagopsis armata	Bonnemaisoniales	EtOH-Hex:EtOAc	*L. donovani* p.	10 µg/ml	—	[8]
Asparagopsis taxiformis		EtOH-Hex:EtOAc	*L. donovani* p.	14 µg/ml	—	[8]
Bostrychia tenella	Ceramiales	Hexane fraction H02	*T. cruzi* t.	15.8 µg/ml	—	[9]
		Hexane fraction H02	*L. amazonensis* p.	1.5 µg/ml	—	
		Hexane fraction H03		2.7 µg/ml	—	
		DCM	*T. cruzi* t.	19.1 µg/ml	—	
		DCM fraction D01	*L. amazonensis* p.	4.4 µg/ml	—	
		DCM fraction D02		4.3 µg/ml	—	
Botryocladia leptopoda	Rhodymeniales	95% EtOH	*A. vitae*	62.5 µg/ml – LC_{100}	—	[10]
			L. sigmodontis	31.3 µg/ml – LC_{100}	—	
			B. malayi	125 µg/ml – LC_{100}	—	
		n-Hexane fraction	*A. vitae*	62.5 µg/ml – LC_{100}	—	
			L. sigmodontis	62.5 µg/ml – LC_{100}	—	
			B. malayi	31.3 µg/ml – LC_{100}	—	
		$CHCl_3$ fraction	*B. malayi*	31.3 µg/ml – LC_{100}	—	

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Calliblepharis jubata	Gigartinales	EtOAc (10%)	*P. falciparum*	5 µg/ml	14	[11]
			T. brucei rh.	23.3 µg/ml	3.1	[12]
Callophycus serratus		Callophycol A	*P. falciparum*	35.7 µM	—	[13]
		Bromophycoic acid C		8.7 µM	>4.0	[14]
		Bromophycolide A		0.5 µM	—	[15]
		18 Acetylbromo-phycolide A		0.24 µM	—	[15]
		Bromophycolide D		0.3 µM	—	[16]
		Bromophycolide E		0.8 µM	—	[16]
		Bromophycolide M		0.5 µM	—	[16]
Ceramium rubrum	Ceramiales	MeOH	*P. falciparum*	16.13 µg/ml	>5.6	[17]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	27.15 µg/ml	>3.3	
			L. donovani a.	16.76 µg/ml	>5.4	
Ceramium virgatum		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	5.4 µg/ml	>16.7	
			L. donovani a.	25.6 µg/ml	>3.5	
Chondria atropurpurea		Chondriamide A	*N. braziliensis*	0.26 mM – EC_{80}	—	[19]
		Chondriamide C		0.09 mM – EC_{80}	—	
		3-Indoleacryl-amide		2.37 mM – EC_{80}	—	
		Chondriamide A derivative		0.3 mM – EC_{80}	—	

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Chondrus crispus	Gigartinales	EtOAc (10%)	*P. falciparum*	2.9 µg/ml	29.0	[11]
			T. brucei rh.	13.6 µg/ml	6.2	[12]
Chylocladia verticillata	Rhodymeniales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	13.9 µg/ml	>6.5	
			L. donovani a.	47.3 µg/ml	>1.9	
Claviclonium ovatum	Gigartinales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	14.3 µg/ml	>6.3	
			L. donovani a.	61.2 µg/ml	>1.5	
Corallina granifera	Corallinales	MeOH	*P. falciparum*	16.26 µg/ml	>5.5	[17]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	41.41 µg/ml	>2.2	
			L. donovani a.	35.02 µg/ml	>2.6	
Corallina officinalis		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	4.8 µg/ml	>18.5	
			L. donovani a.	22.7 µg/ml	>3.9	
Dasya pedicellata	Ceramiales	MeOH	*P. falciparum*	0.38 µg/ml	38.7	[17]
			T. cruzi t.	62.02 µg/ml	0.2	
			T. brucei rh.	0.37 µg/ml	39.7	
			L. donovani a.	23.04 µg/ml	0.6	
Dilsea carnosa	Gigartinales	EtOAc (10%)	*P. falciparum*	3.9 µg/ml	19.0	[11]
			L. donovani a.	9.5 µg/ml	7.8	
			T. brucei rh.	15.3 µg/ml	4.8	[12]
Eucheuma isiforme		DCM:MeOH (7 : 3)	*T. vaginalis*	13 µg/ml	1293	[20]
Furcellaria lumbricalis		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	12 µg/ml	>7.5	
			L. donovani a.	43.3 µg/ml	>2.1	

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Gelidium crinale	Gelidiales	MeOH	*P. falciparum*	19.48 µg/ml	>4.6	[17]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	36.25 µg/ml	>2.5	
			L. donovani a.	19.95 µg/ml	>4.5	
Gelidium latifolium		EtOAc (10%)	*P. falciparum*	3.4 µg/ml	18.0	[11]
			T. brucei rh.	20.5 µg/ml	3.0	[12]
Gelidium pulchellum		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	8.1 µg/ml	>11.1	
			L. donovani a.	32.5 µg/ml	>2.8	
Gracilaria caudata	Gracilariales	DCM:MeOH (7 : 3)	*T. vaginalis*	7.9 µg/ml	20	[20]
Gracilaria corticata		EtOH	*L. major* p.	37.5 µg/ml	—	[21]
		hot H_2O	*L. major* p.	≤38 µg/ml	—	[22]
Gracilaria cervicornis		DCM:MeOH (7 : 3)	*T. vaginalis*	10.5 µg/ml	4.6	[20]
Gracilaria damaecornis		DCM:MeOH (7 : 3)	*T. vaginalis*	3.6 µg/ml	274.9	[20]
Gracilaria gracilis		EtOAc (10%)	*P. falciparum*	3.3 µg/ml	21	[11]
			T. brucei rh.	21.5 µg/ml	3.3	[12]
Gracilaria salicornia		Hot H_2O	*L. major* p.	≤46 µg/ml	—	[22]
Gracilaria sp.		DCM:MeOH (7 : 3)	*T. vaginalis*	5.6 µg/ml	14	[20]
Gracilaria verrucosa		EtOH:H_2O (3 : 1)	*P. falciparum*	5.6 µg/ml	—	[23]

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Grateloupia turuturu	Halymeniales	EtOAc (10%)	*P. falciparum* *T. brucei* rh.	3.1 µg/ml 10.8 µg/ml	23 6.6	[11] [12]
Halopitys incurvus	Ceramiales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 8.1 µg/ml 16.5 µg/ml	n.d. >11.1 >5.5	[18]
Halurus flosculosus		EtOAc (10%)	*P. falciparum* *T. brucei* rh.	4.6 µg/ml 22.4 µg/ml	12 2.6	[11] [12]
Heterosiphonia gibbesii		DCM:MeOH (7 : 3)	*T. vaginalis*	4.8 µg/ml	1090	[20]
Hypnea espera	Gigartinales	EtOH:H_2O (3 : 1)	*P. falciparum*	8.9 µg/ml	—	[23]
Jania rubens	Corallinales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 15.8 µg/ml 60.7 µg/ml	n.d. >5.8 >1.5	[18]
Laurencia corallopsis	Ceramiales	EtOH	*P. falciparum*	14.8 µg/ml	—	[24]
Laurencia dendroidea		Elatol	*T. cruzi* e. *T. cruzi* t. *T. cruzi* a. *L. amazonensis* p. *L. amazonensis* a. *L. amazonensis* p. *L. amazonensis* a.	45.4 µM 1.38 µM 1.01 µM 4.0 µM 0.45 µM 9.7 µg/ml 4.5 µg/ml	— 20 26.7 0.35 3.1 11.6 25.1	[25] [26] [27]
		Obtusol	*L. amazonensis* p. *L. amazonensis* a.	6.2 µg/ml 3.9 µg/ml	21.5 34.2	[27]
Laurencia microcladia		DCM:MeOH (7 : 3)	*L. mexicana* p.	16.3 µg/ml	—	[28]

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Laurencia papillosa		*p*-Hydroxybenzaldehyde	*P. falciparum*	7.3 µg/ml (D6) 5.6 µg/ml (W2)	>2.7 >3.6	[29]
Laurencia obtusa		(8*R**)-8-bromo-10-*epi*-β-snyderol	*P. falciparum*	2.7 µg/ml (D6) 4.0 µg/ml (W2)	— —	[30]
Laurentia pinnatifida		EtOH	*L. major* p.	6.25 µg/ml	—	[21]
Laurencia scoparia		DCM	*N. braziliensis*	<100 µM (EC_{50})	—	[31]
		β-Bisabolene		111 µM (EC_{50})	—	[32]
Mastocarpus stellatus	Gigartinales	EtOAc (10%)	*P. falciparum*	2.8 µg/ml	>30	[11]
			T. brucei rh.	19.5 µg/ml	3.5	[12]
		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[18]
			T. brucei rh.	11.1 µg/ml	>8.1	
			L. donovani a.	44.1 µg/ml	>2.0	
Melanothamnus afaqhusainii	Ceramiales	EtOH	*L. major* p.	32.6 µg/ml	—	[21]

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Osmundea pinnatifida		EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 8.6 µg/ml 32.7 µg/ml	n.d. >10.5 >2.8	[18]
Polyides rotundus	Gigartinales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 12.9 µg/ml 57.3 µg/ml	n.d. >7.0 >1.6	[18]
Porphyra linearis	Ceramiales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 9.4 µg/ml 55.5 µg/ml	n.d. >9.6 >1.6	[18]
Scinaia furcellata	Nemaliales	EtOH	*P. falciparum* *T. cruzi* t. *T. brucei* rh. *L. donovani* a.	41.3 µg/ml >90 µg/ml 17.9 µg/ml 64.4 µg/ml	>2.2 n.d. >5 >1.4	[33]
Scinaia hatei		EtOH	*L. major* p.	14.1 µg/ml	—	[21]
Scinaia indica				59.6 µg/ml	—	
Brown algae (Ochrophyta/Phaeophyceae)						
Bifurcaria bifurcata	Fucales	Eleganolone EtOAc (10%) EtOAc (10%) EtOAc:MeOH (1 : 1)	*P. falciparum* *P. falciparum* *L. donovani* a. *T. brucei* rh. *T. cruzi* t. *T. brucei* rh. *L. donovani* a.	7.9 µM >5 µg/ml 3.8 µg/ml 0.5 µg/ml 34.7 µg/ml 1.9 µg/ml 6.4 µg/ml	21.6 >1.2 1.6 12.4 0.9 17.2 5.1	[12] [11] [12] [34]
Canistrocarpus cervicornis	Dictyotales	(4*R*,9*S*,14*S*)-4α-Acetoxy-9β,14α-dihydroxydolast-1(15),7-diene	*L. amazonensis* p. ax. a. int. a.	2.0 µg/ml 12 µg/ml 4.0 µg/ml	93 — —	[35]

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Chorda filum	Laminariales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 8.5 µg/ml 21.1 µg/ml	n.d. >10.6 >4.3	[34]
Cystoseira baccata	Fucales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	79.3 µg/ml 8.8 µg/ml 15.7 µg/ml	>1.1 >10.2 >5.7	[34]
Cystoseira barbata		MeOH	*P. falciparum* *T. cruzi* t. *T. brucei* rh. *L. donovani* a.	18.32 µg/ml >90 µg/ml 35.21 µg/ml 23.46 µg/ml	>4.9 n.d. >2.6 >3.8	[17]
Cystoseira tamariscifolia		EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	61.0 µg/ml 6.6 µg/ml 19.6 µg/ml	1.0 9.5 3.2	[34]
Desmarestia menziesii	Desmarestiales	Desmarestial	*N. braziliensis*	60 µg/ml (EC_{50})	18	[36]
Dictyopteris polypodioides	Dictyotales	EtOAc (10%)	*L. donovani* a.	10.8 µg/ml	8	[11]
Dictyota caribaea		DCM:MeOH (7 : 3)	*L. mexicana* p. *T. vaginalis*	24.4 µg/ml 5.4 µg/ml	— 97	[28] [20]
Dictyota dichotoma		EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 11.8 µg/ml 42.4 µg/ml	n.d. >7.6 >2.1	[34]

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		EtOAc (10%)	*P. falciparum*	3.1 µg/ml	9.0	[11]
			L. donovani a.	8.8 µg/ml	3.0	
			T. brucei rh.	5.8 µg/ml	4.8	[12]
Dictyota mertensii		DCM:MeOH (2 : 1)	*L. amazonensis* p.	71.6 µg/ml	3.3	[37]
Dictyota pfaffii		Dolabelladienetriol	*L. amazonensis*	44 µM	—	[38]
Fucus ceranoides	Fucales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	10.2 µg/ml	>8.8	
			L. donovani a.	25.3 µg/ml	>3.6	
Fucus serratus		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	14.8 µg/ml	>6.1	
			L. donovani a.	34.1 µg/ml	>2.6	
Fucus spiralis		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	7.2 µg/ml	>12.5	
			L. donovani a.	34.3 µg/ml	>2.6	
Fucus vesiculosus		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	8.9 µg/ml	>10.1	
			L. donovani a.	33.0 µg/ml	>2.7	
Halidrys siliquosa		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	34.6 µg/ml	1.3	[34]
			T. brucei rh.	1.2 µg/ml	37.5	
			L. donovani a.	8.6 µg/ml	5.2	
Himanthalia elongata		EtOAc (10%)	*P. falciparum*	3.5 µg/ml	25	[11]
			T. brucei rh.	30.3 µg/ml	2.9	[12]
Lobophora variegata	Dictyotales	CH_3Cl	*G. lamblia*	0.5 µg/ml	175	[39]
		EtOAc		0.8 µg/ml	625.1	
		n-Hexane		1.0 µg/ml	65	
		CH_3Cl	*E. histolytica*	6.2 µg/ml	14.1	

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
		EtOAc		1.7 µg/ml	294.2	
		n-Hexane		2.8 µg/ml	23.2	
		CH_3Cl	*T. vaginalis*	3.7 µg/ml	23.6	
		EtOAc		11.7 µg/ml	42.7	
		n-Hexane		13.4 µg/ml	4.9	
		SQDG[b)]	*G. lamblia*	20.0 µg/ml	4.1	
			E. histolytica	3.9 µg/ml	21.9	
			T. vaginalis	8.0 µg/ml	10.7	
		DCM:MeOH (7 : 3)	*T. vaginalis*	1.3 µg/ml	328	[20]
Padina perindusiata		DCM:MeOH (7 : 3)	*T. vaginalis*	7.7 µg/ml	794	[20]
Padina sp.		Acetone	*L. braziliensis* a.	40 µg/ml	7.5	[40]
Pelvetia canaliculata	Fucales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	10.8 µg/ml	>8.3	
			L. donovani a.	35.7 µg/ml	>2.5	
Saccorhiza polyschides	Tilopteridales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	10.7 µg/ml	>8.4	
			L. donovani a.	31.8 µg/ml	>2.8	
Sargassum fluitans	Fucales	DCM:MeOH (7 : 3)	*T. vaginalis*	2.8 µg/ml	39.5	[20]
Sargassum heterophyllum		Sargaquinal	*P. falciparum*	2.0 µM	11	[41]
		Fucoxanthin	*P. falciparum*	1.5 µM	54	

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Sargassum muticum		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	10.8 µg/ml	>8.3	
			L. donovani a.	34.7 µg/ml	>2.6	
		EtOAc (10%)	*P. falciparum*	2.9 µg/ml	11	[11]
Sargassum natans		EtOH	*P. falciparum*	18.1 µg/ml	>5	[33]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	>7.4 µg/ml	>12.2	
			L. donovani a.	90.9 µg/ml	1	
Stypocaulon scoparium	Sphacelariales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[34]
			T. brucei rh.	12.1 µg/ml	>7.4	
			L. donovani a.	30.4 µg/ml	>3.0	
Turbinaria turbinata	Fucales	DCM:MeOH (7 : 3)	*L. mexicana* p.	10.9 µg/ml	—	[28]
		DCM:MeOH (7 : 3)	*T. vaginalis*	3.4 µg/ml	6.8	[20]
Green algae (Chlorophyta/Ulvophyceae)						
Anadyomene saldanhae	Cladophorales	Acetone	*L. braziliensis* a.	24 µg/ml	12.3	[40]
Chaetomorpha indica		MeOH:H_2O (3 : 1)	*P. falciparum*	26.73 µg/ml	—	[42]
Caulerpa faridii	Bryopsidales	EtOH	*L. major* p.	34 µg/ml	—	[21]
Caulerpa racemosa		EtOH	*L. major* p.	37.5 µg/ml	—	[21]
		MeOH	*P. falciparum*	3.12 µg/ml	>28.8	[17]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	29.51 µg/ml	>3.0	
			L. donovani a.	22.66 µg/ml	>4.0	

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Table 2.1 (*continued*)

Species	Order	Investigated product	Organism	IC_{50}	SI[a)]	References
Cladophora rupestris	Cladophorales	EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	80.8 µg/ml 3.7 µg/ml 20.2 µg/ml	>1.1 >24.3 >4.5	[43]
Codium bursa	Bryopsidales	MeOH	*P. falciparum* *T. cruzi* t. *T. brucei* rh. *L. donovani* a.	1.38 µg/ml >90 µg/ml 21.36 µg/ml 31.71 µg/ml	>65.2 n.d. >4.2 >2.8	[17]
Codium flabellatum		EtOH	*L. major* p.	34.0 µg/ml	—	[21]
Codium fragile ssp. *tomentosoides*		EtOAc:MeOH (1 : 1)	*T. cruzi* t. *T. brucei* rh. *L. donovani* a.	>90 µg/ml 8.9 µg/ml 16.6 µg/ml	n.d. >10.1 >5.4	[43]
Penicillus dumetosus		DCM:MeOH (7 : 3)	*L. mexicana* p. *T. vaginalis*	≥100 µg/ml 2.9 µg/ml	— 21.3	[28] [20]
Penicillus lamourouxii		DCM:MeOH (7 : 3)	*L. mexicana* p. *T. vaginalis*	≥100 µg/ml 7.6 µg/ml	— 8.9	[28] [20]
Udotea conglutinata		DCM:MeOH (7 : 3)	*L. mexicana* p. *T. vaginalis*	≥100 µg/ml 1.6 µg/ml	— 29.8	[28] [20]

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Udotea flabellum		DCM:MeOH (7 : 3)	*L. mexicana* p.	≥100 µg/ml	—	[28]
			T. vaginalis	7.7 µg/ml	7.4	[20]
Ulva intestinalis	Ulvales	EtOAc:MeOH (1 : 1)	*T. cruzi* t.	>90 µg/ml	n.d.	[43]
			T. brucei rh.	11 µg/ml	>8.2	
			L. donovani a.	14.9 µg/ml	>6	
Ulva lactuca		EtOH	*P. falciparum*	48.8 µg/ml	>1.8	[33]
			T. cruzi t.	>90 µg/ml	n.d.	
			T. brucei rh.	22.3 µg/ml	>4	
			L. donovani a.	5.9 µg/ml	>15.3	
		EtOAc:MeOH (1 : 1)	*T. cruzi* t.	34.9 µg/ml	>2.6	[43]
			T. brucei rh.	18 µg/ml	>5.0	
			L. donovani a.	12 µg/ml	>7.5	
Microalgae						
Dinoflagellate (Miozoa/Dinophyceae)						
Amphidinium sp.	Gymnodiniales	Karatungiol A	*T. foetus*	1 µg/ml	—	[44]

Abbreviations for the various parasite forms: promastigotes (p.), trypomastigotes (t.), amastigotes (a.), epimastigotes (e.), axenic amastigotes (ax. a), and intracellular amastigotes (int. a.). *T. brucei rhodesiense* is abbreviated as (rh.).

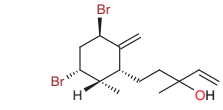
a) Selectivity index (SI) defined as the ratio of cell cytotoxicity to parasite cytotoxicity; n.d. = not determinable.

b) SQDG = sulfoquinovosyl diacylglycerol (mixture of 1-*O*-palmitoyl-2-*O*-myristoyl-3-*O*- (major compound), 1,2-di-*O*-palmitoyl-3-*O*- (minor compound), and 1-*O*-palmitoyl-2-*O*-oleoyl-3-*O*-(6‴-sulfo-α-D-quinovopyranosyl)-glycerol (minor compound)).

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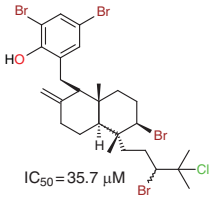
[29]. Brominated sesquiterpenes have been purified from a chloroform/methanol extract of a further member of the genus *Laurencia*, *L. obtusa*. Of these, only (8*R**)-8-bromo-10-*epi*-*β*-snyderol showed a significant antimalarial activity with an IC_{50} of 2.7 μg/ml against the strain D6 and an IC_{50} of 4.0 μg/ml against the *P. falciparum* strain W2 (Table 2.1, Figure 2.1) [30]. A further member of the Rhodophyta, the Fijian red alga *Callophycus serratus*, has turned out to be a rich source for antimalarial agents. Compounds isolated from this alga include the callophycoic acids (A–H), the callophycols (A and B), and the bromophycolides (A–I, J–Q, R–U) [13, 16, 45]. The callophycoic acids and the callophycols were the first examples of diterpene–benzoic acids (C27) and diterpene–phenols (C26, halogenated) isolated from macroalgae. They exhibit only a moderate antimalarial activity in a medium micromolar range with callophycol A being the most effective compound exhibiting an IC_{50} of 35.7 μM (Table 2.1; Figure 2.1) [13]. In a later publication, bromophycoic acid C was found to be more potent against *P. falciparum* with an IC_{50} of 8.7 μM. It is the first nonmacrocyclic diterpene–benzoate metabolite showing an appreciable activity against the malaria parasite (Table 2.1; Figure 2.1) [14]. In contrast to the nonmacrocyclic diterpene–benzoate, the macrocyclic metabolites, bromophycolides A to U, isolated from the same algal species exhibited activities against *P. falciparum* in the low micromolar range with the exception of bromophycolide C and K [16, 46, 45]. Five out of the seventeen bromophycolides even had improved IC_{50} values in the nanomolar range lying between 0.3 and 0.9 μM (Table 2.1; Figure 2.1) [16]. Investigations on compounds lacking the bromide atoms have so far not given a decisive clue whether, and if yes to which extent, bromide is required for the antimalarial activity [7]. To study the mode of action of bromophycolides, bromophycolide A (IC_{50} of 0.5 μM) was acetylated at the C18 phenol (Figure 2.1). The acetylated form of bromophycolide A was even more effective than its parent compound exhibiting an IC_{50} of 0.24 μM (Table 2.1). It colocalized with a neutral lipid stain, suggesting that bromophycolides may target heme biomineralization inside the neutral lipid nanospheres [15, 47]. Bromophycolide A and its acetylated form, 18-acetylbromophycolide A, inhibited heme polymerization with an IC_{50} value of 2.5 and 2.0 M equiv. of compounds to heme (IC_{50} value of amodiaquine was 1.2), respectively, further substantiating a possible function in the inhibition of heme biomineralization [15]. Spectrophotometric and circular dichroism spectroscopic analyses of the interaction of bromophycolide A with heme suggest a hydroxy group coordination to the ion center of heme [15]. Trials to identify possible protein targets of bromophycolide A were not successful [15]. The molecular structure of bromophycolides differs significantly from that of other inhibitors of heme biomineralization such as the well-known natural compounds quinine and artemisinin as well as the xanthones and terpene isonitriles isolated from marine sponges indicating that the bromophycolides are a novel class of therapeutic motifs exploitable for the design of heme biomineralization inhibitors [15, 48].

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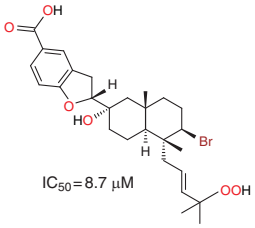
IC_{50} = 2.7 μg/ml

(8*R**)-8-Bromo-10-*epi*-β-snyderol



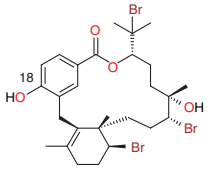
IC_{50} = 35.7 μM

Callophycol A



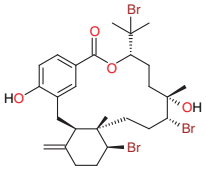
IC_{50} = 8.7 μM

Bromophycoic acid C



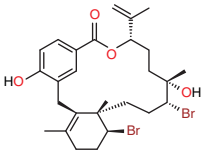
IC_{50} = 0.5 μM

Bromophycolide A



IC_{50} = 0.3 μM

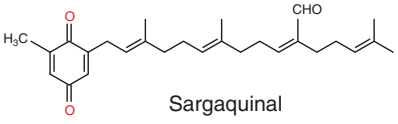
Bromophycolide D



IC_{50} = 0.5 μM

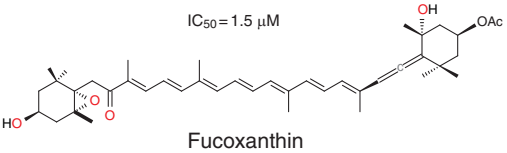
Bromophycolide M

IC_{50} = 2.0 μM



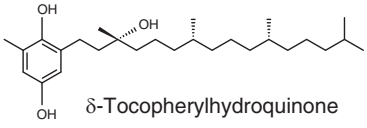
Sargaquinal

IC_{50} = 1.5 μM



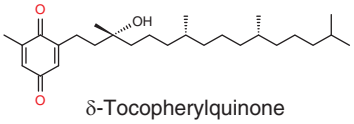
Fucoxanthin

IC_{50} = 220 μM



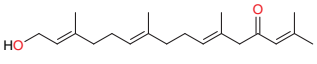
δ-Tocopherylhydroquinone

IC_{50} = 10 μM



δ-Tocopherylquinone

IC_{50} = 7.9 μM



Eleganolone

Figure 2.1 Marine natural products with antiplasmodial activity. IC_{50} values are given next to the chemical formula.

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Another source for antimalarials is the brown alga *Sargassum heterophyllum* [41]. Four active compounds have been isolated from organic extracts of *S. heterophyllum*. Three of them have been identified as tetraprenylated toluquinols and one as fucoxanthin, a carotenoid pigment. The most active compounds are sargaquinal and fucoxanthin with IC_{50} values of 2.0 and 1.5 μM and a selectivity index (SI) of 11 and 54, respectively (Table 2.1; Figure 2.1) [41]. Although it has been discussed that the antiplasmodial activities of these compounds may be related to their antioxidant properties, it would also be conceivable that they interfere with other parasite processes, for example, carotenoid biosynthesis [49].

Two known tocopherols, β-tocopherylhydroquinone and δ-tocopherylhydroquinone, were isolated from the red alga *Amphiroa crassa* [7]. Although their antimalarial activity was negligible, the oxidation of the hydroquinone moiety of δ-tocopherylhydroquinone to quinone in δ-tocopherylquinone resulted in a 20-fold increase of the antimalarial activity, suggesting that the quinone is crucial for the antimalarial activity of the tocopherol-related compounds (Table 2.1; Figure 2.1) [7]. Unfortunately, the authors did not have enough material of β-tocopherylhydroquinone to investigate whether the oxidation of this substance would result in a similar increase in antimalarial activity [7]. A quinone structure similar to that of δ-tocopherylquinone is also present in sargaquinal (Figure 2.1) [41].

Furthermore, the diterpene, eleganolone, from the brown alga *Bifurcaria bifurcata* has been reported to have antimalarial activity with an IC_{50} of 7.9 μM and a selectivity index of 21.6, using the rat myoblast cell line L6 (Table 2.1; Figure 2.1). Eleganolone has been isolated by bioassay-guided fractionation of the ethyl acetate extract of *B. bifurcata* using a polarity gradient [12].

To identify macrophyte extracts with antiprotozoal activity, Orhan and coworkers screened ethanolic extracts of the marine brown algae *Halopteris scoparia*, *Dictyota dichotoma*, and *Sargassum natans*, the marine red alga *Scinaia furcellata*, the marine green alga *Ulva lactuca*, and the freshwater green alga *Cladophora glomerata* for their antiprotozoal activities on *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Leishmania donovani*, and *P. falciparum* [33]. Antimalarial activity of these extract was found to be in the medium micromolar range (18.1 (*S. natans*; (Table 2.1)) to >50 μg/ml). Ethanolic extracts of the marine green alga *U. lactuca* and the freshwater green alga *C. glomerata* were found to inhibit the recombinant FabI enzyme of *P. falciparu*m, which is crucial for type II fatty acid biosynthesis during sporozoite development in the midgut oocyst [50]. IC_{50} values for FabI inhibition were 1.0 and 4.0 μg/ml for the extracts of *C. glomerata* and *U. lactuca*, respectively [33].

In the following part, screens of macroalgae for antiplasmodial activity are reported. A large-scale screen has been performed by Vonthron-Sénécheau and coworkers with 20 seaweed species, 8 being brown, 9 red, and 3 green algae, collected from rocky habitats along the Normandy coast of France [11]. Ethyl acetate (EtOAc (10% w/v)) and hydroalcoholic (70% EtOH) extracts were prepared and tested against *P. falciparum* erythrocytic blood stages, *L. donovani* amastigotes and *T. cruzi* trypomastigotes. While the hydroalcoholic extracts were mainly

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inactive at concentrations of 1.6 and 9.7 μg/ml, most of the EtOAc extracts were active. In this section, only the antiplasmodial activities are discussed, whereas the trypanocidal effects will be discussed in the chapter on Kinetoplastida. Briefly, half of the ethyl acetate extracts exhibited activities against *P. falciparum* in the low micrograms per milliliter range. The EtOAc extract of *Mastocarpus stellatus* had the best efficacy with an IC_{50} of 2.8 μg/ml followed by *Chondrus crispus* and *Sargassum muticum* with IC_{50} of 2.9 μg/ml, *D. dichotoma* and *Grateloupia turuturu* with IC_{50} of 3.1 μg/ml, *Gracilaria gracilis* with an IC_{50} of 3.3 μg/ml, *Gelidium latifolium* with an IC_{50} of 3.4 μg/ml, *Himanthalia elongata* with an IC_{50} of 3.5 μg/ml, *Dilsea carnosa* with an IC_{50} of 3.9 μg/ml, *Halurus flosculosus* with an IC_{50} of 4.6 μg/ml, and *Calliblepharis jubata* with an IC_{50} of 5 μg/ml (Table 2.1) [11]. The IC_{50} values of these extracts are in similar range as the ethanolic crude extracts of *Artemisia annua* (qīnghāo) and of *Azadirachta indica* (Neem) [51, 52]. *Plasmodia* contain a rudimentary plastid, the apicoplast that originates from secondary endosymbiosis through the engulfment of a red algal species by a free-living progenitor of the Apicomplexa [53]. Interestingly, the red algal extracts were the ones with the highest antiplasmodial activity, leading the authors to speculate that algal compounds of these extracts inhibit parasite growth by interfering with common pathways or processes [11].

As part of the continuous investigations of the Tasdemir group on the antiparasitic activity of macroalgae, Turkish seaweeds (two green, three brown, and six red algae) from the Marmara, Black, Aegean, and Mediterranean Seas were examined for antiprotozoal activity against *P. falciparum*, *T. brucei rhodesiense*, *T. cruzi*, *and L. donovani* [17]. Most extracts exhibited antimalarial activity. The most potent ones were the extracts of *Dasya pedicellata* (IC_{50} 0.38 μg/ml), *Codium bursa* (IC_{50} 1.38 μg/ml), and *Caulerpa racemosa* (IC_{50} 3.12 μg/ml) (Table. 2.1). Four additional extracts had IC_{50} values below 20 μg/ml with the *Ceramium rubrum* extract showing an IC_{50} of 16.13 μg/ml, the *Corallina granifera* extract with an IC_{50} of 16.26 μg/ml, the *Cystoseira barbata* (CybB-I) with an IC_{50} of 18.32 μg/ml, and the *Gelidium crinale* extract with an IC_{50} of 19.48 μg/ml (Table 2.1). The antiparasitic activity on the Kinetoplastida is described in the respective paragraph on this parasite group (see below).

In addition to the conventional approaches for the identification of novel marine natural products being based on extract preparation of single species, a high-throughput, high-content live-cell imaging approach was applied by Cervantes and coworkers, during which 2685 crude extracts from marine organisms including red, green, and brown macroalgae, cyanobacteria, cnidarians, and sponges collected from the Fiji Islands were investigated for novel antimalarial compounds [54]. Of the initial library, 27 extracts inhibited parasite growth at concentrations below 11 μg/ml. These extracts were submitted to a second high-content live-cell imaging screen enabling the evaluation of parasitemia, parasite morphology, and parasite transcriptional activity as well as the determination of toxic effects on host erythrocyte morphology. In fact, 10 of the 27 extracts had only a limited toxicity on the host erythrocyte. Of these, three were extracted from the red alga *C. serratus* containing the bromophycolides A–U as antiplasmodial compounds

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and exhibited IC_{50} values of 0.68–0.92 µg/ml [16, 46, 45, 54]. The two most potent extracts originated, however, from a cyanobacterium and from a sponge [54].

Further, data on the algal extracts from *Gracilaria verrucosa* and *Hypnea espera* with antimalarial activity are included in Table 2.1 without further description in the text, since only the extracts with the best efficacy are discussed [23]. The review by Garcia and Monzote provides more details on less effective extracts and on extracts of algal species that did not show any activity [6].

Kinetoplastida – *Trypanosoma brucei sp.*, *Trypanosoma cruzi*, and *Leishmania* sp.

Kinetoplastida are a widespread and very important group of obligatory protozoan parasites. At least during one stage in their life cycle, all members develop into slender and highly flexible cells equipped with one or two flagella, arising from a prominent flagellar pocket. The major human diseases caused by this class of pathogens include sleeping sickness and Chagas disease caused by species of the *Trypanosoma* and leishmaniasis caused by species of *Leishmania*.

T. brucei rhodesiense from East Africa and *Trypanosoma brucei gambiense* from West Africa are the causatives of human African sleeping sickness. Trypanosomiasis is a fatal disease that is largely neglected, affecting a million people in Africa [55]. *T. brucei* sp. is transmitted to humans by tsetse flies. *T. cruzi* is the causative of Chagas disease, which is endemic in 21 countries of Latin America with millions of people at risk. It is transmitted to humans by bloodsucking insects but can also be acquired by blood transfusion and organ transplantation. Leishmaniasis is a sandfly-borne infection that affects 14 million people worldwide. The most severe form of the disease is visceral leishmaniasis (kala-azar), which is caused by *L. donovani* and, if left untreated, can be fatal [56]. Therapy for these diseases remains a major challenge, since available drugs are outdated, have many drawbacks such as severe side effects, and are complicated to administer (i.e., daily parenteral administration) or are inadequate in chronic phases [57, 58]. Further resistance to the current drugs has become a major problem so that novel, effective drugs that are safe are urgently required.

There are only a few reports on isolated compounds with antikinetoplastidal activity. The sesquiterpene elatol has been isolated from the Brazilian red seaweed *Laurencia dendroidea* displaying antitrypanosomal activities against *T. cruzi* epimastigotes, trypomastigote, and amastigotes forms in the low micromolar range with IC_{50} of 45.4, 1.38, and 1.01 µM, respectively (Table 2.1, Figure 2.2) [25]. This is a remarkable efficacy in view of the fact that most algal extracts do not inhibit *T. cruzi* growth (the further screens discussed next and in Table 2.1). Elatol treatment of the typical elongated epimastigote and trypomastigote forms of *T. cruzi* resulted in dramatic morphological changes and the appearance of aberrant-shaped cells. All forms when treated contained swollen mitochondria and numerous cytoplasmic vacuoles [25]. Further investigations on the mode of action of elatol revealed depolarization of the mitochondrial membrane, increase

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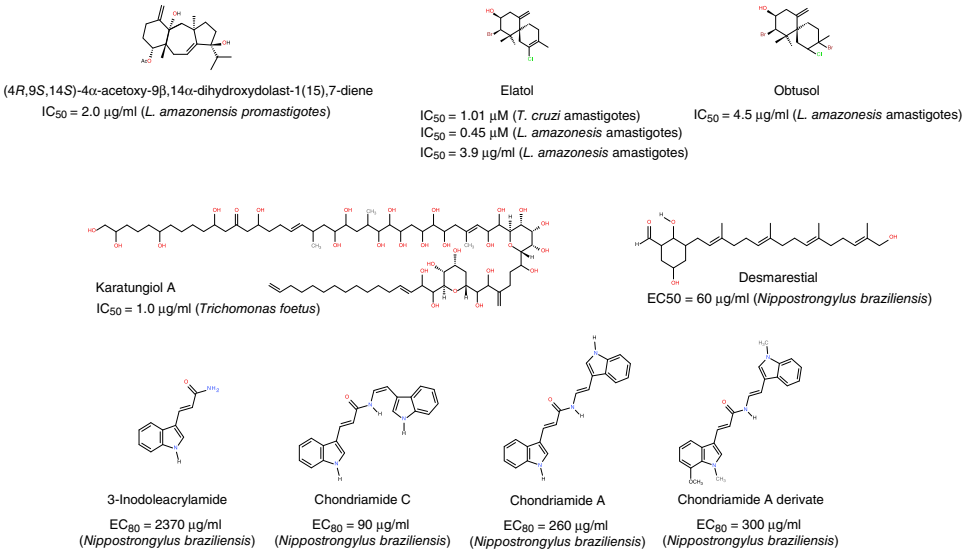


Figure 2.2 Marine natural products with antileishmanial, trypanocidal, trichomonacidal, and anthelmintic activities. IC_{50} values are given below the chemical formula.

in the formation of mitochondrial superoxide anions, and a concomitant loss of cell membrane and DNA integrity [59]. In addition, the formation of autophagic vacuoles and a decrease in cell volume were observed, leading the authors to hypothesize that mitochondrial dysfunction upon elatol treatment induces an increase in the production of reactive oxygen species through the electron transport chain that destabilizes the cell membrane and affects DNA integrity, finally culminating in different types of parasite cell death [59]. Elatol was also active against another member of the Kinetoplastida, namely *Leishmania amazonensis*, with IC_{50} of 4.0 and 0.45 µM for the promastigote and amastigote forms, respectively (Table 2.1, Figure 2.2, [26]). The lethal effects of elatol on the parasite were comparable to those observed for *T. cruzi* and were characterized by a pronounced swelling of the mitochondrion, appearance of concentric structures within the organelle, the destabilization of the plasma membrane, and formation of membranous structures extending from the endoplasmic reticulum being suggestive for an autophagic process [26]. In a parallel study, elatol and a related sesquiterpene obtusol, which also has been isolated from *L. dendroidea*, were tested for antileishmanial activity (Figure 2.2) [27]. In this study, the differences in the antileishmanial activities of elatol on pro- and amastigotes with IC_{50} values of 9.7 and 4.5 µg/ml were less pronounced (roughly twofold) when compared to the ones reported by Dos Santos *et al.*, where the difference was about 10-fold [26]. Obtusol exhibited a comparable antileishmanial activity with IC values of 6.2 versus 3.9 µg/ml on pro- and amastigotes, respectively [27]. Taken together, these data support elatol and obtusol as potent agents against *T. cruzi* trypomastigote

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and amastigote and leishmanial promastigote and amastigote forms with a high potential for antikinetoplastidal chemotherapies.

The next compound, the chemical structure of which has been resolved, is the 4-acetoxydolastane diterpene, (4*R*,9*S*,14*S*)-4α-acetoxy-9β,14α-dihydroxydolast-1(15),7-diene. This compound was obtained from the brown alga *Canistrocarpus cervicornis* (formerly *Dictyota cervicornis*) and tested for its inhibitory potential on *L. amazonensis* promastigotes, axenic amastigotes, and intracellular amastigotes exhibiting IC_{50} values of 2.0, 12.0, and 4.0 μg/ml, respectively, and a selectivity index of 93.0 against promastigotes [35]. To isolate this compound, a dichloromethane (DCM) crude extract of *C. cervicornis* was subjected to a silica gel chromatography. Fractions eluted with DCM were further subjected to a second silica gel chromatography and eluted with *n*-hexane/EtOAc (3 : 1), increasing the polarity until 100% EtOAc was reached. Fractions were analyzed by thin-layer chromatography, combined and separated on a third silica column with *n*-hexane/EtOAc (4 : 1), increasing the polarity until 100% EtOAc was obtained. One of the fractions contained the pure compound (4*R*,9*S*,14*S*)-4α-acetoxy-9β,14α-dihydroxydolast-1(15),7-diene [35]. This diterpene induced pronounced mitochondrial damage in *L. amazonensis* promastigotes, suggesting that it may interfere with the mitochondrial membrane potential and that it leads to lipid peroxidation in *L. amazonensis* amastigotes [35].

Further, dolabelladienetriol, a compound isolated from *Dictyota pfaffii*, inhibited the growth of *L. amazonensis* amastigotes with an IC_{50} value of 44 μM (Table 2.1). Although the selective index has not been determined, mouse peritoneal macrophages and human monocyte-derived macrophages were examined for mitochondrial cell damage, and in addition, the mouse macrophages were inspected for membrane integrity and decrease of phagocytosis [38]. None of these parameters was affected.

Orhan and coworkers examined the ethanolic extracts of marine macrophytes and of the freshwater green alga *C. glomerata* for their antiprotozoal activities on *T. brucei rhodesiense*, *T. cruzi*, and *L. donovani* [33]. Among these were extracts from the brown algae *H. scoparia*, *D. dichotoma* and *S. natans*, the red alga *S. furcellata,* and the green alga *U. lactuca*. None of these extracts exhibited activity against *T. cruzi*. All extracts were active against the trypomastigote form of *T. brucei rhodesiense* in the lower to medium micromolar range, from 7.4 to 43.9 μg/ml, with the extract of *S. natans* producing the best results (Table 2.1) [33]. Except for the extract of *U. lactuca*, antileishmanial activity on axenic amastigotes was not pronounced, ranging from IC_{50} values of 39.2 – 90.9 μg/ml. However, the *U. lactuca* extract exhibited a significantly stronger activity with an IC_{50} of 5.9 μg/ml (Table 2.1) [33].

In a survey by Freile-Pelegrin and coworkers, aqueous and organic extracts of 27 marine algae species (14 Rhodophyta, 5 Phaeophyta, and 8 Chlorophyta) collected from the Gulf of Mexico and the Caribbean coast of Yucatan were tested for their antileishmanial activity against promastigote forms of *Leishmania mexicana*, the form of the parasite that either actively invades host macrophages or granulocytes or is phagocytosed [28]. From the 27 algal extracts, only 4 showed antileishmanial

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activity with IC_{50} values ranging from 10.9 to 49.9 µg/ml. Extracts with the highest antileishmanial activity were obtained from the brown alga *Turbinaria turbinata*, red alga *Laurencia microcladia*, and brown alga *Dictyota caribaea* with IC_{50} values of 10.9, 16.3, and 24.4 µg/ml, respectively (Table 2.1) [28].

Ethyl acetate:methanol extracts (EtOAc:MeOH (1 : 1)) of four green algae (*Cladophora rupestris*, *Codium fragile* ssp. *tomentosoides*, *Ulva intestinalis*, and *U. lactuca*) collected from the Dorset area of England were tested for their antileishmanial and antitrypanosomal activities [43]. All extracts showed a cidal activity against *T. brucei rhodesiense*, with *C. rupestris* and *C. fragile* ssp. *tomentosoides* yielding the most active extracts with IC_{50} of 3.7 and 8.9 µg/ml, respectively (Table 2.1). All extracts showed a moderate antileishmanial activity with IC_{50} values ranging from 12.0 to 20.2 µg/ml. With IC_{50} values of 12.0 and 14.9 µg/ml, respectively, the *U. lactuca* and *U. intestinalis* extracts exhibited the highest antileishmanial activities (Table 2.1). The *U. lactuca* extract was the only extract with a mentionable activity against *T. cruzi* trypomastigotes (IC_{50} of 34.9 µg/ml) (Table 2.1). The same group of authors performed a second screen, where they tested the EtOAc:MeOH (1 : 1) extracts from 21 brown algae collected from the south coast of England and the west coast of Ireland for antileishmanial and antitrypanosomal activities [34]. All extracts exhibited a trypanocidal activity against *T. brucei rhodesiense* trypomastigotes with IC_{50} values ranging from 1.2 to 59.6 µg/ml. The two most potent extracts with IC_{50} of 1.2 – 1.9 µg/ml were obtained from the seaweeds *Halidrys siliquosa* and *B. bifurcata* (Table 2.1). Five extracts from *Chorda filum*, *Fucus spiralis*, *Fucus vesiculosus*, *Cystoseira baccata*, and *Cystoseira tamariscifolia* displayed IC_{50} values below 10 µg/ml, and seven extracts from *D. dichotoma*, *Fucus ceranoides*, *Fucus serratus*, *Pelvetia canaliculata*, *Saccorhiza polyschides*, *S. muticum*, and *Stypocaulon scoparium* displayed IC_{50} values between 10 and 15 µg/ml (Table 2.1). Only the four extracts from *H. siliquosa*, *B. bifurcata*, *Cystoseira tamariscifolia*, and *C. baccata* were active against the trypomastigote stage of the American trypanosome, *T. cruzi*, with moderate to low activities and IC_{50} values ranging from 34.6 to 79.3 µg/ml (Table 2.1). The four seaweeds that exhibit some trypanocidal activity against *T. cruzi* albeit a very low level belong to the Sargassaceae family. All extracts were active against *L. donovani* amastigotes. It is noteworthy that the seaweed extracts from *H. siliquosa and B. bifurcata*, which were most active against *T. cruzi*, were also most active against *L. donovani* amastigotes, however, with four- to fivefold higher IC_{50} values of 6.4 and 8.6 µg/ml (Table 2.1). Extracts of *C. baccata* (IC_{50} 15.7 µg/ml), *C. tamariscifolia* (IC_{50} 19.6 µg/ml), and *C. filum* (IC_{50} 21.1 µg/ml) displayed modest IC_{50} values (Table 2.1), whereas the IC_{50} values of the remaining extracts were all above 25 µg/ml with the majority between 33 and 44 µg/ml. Of the 21 tested extracts, four were active against the three kinetoplastida species investigated representing major members of the particular family. These extracts were from *H. siliquosa*, *B. bifurcata*, *C. tamariscifolia*, and *baccata*, with the *H. siliquosa* and *B. bifurcata* extracts being the most active ones in all the three parasite species tested [34].

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In a continuation of their research efforts, the group of Deniz Tasdemir analyzed the extracts of 23 red algae collected from England and Ireland for their antikinetoplastidal potential. All extracts inhibited the growth of *T. brucei rhodesiense* trypomastigotes with IC_{50} values varying from 4.8 to 57.6 µg/ml. The two most potent extracts with IC_{50} values of 5.4 to 4.8 µg/ml were obtained from the red algae *Ceramium virgatum* and *Corallina officinalis* (Table 2.1) [18]. Extracts from *Gelidium pulchellum*, *Halopitys incurvus*, *Osmundea pinnatifida*, and *Porphyra linearis* displayed IC_{50} values below 10 µg/ml, and extracts from *Chylocladia verticillata*, *Claviclonium ovatum*, *Furcellaria lumbricalis*, *Jania rubens*, *M. stellatus*, and *Polyides rotundus* displayed IC_{50}s between 10 and 16 µg/ml (Table 2.1). None of the extracts was active against the trypomastigote form of the American trypanosome, *T. cruzi*. Activities against *L. donovani* amastigotes ranged from 16.5 to >90 µg/ml and were except for *H. incurvus* in a moderate concentration range (Table 2.1).

The same group investigated the antiparasitic activity of Turkish seaweeds against *P. falciparum*, *T. brucei rhodesiense*, *T. cruzi*, *and L. donovani* as mentioned earlier [17]. All extracts (two green, three brown, and six red algae) were active against *T. brucei rhodesiense.* However, with the exception of the *D. pedicellata* extract, all activities were above 20 µg/ml (Table 2.1). The *D. pedicellata* extract was the most effective extract with an IC_{50} of 0.37 µg/ml against the African trypanosome. The same extract exhibited a weak activity against *T. cruzi* (IC_{50} of 62.02 µg/ml), although this value is clearly above the cytotoxicity (IC_{50} of 14.7 µg/ml) determined using rat skeletal myoblasts (L6 cells), which were used as host cells (Table 2.1). All extracts showed antileishmanial activity in the range from 16.76 µg/ml for the *C. rubrum* extract to 69.98 µg/ml.

In a screen performed by Vonthron-Sénécheau, which has already been reported in the *P. falciparum* section, no activity was detected against *T. cruzi* trypomastigotes and only four of the extracts showed activity against *L. donovani* amastigotes, with the *B. bifurcata* extract exhibiting an IC_{50} value of 3.8 µg/ml, the *D. dichotoma* extract an IC_{50} value of 8.8 µg/ml, the *D. carnosa* extract an IC_{50} value of 9.5 µg/ml, and the *Dictyopteris polypodioides* an IC_{50} value of 10.8 µg/ml [11]. However, a selectivity index of 1.6 points out a general toxicity of the *B. bifurcata* extract.

In a search for new antiprotozoal secondary metabolites from algae, Gallé and coworkers screened 35 polar and apolar extracts from 20 seaweed species from the Normandy coast for trypanocidal activity on trypomastigotes, the mammalian blood stage form of *T. brucei rhodesiense*. Activities (IC_{50}'s) ranged from 0.5 to 30.3 µg/ml with the ethyl acetate extract of the brown alga *B. bifurcata* being the most efficient one exhibiting a selectivity index of 12.4 (Table 2.1) [12]. A SI index above 10 was regarded as an indication for antiprotozoal activity and therefore not attributed to a possible cytotoxicity of the extract. Bioassay-guided fractionation using a polarity gradient on a silica gel resulted in five fractions, of which fraction 2 contained the same strong antitrypanocidal activity as the crude ethyl acetate extract of *B. bifurcata* with a little increase in selectivity to 15.4 [12]. The main constituent of this fraction was determined to be eleganolone (Figure 2.1), which,

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however, with an IC_{50} value of 13.7 µg/ml was significantly less active than fraction 2 (0.5 µg/ml), suggesting that either a minor compound of this fraction or a combination of compounds is required for the strong antitrypanosomal activity [12].

Further, data on the algal extracts from *Asparagopsis armata* and *Asparagopsis taxiformis*, *Bostrychia tenella*, *Codium flabellatum*, *Caulerpa faridii*, *C. racemosa*, *Gracilaria corticata* and *Gracilaria salicornia*, *Laurencia pinnatifida*, *Melanothamnus afaqhusainii, and Scinaia hatei* and *Scinaia indica* with trypanocidal and/or antileishmanial activities are included in Table 2.1 without further description in the text [8, 9, 21, 22]. Only the extracts with the best efficacy are added. In addition, algae extracts from 27 seaweed species (16 Rhodophyta, 7 Phaeophyceae, and 4 Chlorophyta) were assayed for antiprotozoal activity against *Leishmania braziliensis* pro- and amastigotes and *T. cruzi* epimastigotes [40]. Six of these extracts showed a promising growth inhibition of over 50% on *L. braziliensis* promastigotes. Only one of the extracts was effective against *T. cruzi* epimastigotes [40]. IC_{50} values were determined on *L. braziliensis* amastigotes for the extracts of *Anadyomene saldanhae* and *Padina* sp., which were in the range of 24 and 40 µg/ml, respectively. The reviews by Garcia and Monzote and Torres *et al.* provide more details on less effective extracts and on extracts of algae species that did not show activity [4, 6].

With some rare exceptions, evaluation of the antiprotozoal activities of algae extracts on leishmanial and trypanosomal species displayed the same picture. *T. brucei* sp. is the most susceptible parasite throughout the studies followed by the *Leishmania* species and *T. cruzi*, which is the least vulnerable representative (see Table 2.1). One could argue that the *T. brucei* species are more susceptible, since they are freely living forms in the bloodstream and liquor of the host not being sheltered by a host cell, and therefore are more easily accessible to drugs. However, when comparing the efficacy of the extracts on the intracellular amastigote forms of *T. cruzi* and of the *Leishmania* species with the efficacy on the free-living forms of these parasites within their human host, namely trypomastigotes and promastigotes, it is quite obvious that the majority of the extracts displayed a higher activity against the intracellular amastigotes invalidating the above raised line of argumentation (see Table 2.1). One possible explanation why *T. cruzi* is less sensitive than the other kinetoplastidal parasites could reside in its slower growth rates, which in turn is most likely reflected in the reduced metabolism rate compared to the other parasites of Kinetoplastida. This in turn would delay the effect of an active compound. Exceptions from the general susceptibility scheme *T. brucei* sp. < *Leishmania* sp. < *T. cruzi* in which *Leishmania* sp. swapped places with *T. brucei* sp. are reported by Suzgec-Selcuk *et al.* for the MeOH extract of *C. racemosa* (showing IC_{50} values of 29.51, 22.66, >90 µg/ml, respectively), *C. barbata* (with IC_{50} values of 35.21, 23.46, >90 µg/ml, respectively), *C. rubrum* (exhibiting IC_{50} values of 27.15, 16.76, >90 µg/ml, respectively), and *G. crinal*e (with IC_{50} values of 36.25, 19.95, >90 µg/ml, respectively) [17]. The listing of the IC_{50} values refers to the general susceptibility scheme *T. brucei* sp. < *Leishmania* sp. < *T. cruzi*.

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Anaerobic Protozoan Parasites – *Entamoeba histolytica, Giardia lamblia, and Trichomonas vaginalis*

The three anaerobic protozoan parasites *E. histolytica*, *G. lamblia,* and *T. vaginalis* are highly prevalent with a worldwide distribution. Despite major differences in the life cycle and pathogenic properties, they are usually grouped together since they lack a mitochondrion and share similarities in their carbohydrate metabolism [60]. Enteric infections induced by *G. lamblia* and *E. histolytica* are a significant cause of morbidity and mortality in developing countries. Typical signs of a giardiasis or amebiasis are diarrhea and abdominal symptoms including stomachache, cramps, bloating, and tenderness. *E. histolytica* is thought to be responsible for an estimated 50 000 000 diarrheal infections and 100 000 deaths per year [61]. *G. lamblia* is the most common intestinal parasite of humans in developed countries [62]. In Asia, Africa, and Latin America, there are about 200 million people with symptomatic giardiasis with roughly 500 000 new cases reported each year [63]. Trichomonas is supposed to be the most common, nonvirally sexually transmitted infection in the world [64]. Common sites of infections include the vagina, urethra, and endocervix, and the infections are associated with vaginitis, cervicitis, urethritis, prostatitis, epididymitis, cervical cancer, infertility, and pelvic inflammatory disease [65]. The WHO estimated 276.4 million cases in 2008 [64].

An investigation of selected tropical seaweeds (12 Rhodophyta, 5 Phaeophyta, and 8 Chlorophyta) from the coast of Yucatan revealed an unexpected high antitrichomonal activity in the low micrograms per milliliter range [20]. Due to the lack of data on antitrichomonal activities of natural product extracts in the literature, the authors defined IC_{50} values $\leq$5 μg/ml as high, IC_{50} values between 5.1 and 10 μg/ml as moderate, and IC_{50} values $\geq$10 μg/ml as low activity. Ten of the extracts corresponding to roughly 40% of the samples were highly active. The extracts of *Lobophora variegata*, *Udotea conglutinata*, *Sargassum fluitans, Penicillus dumetosus,* and *T. turbinata* were the most active ones with IC_{50} values of 1.3 μg/ml (SI 327.6), 1.6 μg/ml (SI 29.8), 2.8 μg/ml (SI 39.5), 2.9 μg/ml (SI 21.3), and 3.4 μg/ml (SI 6.8), respectively (Table 2.1) [20]. However, in this group, the extract with the highest selective index of 1090 has been obtained for *Heterosiphonia gibbesii* and exhibited an IC_{50} of 4.8 μg/ml. The extracts of *Bryothamnion triquetrum*, *Ceramium nitens*, *Gracilaria caudata*, *Gracilaria* sp., *Jania capillacea*, *D. caribaea*, *Padina perindusiata*, *Avrainvillea* cf *digitata*, *Halimeda tuna*, *Penicillus lamourouxii*, and *Udotea flabellum* were in the moderate activity range with *P. perindusiata* displaying the highest SI of 793.6 and an IC_{50} of 7.7 μg/ml [20]. Only three of the extracts from *Eucheuma isiforme*, *Gracilaria cervicornis*, and *Hydropuntia cornea* displayed a low antitrichomonal activity, of which the extract of *E. isiforme* with an IC_{50} of 13 μg/ml showed an impressive SI of 1293 on Madin–Darby canine kidney epithelial cells (MDCK) (Table 2.1) [20]. Interestingly, the authors investigated the species that belong to the same genus such as *U. conglutinata* and *U. flabellum*, *P. dumetosus* and *P. lamourouxii,* and *Gracilaria damaecornis*, *Gracilaria* sp., *G. caudata*, and *G. cervicornis* allowing

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for a comparison of species-specific activities in the genus context. For the pairs *U. conglutinata* and *U. flabellum* and *P. dumetosus* and *P. lamourouxii,* the IC_{50} values and selectivity indices correlate quite nicely within the genus context, being 1.6 μg/ml (SI 29.8) and 7.7 μg/ml (SI 7.4) for the *Udotea* species and 2.9 μg/ml (SI 21.3) and 7.6 μg/ml (SI 8.9) for the *Penicillus* species, implying that the active substance(s) within a given genus might be the same and that the differences observed in the inhibitory potential of the extracts may be due to variations in the concentration of the active compound(s) produced by the individual species. When comparing the extracts of the *Gracilaria* species, this assumption no longer holds true. Here, the extract of *G. damaecornis* (IC_{50} 3.6 μg/ml; SI 274.9) is strikingly different from that of the other species (*G. caudata* (IC_{50} 7.9 μg/ml; SI 20), *G. cervicornis* (IC_{50} 10.5 μg/ml; SI 4.6), *Gracilaria* sp. (IC_{50} 5.6 μg/ml; SI 14)) having a 13.8- to 60-fold higher selectivity index, and even among the others (*G. caudata*, *G. cervicornis*, and *Gracilaria* sp.), no correlation can be found, implying that these algal species may contain distinct active ingredients.

As a continuation of the study just described, extracts of the tropical brown alga *Lobophora variegate* were further examined for their inhibitory potential on the growth of the enteric parasites *G. lamblia* and *E. histolytica* and reexamined for the sexually transmitted parasite *T. vaginalis* [39]. The nonpolar chloroform, ethyl acetate, and hexane fractions exhibited a pronounced activity against *G. lamblia* with IC_{50} values of 0.5, 0.8, and 1.0 μg/ml and selectivity indices of 175, 625.1, and 65, respectively, using MDCK cells (Table 2.1). With IC_{50} values of 1.7 (EtOAc), 2.8 (hexane), and 6.2 μg/ml (CH_3Cl) and selectivity indices of 294.2, 23.2, and 14.1, activity against *E. histolytica* was less pronounced (Table 2.1). Weakest activities were found against *T. vaginalis*, where the whole extract showed the highest activity with an IC_{50} of 3.2 μg/ml [39]. The major constituent of the chloroform fraction was identified as 1-*O*-palmitoyl-2-*O*-myristoyl-3-*O*-(6‴-sulfo-α-D-quinovopyranosyl)-glycerol together with small amounts of 1,2-di-*O*-palmitoyl-3-*O*-(6‴-sulfo-α-D-quinovopyranosyl)-glycerol and a new compound identified as 1-*O*-palmitoyl-2-*O*-oleoyl-3-*O*-(6‴-sulfo-α-D-quinovopyranosyl)-glycerol [39]. The mixture of these three sulfoquinovosyldiacylglycerols (SQDGs) showed a good activity against *E. histolytica* with an IC_{50} of 3.9 μg/ml; a moderate activity against *T. vaginalis* with an IC_{50} of 8.0; and the least activity against *G. lamblia* with an IC_{50} of 20.9 [39]. The last finding is somehow puzzling, since the chloroform extract was most active against *Giardia*. Thus, it may be that it contains (an) additional compound/s responsible for the inhibition growth of *G. lamblia*.

Helminths

Helminths represent a huge group of multicellular worm-like organisms that are commonly known as *parasitic worms*. Helminths consist of the plathelminthes with the cestodes and trematodes as major groups, the Acanthocephala, the

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nemathelminthes with the nematodes, and the Annelida with ectoparasites such as leeches. Despite their enormous impact on health and child development, studies on the evaluation of the anthelminthic activities of algae extracts are rather rare [66].

Wuchereria bancrofti, *Brugia malayi*, and *Brugia timori* are the causatives of lymphatic filariasis also known as elephantiasis tropica in various tropical and subtropical regions in the world. *W. bancrofti* is the most common filarial parasite causing 90% of the infections. The worms are transmitted by the bites of infected mosquito. Infections are usually acquired during childhood and in many cases stay without symptoms. If disease develops, the infection damages the lymphatic systems and results in an enormous swelling of arms, legs, or genitals. About 120 million people are infected with filarial parasite, of which 40 million are disfigured and disabled by the infection. Roughly 1.23 billion people in 58 countries are living in endemic regions and require large-scale treatment as preventive chemotherapy [67].

To globally eradicate lymphatic filariasis, the antifilarials ivermectin (in Africa) and diethylcarbamazine (outside Africa) alone or in combination with albendazole are administered to all eligible people in an area or a region where the infection is endemic. Although the drugs are safe, they only kill the microfilariae form from the bloodstream, thereby preventing the spread of the parasite to its vector. In adult worms, ivermectin and diethylcarbamazine cause a temporary infertility of the female worm, but within 1 year, microfilariae return to levels high enough for transmission to continue [68]. Thus, the recommended large-scale treatment strategy, also called *preventive chemotherapy,* requires an annual dose for 4–6 years in succession to interrupt the transmission cycle [67].

Extracts of the red alga *Botryocladia leptopoda* were tested for antimicrofilarial activity against the animal filarial species *Litomosoides sigmodontis* and *Acanthocheilonema vitae* and the human parasite *B. malayi* [10]. The *n*-hexane and the chloroform fractions were most active against *B. malayi* exhibiting absolute lethal concentration values (LC_{100}) of 31.3 µg/ml, whereas the EtOH (95%) fraction was four times less active. However, this fraction proved to be the most active fraction against *L. sigmodontis* with an LC_{100} of 31.3 µg/ml followed by the *n*-hexane fraction with an LC_{100} of 62.5 µg/ml. Activities (LC_{100} values) of both fractions on *A. vitae* were 62.5 µg/ml. The only fraction being active against adult worms was the hexane fraction, which killed significant portions of *L. sigmodontis* and *A. vitae* adult worms. Treatment of *B. malayi* resulted in sterilization of 71.05% of the adult females [10].

Further studies have been performed using *Nippostrongylus braziliensis*, a test system for parasite production. Due to its quite simple life cycle, *N. braziliensis* is one of the most widely studied helminth parasites. It is a natural parasite of rats and closely related to human hookworms and prevalent livestock parasites [69]. In a systematic screen of marine organisms from the Uruguayan coast for anthelminthic activity, several novel chemical entities were identified from the red alga *Chondria atropurpurea* and the brown alga *Desmarestia menziesii* exhibiting anthelminthic activities [19, 36]. Desmarestial isolated from

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D. menziesii showed a moderate anthelminthic activity with an half maximal effective concentration (EC_{50}) of 60 µg/ml and an SI value of 18 using *Artemia salina* as cytotoxicity model (Table 2.1, Figure 2.2 [36]). New indole derivatives isolated from the red alga *C. atropurpurea* displayed a moderate anthelminthic activity with chondriamide C, a novel (bis)indole amide, exhibiting the highest activity with an EC_{80} value of 0.09 mM (Table 2.1, Figure 2.2) [19]. In addition, new sesquiterpene derivatives with moderate anthelmintic activities below 100 µM were purified from the red alga *Laurencia scoparia* [31]. Unfortunately, no precise activities were shown for the individual compounds. However, it is striking that the *Z*-isomer, (+)-3-(*Z*)-bromomethylidene-10β-bromo-β-chamigrene, is 30-fold more active than the *E*-isomer, (−)-3-(*E*)-bromomethylidene-10β-bromo-β-chamigrene [31]. The same group identified novel halogenated β-bisabolene sesquiterpenoids from the same algal species, which showed only a moderate anthelminthic activity with an EC_{50} of 111 µM [32].

Microalgae

Despite the considerable amount of work performed on macroalgae, microalgae have so far escaped the general awareness. To our knowledge, there is only one report dealing with the antiparasitic activity of these algae species. The respective study is on the compound karatungiol, which has been isolated from the dinoflagellate *Amphidinium* sp. living in symbiosis with an unidentified marine acoel flatworm. Karatungiol consists of a C96 linear chain with a ketone, 25 hydroxyl groups, and 2 tetrahydropyran rings (Figure 2.2) and exhibits an antiprotozoan activity against *Trichomonas foetus* of 1 µg/ml [44].

Conclusion

For some time, the deeper understanding on the pathogenic mechanisms of diseases and the development of high-throughput screening technologies shifted the attention of drug developers from natural products to purely synthetic drugs very often directed against a specific molecular target [70]. Combinatorial chemistry supported this development, allowing the fast supply of large compound libraries. However, most natural molecules such as DNA, proteins, lipids, carbohydrates, and nucleosides and a number of alkaloids and hormones are unique asymmetric molecules without a superimposable mirror image [71]. This so-called chirality defines the precise spatial arrangement of a given molecule or, in other words, its stereochemistry. Chiral molecules are uniquely predestined to interact with other chiral molecules. Thus, it is not astonishing that about 40% of all new medications originate from natural products or are designed upon natural products' structures [72]. Moreover, half of the 20 top-selling drugs that are based on chemicals and not on proteins are related to natural products [73], indicating that natural compounds especially with regard to their chiral

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structures (possessing a well-defined spatial organization) are superior to the combinatorial chemistry approach [70]. In line of this, 40% of the chemical scaffolds in the Dictionary of Natural Products occupy a chemical space that has never been seen before and that is not covered by synthetic compounds [74]. In the past years, the efforts on antiparasitic drug discovery from marine sources have increased significantly. Among the marine organisms, marine macroalgae, invertebrates, and microorganisms have been the focus of attention, and the respective studies provided some promising candidates for drug development [4, 6, 75]. With respect to algae species, extracts and pure compounds isolated from macroalgae have taken the center stage. These investigations resulted in some promising products demonstrating their potential as source for novel antiparasitics. Astonishingly, microalgae have been entirely neglected. The major focus of the drug discovery efforts from algae has concentrated on *P. falciparum*, the malaria pathogen, and on the neglected tropical diseases such as leishmaniasis, Chagas disease, and African trypanosomiasis caused by the Kinetoplastida. There exist only a very limited number of studies on the anaerobic parasites, *E. histolytica*, *G. lamblia*, and *T. vaginalis*, whereas helminth infections have almost been neglected so far. Summed up, the discovery of novel molecules with a high antiparasitic potential from algae is heading in the right direction; however, it has to be extended to the so far neglected parasite species, especially the enteric parasites and helminths, and to the underinvestigated microalgae species as source of novel antiparasitics.

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3
Contribution of Natural Products to Drug Discovery in Tropical Diseases

Frederick Annang, Olga Genilloud, and Francisca Vicente*

Abstract

Malaria, human African trypanosomiasis, Chagas and leishmaniasis are four protozoan tropical infectious diseases that remain a global public health problem with reports of millions of new infections and deaths per year in endemic countries. There is a huge deficit and an urgent need for safe and affordable potent novel drugs against these diseases since the existing drugs are out of date and have problems with parasite resistance, toxicity, and the ways in which they are administered. In the search for inspiring bioactive molecules to serve as templates for the development of the next generation antiprotozoal drugs, natural products remain the number one source of diversity and a highly underexplored chemical space. This review discusses recent collaborative and explorative efforts in terrestrial and marine natural product research that have led to the discovery of inspiring chemotypes such as flinderoles, discorhabdins, simalikalactones and lepadins as potent antiprotozoal scaffolds that could be developed into future drugs. Advancing collaborative research efforts in this area that leverage the technological advancements in microbiology, biotechnology, genomics, and high-throughput screening platforms will lead to the eventual harnessing of the full potential of natural products in response to the urgent medical needs in tropical diseases and beyond.

Introduction

Malaria, human African trypanosomiasis (HAT), Chagas, and leishmaniasis are four protozoan tropical infectious diseases that remain a global public health problem, with reports of millions of new infections and deaths per year in endemic countries [1]. Malaria alone causes over 1 million deaths per year, of which 85% involve children under 5 years of age and pregnant women in sub-Saharan Africa [1, 2]. The disease is caused by parasites of the genus *Plasmodium* and transmitted to humans when bitten by an infected female *Anopheles*

*Corresponding author.

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mosquito [2]. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* are the five main parasite species implicated in human malaria, and of these, *P. falciparum* is the most prevalent and most dangerous, accounting for 90% of all reported cases [3]. The two main strategies to control malaria involve parasite and vector chemotherapy as vaccines development have not been shown to efficiently prevent infection so far. These strategies are, however, limited by two main challenges: (i) parasite/vector resistance development and (ii) lack of novel chemical classes of compounds in the drug development pipeline. Recent reports of delayed parasite clearance against the World Health Organization (WHO)-recommended artemisinin-combination therapies along the Thai–Cambodia boarder make it a race against time in finding the next generation of antimalarial drugs [3]. HAT is a kinetoplastid disease caused by parasites of the genus *Trypanosoma*. Two different subspecies of the parasite cause two different forms of the disease in humans. *Trypanosoma brucei gambiense* causes the chronic West African HAT, which contributes to about 90% of all reported HAT cases and is localized to the Central-Western part of Africa, while the remaining 10% acute form is caused by *Trypanosoma brucei rhodesiense*, a parasite local to the Eastern and Southern areas of Africa [4]. The African continent is estimated to be losing a total of about US$ 1.5 billion in global annual revenue as a result of HAT-related mortality and morbidity in both humans and livestock [5]. Two stages of both the acute and chronic types of HAT exist. The first stage of *T. b. gambiense* HAT can be treated locally by intramuscular pentamidine and that of *T. b. rhodesiense* HAT can be treated with intravenous suramin [6, 7]. The second stage of a *T. b. gambiense* HAT can be treated by intravenous coadministration of nifurtimox–eflornithine combination therapy (NECT) [6, 7]. However, this drug is ineffective against the second stage of *T. b. rhodesiense* HAT, which has to be treated by intravenous administration of melarsoprol, a highly toxic drug with up to 9% drug-induced mortality due to reactive encephalopathies [6–8]. The drugs for treating HAT are expensive, logistically difficult to administer in very rural environments where they are most needed, and have highly undesirable side effects. Chagas (American trypanosomiasis) is a parasitic protozoal disease caused by the *Trypanosoma cruzi* that affects about 10 million people and is endemic in South and Central America [9]. It is estimated that 30–40% of the affected individuals will develop cardiomyopathies or mega digestive syndrome, which leads to 12 500 patient deaths per year [10]. About 25 million people are estimated to live in endemic areas and 200 000 new cases are reported annually [11]. The disease manifests in two forms, an asymptomatic short acute phase and a patent parasitemia, which when untreated further develops into a chronic phase decades later. Without any available vaccines for the prevention of Chagas, its chemotherapeutic intervention is limited to the two nonideal nitroheterocyclic compounds, benznidazole and nifurtimox, both requiring long treatment periods, frequent adverse side effects, poor activity against the chronic stage, and resistance problems [11].

Leishmaniasis is also a kinetoplastid disease that largely affects populations of the developing world. In humans, *Leishmania* spp. cause a variety of clinical

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diseases due to the ability of the organism to proliferate in deep tissue or close to the skin's surface at low temperatures. According to the WHO, leishmaniasis can be classified into four main forms: visceral leishmaniasis (mortal), cutaneous leishmaniasis (common form, skin lesions), mucocutaneous leishmaniasis (tissue destruction), and diffuse cutaneous leishmaniasis (skin lesions difficult to cure) [5]. Certain species of the parasite have been associated with different clinical forms of the disease. Historically, the chemotherapy of leishmaniasis has been based on the use of toxic heavy metals, particularly antimony compounds such as stibogluconate and meglumine antimoniate. Whenever these kinds of drugs are no longer effective, some others are used, including pentamidine and amphotericin B. These chemicals have to be injected and clinical care or hospitalization during treatment may be necessary due to possible side effects [12]; thus, other treatments are needed. While approximately 600 000 infections are officially reported each year, it is estimated that 2 million new cases occur annually and that 12 million people are currently infected worldwide [13].

Natural products represent one of the most diverse underexplored chemical space and have represented one of the major sources for unearthing potent bioactive compounds that have been used as drugs in human history [14–16]. An estimated 60% of commercially available drugs, including household names such as penicillin, camptothecin, paclitaxel, lovastatin, maytansine, silibinin, and reserpine, were originally obtained or inspired from natural product sources [14–16]. In fact, the history of antiprotozoal drug discovery is rife with natural products and derivatives such as quinine, chloroquine and its derivatives, artemisinin, hydroxynaphthoquinones, and doxycycline. As things stand now, however, there are no new compounds from natural product sources in preclinical or later stages of the drug development pipeline for any of the aforementioned diseases [2]. The explosion of high-throughput technology and its adaptation to natural product research have made it possible to screen and search for new antiprotozoal compounds from this source and to fill the gap left in drug development pipelines. A search through the literature brings up many metabolites from natural product sources that have exhibited powerful antiprotozoal activities but which, for various bottlenecks, have not yet progressed to viable hit-to-lead projects [1]. This review brings together interesting antiprotozoal chemical scaffolds from natural products isolated from plant and marine sources reported in the literature against *Plasmodia*, *Trypanosoma*, and *Leishmania* parasites (Table 3.1) and the prospects of some of these scaffolds becoming viable antiprotozoal drug development pipeline projects in the future.

Antiparasitic Natural Product Compound Classes

The use of traditional plant medicine for the treatment of tropical diseases and general fever extends to several countries on the African, American, and Asian continents [2, 70]. In addition, the marine environment also teems with both microorganisms including algae, bacteria, and fungi and marine invertebrates

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Table 3.1 Natural products with antiprotozoan parasitic activity.

Compound	Activity (IC_{50})				Source	References
	P. falciparum	*T. cruzi*	*T. brucei*	*L. donovani*		
Alkaloids						
Quinidine	—	—	0.8 μM	—	*Cinchona* sp.	[17]
Cinchonine	—	—	1.2 μM	—	*Cinchona* sp.	[17]
Quinine	—	—	4.9 μM	—	*Cinchona* sp.	[17]
Cinchonidine	—	—	7.1 μM	—	*Cinchona* sp.	[17]
Ancistroealaines A	—	—	5 μM		*Ancistrocladus ealaensis*	[18]
Ancistroealaines B	—	—	8 μM	—	*Ancistrocladus ealaensis*	[18]
Actinodaphnine	—	—	3.2 μM	—	*Cassytha filiformis*	[19]
Cassythine	—	—	6.0 μM	—	*Cassytha filiformis*	[19]
Dicentrine	—	—	14.6 μM	—	*Cassytha filiformis*	[19]
Isoborreverine	0.32 μM	—	—	—	*Flindersia acuminate*	[20, 21]
Dimethylisoborreverine	0.08 μM	—	—	—	*Flindersia amboinensis*	[20, 21]
Flinderole B	0.15 μM	—	—	—	*Flindersia amboinensis*	[20, 21]
Cassiarin A	0.02 μM	—	—	—	*Cassia siamea*	[22]
Prenylated hydroquinone	—	6.10 μg/ml	—	—	*Piper crassinervium*	[23]
8,9-*Z*-dehyropellitorine	—	—	2.0 μg/ml	—	*Achillea ptarmica* L.	[24]

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Pellitorine	3.3 μg/ml	—	—	—	*Achillea ptarmica* L.	[24]
Saropeptate	—	—	3.63 μM	—	*Zapoteca portoricensis* (Jacq) HM Hernández	[25]
Anabellamide	—	—	12.21 μM	—	*Zapoteca portoricensis* (Jacq) HM Hernández	[25]
Piperine	—	4.91 μM	—	—	*Piper* sp.	[26]
Discorhabdins A	0.053 μM	—	—	—	*Latrunculia* sp.	[27]
Discorhabdins C	2.8 μM	—	—	—	*Latrunculia* sp.	[27]
Dihydrodiscorhabdin	0.17 μM	—	—	—	*Latrunculia* sp.	[27]
Psammaplysin F	1.4 μM	—	—	—	*Hyattella* sp.	[28]
Psammaplysin G	0.87 μM	—	—	—	*Hyattella* sp.	[28]
Dispacamide B	1.34 μg/ml	—	—	—	*Axinella* and *Agelas* spp.	[29]
Spongiacidin B	1.09 μg/ml	—	—	—	*Axinella* and *Agelas* spp.	[29]
Bromopyrrolohomoarginin	20 μg/ml	—	—	—	*Axinella* and *Agelas* spp.	[29]
(+)-8-Hydroxymanzamine A (+)-Manzamine A (+)-8-Hydroxymanzamine A hydrochloride (+)-Manzamine A hydrochloride	0.006–0.036 μg/ml	—	—	0.006–0.036 μg/ml	*Acanthostrongylophora ingens*	[30]

(*continued overleaf*)

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Table 3.1 (*continued*)

Compound	Activity (IC_{50})				Source	References
	P. falciparum	*T. cruzi*	*T. brucei*	*L. donovani*		
12-Deoxyascididemin	—	—	0.077 μM	—	*Polysyncraton echinatum*	[31]
Ascididemin	—	—	0.032 μM	—	*Polysyncraton echinatum*	[31]
Eilatin	—	—	1.33 μM	—	*Polysyncraton echinatum*	[31]
Lepadins D, E, and F	—	—	<1 μM	—	*Didemnum* spp.	[32]
Terpenoids						
Monoterpene hydroperoxide A	—	23 μM[a)]	—	—	*Chenopodium ambrosioides*	[33]
Monoterpene hydroperoxide B	—	1.2 μM[a)]	—	—	*Chenopodium ambrosioides*	[33]
Monoterpene hydroperoxide C	—	1.6 μM[a)]	—	—	*Chenopodium ambrosioides*	[33]
Monoterpene hydroperoxide D	—	3.1 μM[a)]	—	—	*Chenopodium ambrosioides*	[33]
Endoperoxide ascaridole	—	0.8 μM[a)]	—	—	*Chenopodium ambrosioides*	[33]
Hydroperoxide monoterpene	—	1.4 μM[a)]	—	—	*Laurus nobilis*	[33]
Dehydrocostus lactone	—	6.3 μM[a)]	—	—	*Laurus nobilis*	[33]
Zaluzanin	—	2.5 μM[a)]	—	—	*Laurus nobilis*	[33]
Luteolin glucopyranoside	2.5 μg/ml	—	—	—	*Phlomis brunneogaleata*	[34]

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Chrysoeriol glucopyranoside	5.9 µg/ml	—	—	—	*Phlomis brunneogaleata*	[34]
Vernolide	1.87 µg/ml	—	—	—	*Vernonia colorata*	[35]
Vernodalin	0.52 µg/ml	—	—	—	*Vernonia colorata*	[35]
Okundoperoxide	0.470 µg/ml	—	—	—	*Scleria striatinux*	[36]
Globiferin and analogs	0.8–13.9 µM	—	—	—	*Cordia globifera*	[37]
Terpinen-4-ol	—	—	0.13 µM	—	*Juniperus communis*	[18]
Helenalin	—	—	0.05 µM		*Arnica* and *Inula* spp.	[38]
Mexicanin I	—	—	0.32 µM		*Arnica* and *Inula* spp.	[38]
Komaroviquinone	—	0.4 µM[a)]	—	—	*Dracocephalum komarovi*	[39–41]
Lychnopholide	—	2.0 mg/kg/day[b)]	—	—	*Lychnophora trichocarpha*	[11, 42]
Psilostachyin C	—	0.6 µg/ml	—	—	*Ambrosia scabra*	[43]
Parthenolide	—	0.5 µg/ml	—	—	*Tanacetum parthenium*	[44, 45]
Simalikalactone D	0.01 µM	—	—	—	*Quassia amara*	[46, 47]
Simalikalactone E	0.5 mg/kg[b)]	—	—	—	*Quassia amara*	[46, 47]
Delaumonones A	0.6 µM	—	—	—	*Laumoniera bruceadelpha*	[48]
Delaumonones B	1.2 µM	—	—	—	*Laumoniera bruceadelpha*	[48]
Isobrucein A	0.05 µM	—	—	—	*Laumoniera bruceadelpha*	[48]
Salviandulin E	—	—	0.72 µg/ml	—	*Salvia leucantha*	[49]

(*continued overleaf*)

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Table 3.1 (*continued*)

Compound	Activity (IC_{50})				Source	References
	P. falciparum	*T. cruzi*	*T. brucei*	*L. donovani*		
Butanoyl 3,4-dihydrosalviandulin E	—	—	0.055 μg/ml	—	*Salvia leucantha*	[49]
Cryptotanshinone, 1β-hydroxycryptotanshinone, 1-oxocryptotanshinone, 1-oxomiltirone	—	—	—	18–47 μM	*Perovskia abrotanoides*	[50]
Karavoate M	0.6 μM	—	—	—	*Momordica balsamina*	[51]
Four isonitrile diterpenes	<1 μM	—	—	—	*Ciocalapata* spp.	[52]
4-Acetoxy-dolastane diterpene	—	—	—	2.0 μg/ml	*Canistrocarpus cervicornis*	[53]
Flavonoids						
O-Acetyl derivatives	—	—	—	11.18–10.53 μM	*Consolida oliveriana* (DC) Schrod	[54]
Octa-O-acetylhyperoside	—	—	—	6.21–7.35 μM	*Consolida oliveriana* (DC) Schrod	[54]
(−)-Epigallocatechin-3-*O*-gallate	—	—	—	1.6 μM	Green tea	[55]
8-Prenylmucronulatol	—	—	—	Low micromolar range	*Smirnowia iranica*	[56–59]
Smiranicin	—	—	—	Low micromolar range	*Smirnowia iranica*	[56–59]

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Polyketides and peptides						
Gracilioethers A–C	0.5–10 µg/ml	—	—	—	*Agelas gracilis*	[60]
Dragonamide E	—	—	—	5.1 µM	*Lyngbya majuscula*	[61, 62]
Dragonamide A	—	—	—	6.5 µM	*Lyngbya majuscula*	[61, 62]
Herbamide B	—	—	—	5.9 µM	*Lyngbya majuscula*	[61, 62]
Preussomerin EG1	—	—	—	0.12 µM	*Edenia* sp.	[63]
Palmarumycin CP2	—	—	—	3.93 µM	*Edenia* sp.	[63]
Palmarumycin CP17	—	—	—	1.34 µM	*Edenia* sp.	[63]
Palmarumycin CP18	—	—	—	0.62 µM	*Edenia* sp.	[63]
CJ-12,371	—	—	—	8.40 µM	*Edenia* sp.	[63]
Other compounds						
Jacaranone	7.82 µg/ml	13 µg/ml	—	11.86 µg/ml	*Pentacalia desiderabilis*	[64]
Eupomatenoid-5	—	7 µg/ml	—	5.0 µg/ml	*Piper regnellii* var. *pallescens*	[65–68]
Eupomatenoid-6	—	7.5 µg/ml	—	—	*Piper regnellii* var. *pallescens*	[65–68]
Conocarpan	—	8 µg/ml	—	—	*Piper regnellii* var. *pallescens*	[65–68]
Sulfated polysaccharide Cr	—	—	—	34.5 µg/ml	*Caulerpa racemosa* (Cr)	[69]
Sulfated polysaccharide Bo	—	—	—	63.7 µg/ml	*Botryocladia occidentalis* (Bo)	[69]
Sulfated polysaccharide Sf	—	—	—	137.4 µg/ml	*Solieria filiformis* (Sf)	[69]

a) Activity estimated by minimum lethal concentrations (MLC).
b) *In vivo* activity.

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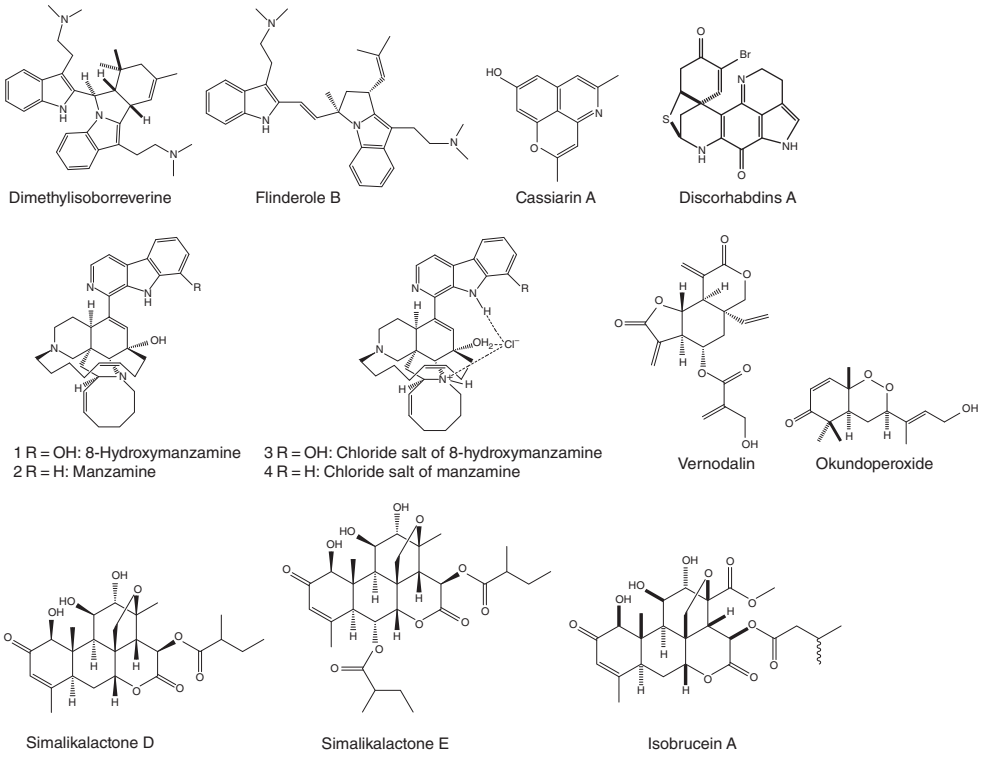


Figure 3.1 Most potent natural product compounds against *Plasmodium* ($IC_{50} \leq 0.2\,\mu M$ or $\leq 0.5\,\mu g/ml$).

such as sponges, corals, and ascidians, which are a rich potential source of novel metabolites with possible applications in human health [2, 71]. Some of the alkaloidal, terpenoid, flavonoid, and other interesting antiprotozoal chemical scaffolds discovered from plants and marine organisms are discussed (Table 3.1 and Figures 3.1 and 3.2).

Alkaloids

Alkaloids are mostly basic-nitrogen-containing secondary metabolites occurring abundantly in various forms in plants. Quinidine, cinchonine, quinine, and cinchonidine are quinolone alkaloids from *Cinchona* sp. bark, which have been historically demonstrated to exhibit potent anti-*T. b. brucei* activities with IC_{50} values of 0.8, 1.2, 4.9, and 7.1 μM, respectively [17]. Berberine is a quaternary benzylisoquinoline alkaloid isolated from many plant families. This compound, which has been predicted to act via DNA intercalation and protein synthesis inhibition, exhibits IC_{50} and selectivity values 0.5 μM and 51, respectively, against *T. brucei* [17]. Dioncophyllines A, B, and E are naphthylisoquinolines isolated

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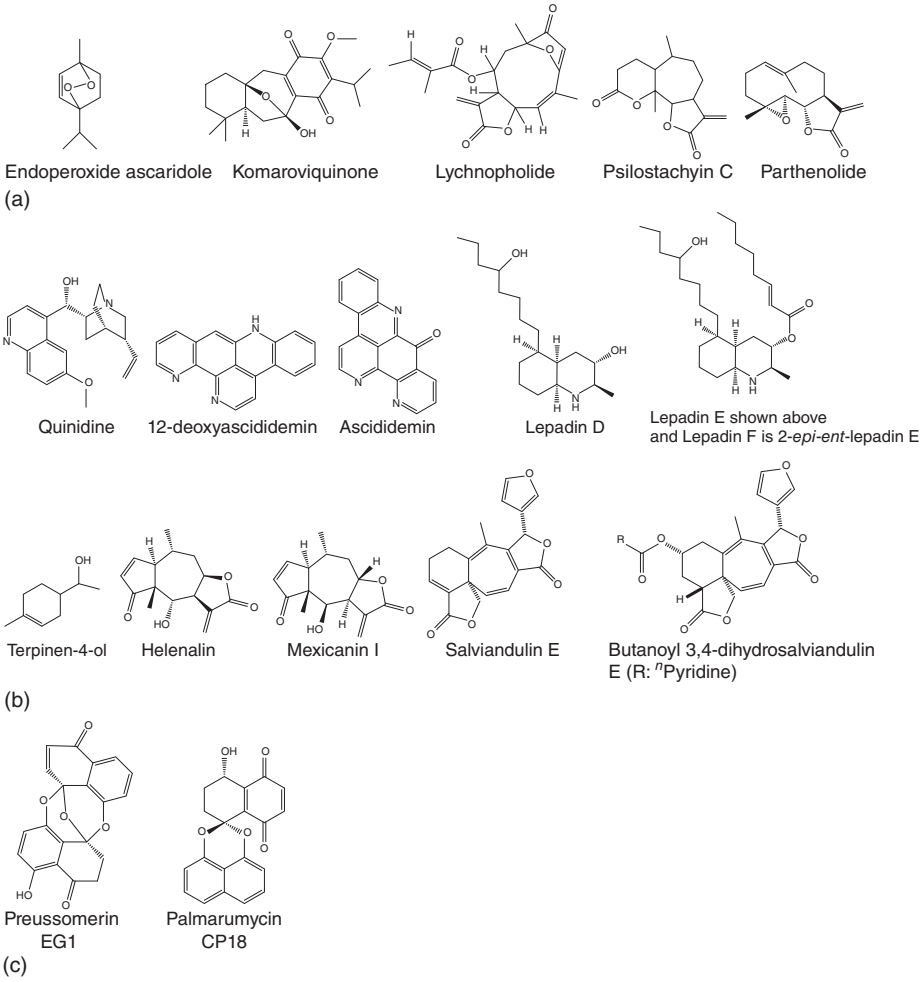


Figure 3.2 Most potent natural product compounds against the kinetoplastids ($IC_{50} \leq 1\,\mu M$ or $\leq 1\,\mu g/ml$). (a) Compounds active against *T. cruzi*. (b) Compounds active against *T. brucei*. (c) Compounds active against *Leishmania* parasites.

from an African plant and have shown anti-*T. b. rhodesiense* IC_{50} values ranging from 2.0 to 3.0 μM [72, 73]. Ancistroealaines A and B have been isolated from the stem bark of *Ancistrocladus ealaensis*, a flowering plant usually found in tropical regions. Both compounds respectively showed anti-*T. b. rhodesiense* IC_{50} values of 8 and 5 μM, and selectivity indices of 26.9 and 44 when tested in rat skeletal muscle cell line [18]. Actinodaphnine, cassythine, and dicentrine are three trypanocidal aporphines with respective IC_{50} values of 3.2, 6.0, and 14.6 μM obtained from *Cassytha filiformis* that seem to work by DNA intercalation and stabilization of the double helix structure and with effect in parasite

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proliferation [19]. In a study investigating the antimalarial activity of extracts from four plants namely, *Flindersia amboinensis*, *Stephania zippeliana*, *Voacanga papuana* from Papua New Guinea, and *Flindersia acuminate* from Australia, eight antimalarial alkaloids, namely indole alkaloids flinderole B, flinderole C, and dimethylisoborreverine from *F. amboinensis*, analog of flinderole A and isoborreverine from *F. acuminate*, liriodenine and xylopine from *S. zippeliana*, and voacamine from *V. papuana*, were isolated and demonstrated to have potent antimalarial activities with IC_{50} values ranging from 0.02 to 25.85 μM against chloroquine-sensitive, chloroquine-resistant, pyrimethamine-resistant, and chloroquine–pyrimethamine-resistant strains of *P. falciparum*. Isoborreverine (0.32 μM IC_{50}), dimethylisoborreverine (0.08 μM IC_{50}), and flinderole B (0.15 μM IC_{50}) were the most potent compounds with good selectivity indices in cell lines [20, 21]. From the leaves of *Cassia siamea*, a legume native to Southeast Asia, a potent antimalarial compound, cassiarin A, was purified with highly selective *in vitro* (IC_{50} of 0.02 μM) and *in vivo* (ED_{50} of 0.17 mg/kg) activities against *P. falciparum* and *Plasmodium berghei* parasites, respectively [22]. Structure–activity relationship (SAR) studies of cassiarin A revealed that its hydroxyl and nitrogen without any further substitutions are critical for its antimalarial and vasodilator activities with the tendency of reducing cytoadherence of parasitized red blood cells to vascular endothelium in severe and cerebral malaria [74]. Of five compounds isolated from *Piper crassinervium* (Piperaceae) leaves, a prenylated hydroquinone was found to be the most active compound against *T. cruzi* epimastigote Y strain with IC_{50} value of 6.10 μg/ml comparable to that of benznidazole (1.60 μg/ml) [23]. From the dichloromethane extract of the flowering aerial parts of *Achillea ptarmica* L., five alkamides were isolated, of which pellitorine and 8,9-*Z*-dehyropellitorine were the major components. Of the two, 8,9-*Z*-dehydropellitorine was found to be *T. b. rhodesiense*-active with IC_{50} of 2.0 μg/ml, while pellitorine exhibited an anti-*P. falciparum* activity with IC_{50} of 3.3 μg/ml [24]. Nwodo *et al.* isolated two dipeptide alkaloids, saropeptate and anabellamide, from the methanol root extract of *Zapoteca portoricensis* (Jacq) HM Hernández, a perennial plant used in the eastern part of Nigeria for tonsillitis. Both saropeptate and anabellamide showed anti-*T. b. rhodesiense* activity with IC_{50} values of 3.6 and 12.2 μM and selectivity indices of 25.3 and 5.83, respectively, against rat muscle cells [25]. Freire-de-Lima *et al.* previously demonstrated the antiproliferative effect of piperine (from the fruit bodies of *Piper* sp.) and its chemical derivatives, as active anti-*T. cruzi* agents. Piperine in its natural form was shown to be more potent in intracellular amastigotes as compared to axenic epimastigotes with respective IC_{50} values of 4.91 and 7.36 μM. In murine peritoneal macrophages, however, piperine did show microbicidal effect on the cells. Sublethal concentrations of piperine induced a reversible cell cycle arrest, which renders parasites rounded with mitochondrion matrix swelling, membrane invaginations, and intracellular vacuolization. Similar to the effects seen when *T. cruzi* epimastigotes are exposed to the antitumor and microtubule stabilizer taxol, multiplication of cell organelles occurred in the piperine-treated parasites; however, division of parasites into daughter cells was inhibited. Piperine

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treatment also did not cause induction of giant multinucleated cell formation as seen in cytokinesis arrestment induced by treating *T. cruzi* epimastigotes with the antimicrotubular vinca alkaloids vincristine and vinblastine, thus suggesting that piperine triggers a unique and selective anti-*T. cruzi* mechanism of action [26]. From a new deepwater Alaskan sponge *Latrunculia* sp., two new brominated pyrroloiminoquinones dihydrodiscorhabdin B and discorhabdin Y, alongside six other known pyrroloiminoquinone alkaloids discorhabdins A, C, E, and L, dihydrodiscorhabdin C and its benzene derivative were isolated. Of these, discorhabdins A, C and dihydrodiscorhabdin C had potent *in vitro* antimalarial activities against *P. falciparum* D6, (IC_{50} values of 0.053, 2.8, and 0.17 μM, respectively) and strain W2 (0.053, 2.0, and 0.13 μM, respectively) but discorhabdins A and dihydrodiscorhabdin C turned out to be toxic *in vivo* when tested on *P. berghei*-infected mice [27]. In a related development, two compounds that were isolated from the marine sponge *Hyattella* sp. turned out to be the known bromotyrosine alkaloid Psammaplysin F and a related new one, Psammaplysin G. The two compounds exhibited respective IC_{50} values of 1.4 and 0.87 μM, in *P. falciparum* Dd2 and 3D7 strains, with the new Psammaplysin G showing 98% *P. falciparum* Dd2 parasite growth inhibition at 40 μM [28]. Thirteen bromopyrrole oroidin analog alkaloids and related derivatives without the imidazole ring were isolated from marine sponge species of the genera *Axinella* and *Agelas*. Of these, dispacamide B and spongiacidin B showed selective potent *P. falciparum* IC_{50} values of 1.34 and 1.09 μg/ml, respectively, as against their respective cytotoxicity IC_{50} values of 90 and 35.65 μg/ml in the rat muscle cell line. Although a third compound, bromopyrrolohomoarginin did not show the same level of antimalarial potency in the whole parasite ($IC_{50} > 20$ μg/ml); this compound was the only one that demonstrated potent inhibition of the enzyme PfFabZ, when tested against the *de novo* FAS-II *P. falciparum* fatty acid biosynthesis pathway enzymes and hence may have possible use in antimalaria prophylaxis [29]. Also, four manzamine alkaloids, (+)-8-hydroxymanzamine A, (+)-manzamine A, (+)-8-hydroxymanzamine A hydrochloride, and (+)-manzamine A hydrochloride, isolated from *Acanthostrongylophora ingens*, a Pacific marine sponge, were demonstrated to have very potent antiplasmodial activity with IC_{50} values between 0.0061 and 0.0365 μg/ml in both chloroquine-resistant and -sensitive *P. falciparum* strains. With the exception of (+)-manzamine A, which showed weak activity against *Leishmania donovani* promastigotes and was also cytotoxic in both cancer and noncancer cell lines, the remaining three compounds were also potent in inhibiting the growth of *L. donovani* promastigotes [30]. From the crude dichloromethane/methanol extract of the Australian ascidian *Polysyncraton echinatum*, Quinn *et al.* purified a new pyridoacridine alkaloid, 12-deoxyascididemin, and two other known analogs, ascididemin and eilatin. These three compounds exhibited anti-*T. b. brucei* activities with IC_{50} values of 0.077, 0.032, and 1.33 μM, respectively [31]. Two synthetic analogs from a series of ascididemin derivatives were active against *T. b. rhodesiense* bloodstream forms with respectively improved trypanocidal activities 2000 and 770 times that of the parent ascididemin (IC_{50} values of 0.007 and 0.018 μM comparable to the IC_{50}

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of the standard drug melarsoprol of 0.003 μM). DNA intercalation and oxidative damage have been predicted as the possible mode of action for these compounds. Although the potency of the aforementioned derivatives of ascididemin seems impressive, the compounds have inherent cytotoxicity problems, which require further investigation [75]. From the marine tunicates genus *Didemnum*, the compounds lepadins D, E, and F were isolated with below 1 μM selective anti-*T. b. rhodesiense* bloodstream trypomastigotes IC_{50} values and selectivity indices in rat skeletal muscle cells between 43 and 80. This group of compounds possess a rare decahydroquinoline skeleton. While lepadins D has a secondary hydroxyl at C3, both lepadins E and F have 2-*E*-octenoic acid ester function at this position instead, and this seems to significantly enhance their antitrypanosomal activity [32].

Terpenoids

Terpenes are a very large and diverse group of organic compounds from which a number of active antiprotozoal compounds have been characterized [76]. From the aerial parts of *Chenopodium ambrosioides*, four monoterpene hydroperoxides and an endoperoxide ascaridole were isolated, and these showed anti-*T. cruzi* epimastigote activity with minimum lethal concentrations (MLCs) of 23, 1.2, 1.6, 3.1, and 0.8 μM, respectively [33]. The endoperoxide ascaridole was shown to almost completely inhibit infection of trypomastigotes in HeLa infection assay at 1 μg/ml (6.5 μM) while the hydroxides inhibited the infection by 63 and 88% at 1 μg/ml (6.0 and 6.5 μM), respectively; however, they did not inhibit the proliferation of amastigotes in infected cells [33]. Another hydroperoxide monoterpene alongside the sesquiterpene lactones, dehydrocostus lactone and zaluzanin isolated from *Laurus nobilis* showed MLC of 1.4, 6.3, and 2.5 μM, respectively [33]. The hydroperoxide monoterpene further showed 98% inhibited inhibition of cells at 4.4 μM and prevented intracellular proliferation of amastigotes at 83% at the same concentration. Whereas dehydrocostus lactone further inhibited both trypomastigote infection cells and amastigote proliferation at 1 μg/ml (4.3 μM) by 75%, the zaluzanin only weakly inhibited amastigote proliferation at 1 μg/ml (3.4 μM) without inhibiting promastigote infection of cells [33]. Although the trypanocidal activities of hydroperoxides largely depend on the presence of this functional group in this class of compounds, the activities of the lactones here depend on the formation of covalent bond between the α,β-unsaturated γ-lactone moiety with nucleophiles that are essential for the parasite life [33, 77]. Of two new compounds, brunneogaleatoside (iridoid glycoside) and its pyrrolidinium derivative and 14 other known metabolites purified from *Phlomis brunneogaleata*, the known flavone glycosides, luteolin glucopyranoside, and chrysoeriol glucopyranoside exhibited antiplasmodial (IC_{50} 2.5 and 5.9 μg/ml, respectively) and antileishmanial (1.1 and 4.1 μg/ml, respectively) activities. Further, the luteolin glucopyranoside was shown to be active at IC_{50} value of 10 μg/ml against enoyl-ACP reductase, a key regulator

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in the synthesis of *P. falciparum* type II fatty acid synthases [34]. Vernolide and vernodalin with antiplasmodial IC_{50} values of 1.87 and 0.52 μg/ml, respectively, were isolated by bioassay-guided purification of antiplasmodial compounds from *Vernonia colorata*, a shrub from west, central, and south tropical Africa widely reported on as containing active metabolites effective in treating various disease conditions [35]. The roots of *Scleria striatinux*, a herbal tea for the treatment of fevers in Cameroon, were the source of a new bicyclofarnesyl sesquiterpene endoperoxide, okundoperoxide, with antiplasmodial IC_{50} values ranging from 0.470 to 1.498 μg/ml against different strains of *P. falciparum* [36]. Globiferin is a new 10-membered ring meroterpene isolated from root extracts of *Cordia globifera*, a flowering plant native to India and other warm regions. Transformations either by acid cyclization or by Cope rearrangement led to eight other derivatives, which, together with the original globiferin, showed *P. falciparum* strain K1 antiplasmodial activity of IC_{50} values from 0.8 to 13.9 μM [37]. While investigating the trypanocidal effect of mono- and sesquiterpenes, terpinen-4-ol, a monoterpene essential component of tea tree oil, showed potent anti-*T. brucei* IC_{50} and selectivity of 0.13 μM and 1000, respectively [18]. Among several sesquiterpene lactones isolated from *Arnica* and *Inula* species, helenalin and mexicanin I were identified as the most potent trypanocidal compounds with anti-*T. b. rhodesiense* bloodstream trypomastigote IC_{50} values of 0.05 and 0.32 μM, respectively [38]. The presence of two α,β-unsaturated carbonyl structures (cyclopentenone and α-methylene-γ-lactone) in the two compounds helenalin and mexicanin I seems to be potential alkylation centers, which may inactivate various enzymes by reacting to their sulfhydryl groups, thus, suggesting the compounds to be causing their respective trypanocidal effects by inactivating trypanothione reductase in the trypanothione metabolism pathway of the parasite and increasing the production of toxic reactive oxygen species (ROS) as a consequence [38, 78]. Komaroviquinone is a novel diterpene icetexane isolated from *Dracocephalum komarovi* with interesting MLC of 0.4 μM against *T. cruzi* epimastigotes [39–41]. The production of ROS is described as the mechanism of the trypanocidal activity of several naturally occurring quinones against *T. cruzi* [79, 80] Komaroviquinone is reduced by *T. cruzi* old yellow enzyme to a semiquinone radical, which further generates superoxide anion radicals responsible for the trypanocidal activity of the compound. Although this compound did not inhibit the intracellular growth of amastigotes even at 3 μM, it potently inhibited both trypomastigote survival and infection of cells with IC_{50} of 0.009 μM. Moreover, the compound was 2200 times more selective for the trypomastigote parasites as against the host HeLa cells [79, 80]. De Oliveira *et al.* first reported on the *in vitro* anti-*T. cruzi* trypomastigote activity of lychnopholide, a sesquiterpene lactone isolated from *Lychnophora trichocarpha* [42]. This compound although active presents other problems such as clastogenicity, cytotoxicity, physicochemical properties such as poor aqueous solubility, high lipophilicity, and potential chemical instability in alkaline media that hinder oral administration and hence its pharmacological application [42]. Branquinho *et al*. later studied the *in vivo* activity of nanocapsule-loaded lychnopholide in *T.*

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cruzi CL strain-infected mice in the acute phase with 2.0 mg/kg/day dosage as against nonencapsulated lychnopholide and other formulations of the drug. They treated the mice with the drug formulations intravenously 24 h after infection for 10 days and found that while the free lychnopholide reduced the parasitemia and improved mice survival, this did not cure the mice of the infection. However, the nanocapsule-loaded lychnopholide at a dosage five times lower than that of the standard drug benznidazole reduced mice parasitemia, increased survival, and cured the mice at a higher rate, thus confirming that with the appropriate formulation, lychnopholide has the potential of serving as a new treatment for Chagas [11]. Psilostachyin C is an anti-*T. cruzi*-active sesquiterpene lactone isolated from *Ambrosia scabra* by Martino *et* al. [43]. It has *in vitro* trypanocidal activity against *T. cruzi* epimastigotes, trypomastigotes, and amastigotes, with IC_{50} values of 0.6, 3.5, and 0.9 μg/ml (and selective indices of 145.8, 25, and 97), respectively. At compound concentrations as low as 0.2 μg/ml, transmission electron microscopy revealed the induction of parasitic ultrastructural alterations in the form of vacuolization and multivesicular body-like structures appearance in parasites. The compound also reduced parasitemia *in vivo* in *T. cruzi*-infected mice treated for 5 days. This compound also demonstrates activity against *Leishmania mexicana* and *Leishmania amazonensis* promastigotes with IC_{50} values of 1.2 and 1.5 μg/ml, respectively; thus, it has the potential of serving as a good template for designing new and more potent trypanocidal drugs [43]. From extracts of *Tanacetum parthenium*, Izumi *et al*. isolated parthenolide, a sesquiterpene lactone, which showed IC_{50}/96 h and IC90/96 h of 0.5 and 1.25 μg/ml, respectively, against *T. cruzi* epimastigotes. At compound concentrations of 2 and 4 μg/ml, there was reduction in the internalization index of *T. cruzi* in LLMCK2 cells by 51% and 96.6%, respectively. Further, transmission electron microscopy scanning showed the compound to cause morphological and ultrastructural alterations in the parasite [44]. In a related work by the same group, parthenolide previously isolated from *Tanacetum vulgare* showed a strong *in vitro* synergy with benznidazole against *T. cruzi* epimastigotes causing a 23-fold reduction in the IC_{50} of benznidazole when used alone (IC_{50} from 1.6 to 0.07 μg/ml). Interestingly, parthenolide had an additive effect against *T. cruzi* trypomastigotes but an antagonistic cytotoxicity effect in the two-combination. Morphological alterations of *T. cruzi* trypomastigote induced by parthenolide in the two-drug combination resulted in rounding, shortening, and loss of plasma membrane integrity in the parasite [45]. In analyzing fractionated methylene chloride extract of *Quassia amara* young leaves (treatment of malaria in French Guiana), a number of quassinoids including neoquassin, quassin, picrasin B, picrasin H, picrasin I (new), picrasin J (new), simalikalactone D, and simalikalactone E were isolated. When examined for their antimalarial potential, simalikalactone D was the most potent with IC_{50} value of 0.01 μM but also cytotoxic with IC_{50} value of 0.02 μM against cell lines. Simalikalactone E, which was less toxic, showed *in vitro P. falciparum* gametocytocidal activity with IC_{50} value sevenfold lower than primaquine. An *in vivo* test of Simalikalactone E via the intraperitoneal routes showed an ED_{50} of 0.5 mg/kg against murine malaria parasite *Plasmodium vinckei petteri* [46,

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47]. In a related work, two new quassinoids, delaumonones A and B, alongside the known quassinoid isobrucein A, were isolated from the bark of *Laumoniera bruceadelpha* from Malaysia. These compounds showed antimalarial IC_{50} values of 0.6, 1.2, and 0.05 μM, respectively, against *P. falciparum* [48]. *In vitro* antimalarial SAR study of a series of 14 quassinoids showed 10 of them to have IC_{50} values less than 0.02 μg/ml [70]. Key to the activity of these series is the presence and nature of the carbon-15 ester functional group. The pattern of the ring A substitution was also found to be critical for activity, for example, glaucarubinone, which had a ring A with an α,β-unsaturated keto functional group, was 10 times more active than glaucarubin, which lacked this functional group in the ring A. Also critical to the antimalarial potency of this series is the oxygen bridge between carbon-20 and either carbon-11 or carbon-13 [70]. Salviandulin E is a rearranged neoclerodane diterpene originally isolated from *Salvia leucantha* and previously showed potent anti-*T. b. brucei* activity with IC_{50} value of IC_{50} 0.72 μg/ml against the GUTat 3.1 strain of the parasite [49]. While analyzing the trypanocidal potency of a series of 19 analogs of this compound, Aoyagi *et al.* found the butanoyl 3,4-dihydrosalviandulin E analog with anti-*T. b. brucei* strain GUTat 3.1 IC_{50} value of 0.055 μg/ml and selectivity index value of 1236 against MRC-5 cells to be the most active, hence, making this analog a possible candidate for further development into an potent selective trypanocidal lead [49]. Danshen (one to three tanshinones) is a dried root of the Chinese red-rooted sage *Salvia miltiorrhiza* Bunge and is clinically useful for the treatment of coronary heart and cerebrovascular diseases and viral hepatitis. In addition to the well-known cryptotanshinone, a quinoid diterpene with a nor-abietane skeleton and three new natural products, 1*β*-hydroxycryptotanshinone, 1-oxocryptotanshinone, and 1-oxomiltirone, were isolated from the roots of the Iranian medicinal plant *Perovskia abrotanoides*, although 1-oxomiltirone was previously obtained as a synthetic material. These compounds exhibited *in vitro* antileishmanial activities with IC_{50} values in the range 18–47 μM. These findings provide a rationale for traditional use of the roots in Iran as a constituent of poultices for the treatment of cutaneous leishmaniasis. The isolated tanshinones also inhibited growth of *P. falciparum* with IC_{50} values in the range 5–45 μM [50]. Structure–activity relationship studies of a cucurbitane-type triterpenoid called *karavilagenin C* previously isolated from *Momordica balsamina* and its generated alkanoyl and monoaroyl/cynamoyl derivatives, karavoates B, D, E, I, and M, found karavoate M to be the most potent of the series displaying IC_{50} values of less than 0.6 μM in *P. falciparum* resistant strains [51]. Most of the esters in these series showed no cytotoxicity in a breast cancer cell line [51]. A wide variety of core terpenoid skeleton compounds ranging from classical monoterpenes, sesquiterpenes, and diterpenes as well as nonclassical sesquiterpene-like spiroaxanes, cadinanes, eudesmanes, and guaianes and diterpene-like bifloranes, amphilectanes, and cycloamphilectanes, with differing spectra of functionalities have been isolated from marine sources. Those of the nitrogen-containing functionalities (isonitrile, isocyanate, and isothiocyanate) have been said to be unique in displaying significant antimalaria and related activities [52, 76]. Marine isonitrile-bearing

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terpenoids are among the best of several classes of bioactive natural products with potency comparable to quinolines and endoperoxides used against various species and strains of the *Plasmodium* genus [52, 76]. Wattanapiromsakul *et al*. [52] isolated four antimalarial isonitrile diterpenes (three known derivatives and a fourth novel derivative) from a sponge species of the genus *Ciocalapata* collected from Ko Tao in the Surat Thani Province of Thailand. Spectroscopic structural characterization of the compounds led to the identification of the three known compounds, 8,15-diisocyano-11(20)-amphilectene, 7-isocyanoamphilecta-11,15-diene, and 8-isocyanoamphilecta-11,14-diene, and a fourth new derivative, 8-isocyanoamphilecta-11,15-diene. The compounds exhibited selective submicromolar activities against the chloroquine-resistant *P. falciparum* strain K1 (IC_{50} values 0.09 – 1.07 μM) as compared to their cytotoxicity in MCF-7 and fibroblast cell lines [52]. A 4-acetoxy-dolastane diterpene obtained from brown alga *Canistrocarpus cervicornis* was shown to exhibit selective dose-dependent activity during 72 h of treatment, with respective IC_{50} values of 2.0, 12.0, and 4.0 μg/ml for promastigote, axenic amastigote, and intracellular amastigote forms of *L. amazonensis*. Additionally, this compound induced ultrastructural changes, including extensive mitochondrial damage and lipid peroxidation in parasite cells. Although the mechanism of action of this compound is still unclear, these findings are promising with respect to the potential development of this compound into an alternative antileishmanial lead [53].

Flavonoids

Groups from universities in Granada and Tenerife investigated the inhibitory effects of flavonoids derived from the aerial parts of *Consolida oliveriana*, a species used medicinally in parts of Anatolia (Turkey), on *Leishmania* parasites. From this work, the inhibitory effects kaempferol, quercetin, trifolin, as well as their acetyl hyperoside and their O-acetyl derivatives and also the inhibitory effects of octa-*O*-acetylhyperoside flavonoid compounds derived from aerial parts of the same plant, have been investigated against the extracellular promastigote and the intracellular amastigote stages of *Leishmania* (V.) *peruviana* and *Leishmania* (V.) *braziliensis* (cutaneous and mucocutaneous leishmaniasis). When the compounds were tested in 72 h of exposure against *L.* (V.) *peruviana*, and also cytotoxicity experiments against macrophage cells, the results showed the acetylated compounds, octa-*O*-acetylhyperoside and *O*-acetyl derivatives, to exhibit IC_{50} values (i.e., IC_{50} of 7.35 and 11.18 to 10.53 μM, respectively) lower than those of the reference drugs (pentostam and glucatim). Further, despite being slightly cytotoxic, these compounds showed 10- to 15-fold higher selectivity against the parasites compared to the cytotoxicity of reference compounds in macrophage cells. It appears that the kaempferol derivatives possessing a mono-substituted B-ring are more active than the quercetin analogs [54]. It has been shown that (−)-epigallocatechin-3-*O*-gallate, the most abundant flavanol in green

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tea, has antiproliferative effects on *T. cruzi*. There are different studies that report the effects of this compound *in vitro* and *in vivo* on *L. amazonensis*. *L. amazonensis*-infected macrophages treated with (−)-epigallocatechin-3-*O*-gallate exhibited a significant reduction of the infection index in a dose-dependent manner, with an IC_{50} value of 1.6 μM. Oral administration of this compound in *L. amazonensis*-infected BALB/c mice (30 mg/kg/day) resulted in a decrease in the lesion size and parasite burden, without altering serological markers of toxicity, which demonstrate the *in vitro* and *in vivo* leishmanicidal effects of this compound [55]. Three unusual, highly oxygenated novel phenylpropanoids and two novel isoflavans, 8-prenylmucronulatol and smiranicin, were isolated from *Smirnowia iranica* together with a previously described isoflavan, glyasperin H. The isoflavans significantly inhibited the growth of extracellular stages of three *Leishmania* species *in vitro*, their activity against the intracellular stages being considerably lower. 8-Prenylmucronulatol showed moderate *in vitro* toxicity against *P. falciparum*, without noticeable erythrocyte membrane effects at the inhibitory concentration. The activities of oxygenated novel phenylpropanoids against various strains of *Leishmania* promastigotes were rather low, the quinone being the most active. Leishmanicidal activity of two novel isoflavans, 8-prenylmucronulatol and smiranicin, was of interest, because compounds with two aromatic rings connected by a carbon spacer, such as chalcones and aurones, exhibit significant leishmanicidal effects [56, 57]. In this sense, the activities of two novel isoflavans, 8-prenylmucronulatol and smiranicin, were determined for promastigotes of various leishmania species and expressed as IC_{50} values in the low micromolar range. These isoflavans are considerably less potent against promastigotes than the reference compound amphotericin B and less potent than many aurones and chalcones studied so far [58, 59].

Polyketides and Peptides

Polyketides and peptides are other natural product compound classes with promising potential that have been identified from marine-dwelling organisms. Examples include gracilioethers A–C, which are three new antiparasitic compounds isolated from the marine sponge *Agelas gracilis*, reported to have antimalarial IC_{50} values between 0.5 and 10 μg/ml against *P. falciparum* ItG strain. Gracilioether B was also shown to be active against leishmania parasites [60]. The International Cooperative Biodiversity Group located in Panama isolated a new modified linear lipopeptide, dragonamide E, as well as two related compounds previously reported in the literature, dragonamide A and herbamide B, from *Lyngbya majuscula*, a field-collected marine cyanobacterium extract found to be active against leishmaniasis in an *in vitro* screening assay [61]. Dragonamides A and E and herbamide B exhibited antileishmanial activity with IC_{50} values of 6.5, 5.1, and 5.9 μM, respectively [62]. Further, the same group described the isolation, structural elucidation, and biological activity of the major compounds from the endophytic fungus *Edenia* sp., which was isolated from a mature leaf

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of *Petrea volubilis* collected from the Coiba National Park in Panama. Bioassay fractionation of extracts from the fermentation broth and mycelium of the fungus *Edenia* sp. led to the isolation of five antileishmanial compounds, preussomerin EG1, palmarumycin CP2, palmarumycin CP17, palmarumycin CP18, and CJ-12,371. Two of these compounds are new natural products. All metabolites caused significant inhibition of the growth of *L. donovani* amastigotes with IC_{50} values of 0.12, 3.93, 1.34, 0.62, and 8.40 μM, respectively. These compounds were inactive when tested against *P. falciparum* or *T. cruzi* at a concentration of 10 μg/ml, indicating that they have selective activity against leishmania parasites. In addition, they showed weak cytotoxicity to Vero cells (IC_{50} of 9, 162, 174, 152, and 150 μM, respectively) [63].

Other Compounds

Morais *et al*. isolated jacaranone from the dichloromethane phase of the extract of the leaves of *Pentacalia desiderabilis* (Brazilian plant). This compound exhibits anti-*T. cruzi* trypomastigotes and *P. falciparum* K1 strain IC_{50} values of 13 and 7.82 μg/ml, respectively. The compound was also 3.5 times more active than benznidazole against *T. cruzi*. Activities of this same compound were also reported against *Leishmania* (L.) *chagasi*, *L.* (V.) *braziliensis*, and *L.* (L.) *amazonensis* with respective IC_{50} values of 17.22, 12.93, and 11.86 μg/ml. Although active against the promastigotes of *Trypanosoma* and *Leishmania* parasites, jacaranone was not active against the clinically relevant amastigote forms *L.* (L.) *chagasi* and *T. cruzi* [64]. From ethyl-acetate leave extracts of *Piper regnellii* var. *pallescens*, Luize *et al.* reported on the *in vitro* anti-*T. cruzi* activities of four neolignans purified. With the exception of eupomatenoid-3, which showed slightly lower activity, the remaining three compounds, eupomatenoid-5, eupomatenoid-6, and conocarpan, respectively showed anti-*T. cruzi* epimastigote IC_{50} values of 7, 7.5, and 8 μg/ml. Methylation of the hydroxyl groups in these active compounds renders them less active, thus showing that these groups are essential to their potency. At a concentration that inhibits growth of *T. cruzi* epimastigote, eupomatenoid-5 was less toxic to Vero cells, did not lyse sheep blood, and was significantly more active than benznidazole [65]. Further, Pelizzaro-Rocha *et al.* demonstrated the anti-*T. cruzi* trypomastigotes activity of this compound (EC_{50} 40.5 μM) and showed it to induce ultrastructural alteration and lipoperoxidation in the cell membrane of the infective stage parasite. Eupomatenoid-5 was also reported as inducing mitochondrial membrane depolarization, lipoperoxidation, and increased activity of glucose-6-phosphate dehydrogenase in *T. cruzi* epimastigotes. These interesting findings suggest the compound's mechanism of action to be stage-dependent, targeting the plasma membrane in trypomastigotes but the mitochondrion in epimastigotes. Thus, in epimastigotes, parasite death may be the result of eupomatenoid-5 causing mitochondrial dysfunction and oxidative damage [66]. Further work into the mechanism of action of eupomatenoid-5 in

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the three main forms of *T. cruzi* showed that the compound induces oxidative imbalance in these parasites, especially in the trypomastigotes, which leads to a decrease in trypanothione reductase activity, thus increasing the formation of cytotoxic ROS. The compound also reduces mitochondrial membrane potential, thus compounding the impairment of the parasitic redox system through increasing ROS and reactive nitrogen species production. Lipid peroxidation and DNA fragmentation are the downstream effects of these oxidative stresses. The network of events mentioned earlier triggers parasite death by pathways that may include apoptosis, necrosis, and autophagy [67]. Vendrametto *et al.* demonstrated that eupomatenoid-5 has dose-dependent anti-*L. amazonensis* activities during 72 h treatment, with IC_{50} values of 9, 13, and 5.0 μg/ml against promastigote, axenic, and intracellular amastigotes, respectively. At 9 μg/ml compound treatment, parasitic ultrastructural alterations include intense flagellar pocket exocytic activity and cytoplasmic myelin-like figures and vacuoles. When the concentration of eupomatenoid-5 was increased to 25 μg/ml, significant mitochondrial and endoplasmic reticulum damages were observed in the parasites. The compound's good selectivity for the different stages of the aforementioned trypanosomatid parasites makes it a candidate for future development into a broad-spectrum trypanocidal agent [68]. Bioassay-guided fractionation of methanol extract of the stem bark of *Calophyllum brasiliense* led to the isolation of soulamarin, a coumarin, whose structure was characterized as 6-hydroxy-4-propyl-5-(3-hydroxy-2-methyl-1-oxobutyl)-6", 6"-dimethylpyrane-[2", 3": 8,7] benzopyran-2-one. Soulamarin showed dose-dependent permeability of the plasma membrane of *T. cruzi*-treated parasites similar to that of fully permeable Triton X-100-treated parasites. Fluorescence microscopy also revealed this compound to induce a strong mitochondrial membrane potential depolarization (about 97%), thus suggesting that this compound to act through parasite plasma membrane damage and mitochondrial dysfunction but without the generation of ROS. Soulamarin may therefore contribute a novel template for designing new selective mitochondrion-active drug candidates against *T. cruzi* infections [81]. Of several sulfated polysaccharides purified from the marine macroalgae *Solieria filiformis* (Sf), *Botryocladia occidentalis* (Bo), *Caulerpa racemosa* (Cr), and *Gracilaria caudata* (Gc), only three were active against *L. amazonensis*. The polysaccharide purified from Cr was the most potent with EC_{50} value of 34.5 μg/ml. The polysaccharides derived from Bo and Sf demonstrated moderate antileishmanial activities with EC_{50} values of 63.7 and 137.4 μg/ml, respectively. An *in vitro* cytotoxic assay using peritoneal macrophages and J774 macrophages showed all the sulfated polysaccharides to decrease cell survival with CC_{50} values of 27.3, 49.3, 73.2, and 99.8 μg/ml for Bo, Cr, Gc, and Sf, respectively. However, none of these compounds reduced the cell growth rate of the peritoneal macrophages. These compounds modulate the growth rate and cell survival of *L.* (L.) *amazonensis* promastigotes *in vitro*, acting on the cell membrane of the parasites. In addition, these showed a higher efficiency against the development of leishmaniasis than alkaloids, terpenoids, and other natural products isolated

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from seaweed [69]. Bioassay-guided fractionation from the less cytotoxic fraction of the lipophilic extract of sponge *Neopetrosia* sp., collected in Southern Japan, afforded an active compound, which was identified as renieramycin A by spectroscopic analysis. As expected, this compound showed moderate selectivity for inhibition against La/egfp proliferation over cytotoxicity against P388 cells. Thus, it inhibited La/egfp with an IC_{50} value of 0.2 µg/ml [82].

Discussion

Natural product scaffolds have historically been the source of novel chemistry that provides medicinal chemists with pharmacophore warheads based on which many potent drugs are developed. Classical examples of this are quinine and artemisinin. In spite of this excellent track record, it is abysmally disappointing to see no recent natural-product-derived scaffolds in the current antiprotozoal drug development portfolios. In the last two decades, the focus of most pharmaceutical industries has shifted from novel natural product chemistry toward the use of combinatorial chemistry to produce libraries that may contain millions of compounds but lack novelty [14, 83]. This is because screening microbial and plant crude extracts for potential natural product drug leads presents certain bottlenecks such as limit in supply from source, low yield, complex chemistry that presents problems in structural identification, modification and total synthesis, and issues surrounding the drug-like properties and toxicities of compounds from this source [14]. Thanks to advances made in multilevel collaborative natural habitat explorative research (including those in deep ocean environments), tailor-made high-throughput screening platforms, and expertise in bioassay-guided isolation and purification of bioactive natural products, most of these bottlenecks are now issues of the past. With the issue of toxicity, for example, recent screening strategies have clearly demonstrated that introducing cytotoxicity and dereplication checks early in the screening program enables efficient weeding out of notorious pan-assay interfering compounds [84, 85]. Thus, hits emerging from such scrutinized screens have better chances of progressing quickly into possible follow-up SAR, *in vivo,* and mode of action programs. The potential for SAR studies to greatly enhance the possibilities of hits is seen in the *T. brucei* active compound ascididemin (Section Alkaloids), in which SAR studies alone led to two analogs, which were 2000 and 770 times more active than the parent compound ascididemin. The critical role that early *in vivo* and drug formulation studies could play here is also highlighted by the interesting example of lychnopholide (Section Terpenoids) in which the original compound's non-drug-like properties such as clastogenicity, cytotoxicity, solubility, lipophilicity, and instability were all overcome and a five-time benznidazole efficacy was attained *in vivo* when an intravenous nanocapsule formulation of lychnopholide was administered at 2.0 mg/kg/day in *T. cruzi* CL strain-infected mice. With the current advanced knowledge and technical know-how in microbiology,

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biotechnology, and genomics, it is possible to manipulate several beneficial properties of microbes in large-scale fermentations in order to greatly enhance natural product compound yields [86]. The technique of metabolic pathway engineering, for example, allows the manipulation of biosynthetic gene clusters of natural-product-producing microbes and may be used to produce drug-like natural product analogs, which may provide focused libraries to facilitate SAR studies [87]. The bacterial genus *Streptomyces* is well known for the production of medically relevant bioactive molecules. Recent whole genome sequencing and gene mining studies have revealed underexplored biosynthetic gene clusters in this genus, and it is estimated that under the right growth conditions, each species in this genus is capable of producing 30 different bioactive molecules [88]. Among the vast array of terrestrial and marine microbial life, it is a known fact that only about 1% is culturable; however, with the speed of research exploring alternative culturing methods, one could never imagine the plethora of unexplored chemistry attainable from this source [89]. Another intrinsically beneficial property of especially plant-based natural products is the fact that in Asian and African countries, for instance, an estimated 80% of the population depends on traditional herbal plants for their primary health care [90, 91]. This situation presents the unique potential advantage of providing relevant observational clinical data generated by regulators and public health bodies in those communities to guide the focus of screening programs since a substantial number of natural product compounds reported in the literature has sprung from the screening of medicinal plants [90, 91]. From such trusted data, projects involving community-based therapeutic herbal plants would start with the confirmation and definition of the clinical activity of the medicinal plant, which will form a solid basis for *in vivo* pharmacological characterization, culminating in the capital- and labor-intense process of isolation and structural characterization of the active components involved. In this way, the paucity observed in natural product hits progressing into viable hit-to-lead projects will be greatly reduced. Clearly, natural product libraries cannot compete with synthetic combinatorial libraries in terms of providing large numbers of preselected structural compound types used in drug discovery screening programs. However, when looking for untapped sources of leads with scaffolds that challenge the imagination of medicinal chemists, natural products remain the number one source. With the high-throughput potential of modern screening methods, critical assay designs, informed selection of sources from which to screen, and integrative modern pharmacodynamic/kinetic expertise, natural products will continue to provide hit templates for antiprotozoal rational drug design. Within the scope of this review, we have presented the structures of 11 anti-plasmodial and 17 anti-kinetoplastid compounds, which in our view offer the most promising potential in terms of activity and novelty in their structures (Figures 3.1 and 3.2). In light of the vast array of research and technological tools available, there is a unique opportunity now to fully unlock this rich source of chemistry in response to meeting the current medical needs.

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Conclusion and Future Perspectives

In this review, we discussed the antimalarial and anti-kinetoplastid natural product compounds from plants and marine sources reported in the literature in recent times. In some of the interesting compounds, follow-up investigations have been done in terms SAR, *in vivo*, and possible modes of action, thus providing the essential information necessary for further development of the said compounds into future drugs. Although many effective compounds have been found against malaria and neglected diseases, most of them present toxic effects that prevent their further development. Another important challenge lies in the cost–effective and efficient delivery of safe drugs to endemic regions. Given that the majority of the infected population lives in countries where the prospects of any financial returns on investment are too low to support market-driven drug discovery and development by industry, alternative approaches are urgently needed. Drug discovery is an iterative process that typically comprises several discrete stages, including different approaches to antiparasitic drug discovery, the promise of introducing high-throughput screening on new molecular targets, and emphasis on the importance of lead optimization. Thus, among the potential approaches for new discovery, microbial and plant secondary metabolites represent the largest repository of new chemical structures, which has been and will continue to be a source of new drugs, directly in their native form or after optimization by synthetic medicinal chemistry. Consequently, "neglected" disease drug discovery is principally field-driven and designed to meet the needs of disease-control programs in the field. This generally means an emphasis on low cost of goods, short treatment regimens, and the ability to use the drug safely in the absence of close medical supervision. Optimization of lead compounds so that they have the characteristics required to meet product profiles is the rate-limiting factor in preclinical drug development. Finally, the inputs of various public–private partnerships have contributed to the discovery process and led to an increase in the numbers of antiparasite drug candidates reaching the clinic for the past 5 years.

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4
Isoxazolines: A Novel Chemotype Highly Effective on Ectoparasites

*Tina Weber and Paul M. Selzer**

Abstract

Efficient control of arthropod ectoparasite infestations has a long-standing history in the agriculture and veterinary sectors, aiming to decrease the parasite burden of affected crops and animals. Ligand-gated chloride channels (LGCCs) modulated by γ-aminobutyric acid (GABA) and glutamate have been identified as suitable molecular targets, and several classes of potent parasiticides have been devised. Due to the increase in cross-resistance and decreased development of new chemical entities, an urgent need for new parasiticides or prevention schemes has emerged. In the past decade, an innovative isoxazoline chemotype appeared to offer promise for inhibiting LGCCs with a new mode of action and distinct binding site from that of historical agents. Considerable efforts have focused on optimizing the antiparasitic activity of isoxazolines and may provide the potential for future human use.

Arthropod Ectoparasites: Burden to the Agricultural and Veterinary Sectors

Safe and efficient control of pests has become pivotal not only for agricultural productivity [1] but also in the veterinary sector, including companion animals and livestock [2]. In this regard, antiparasitic drugs strive to regulate, mitigate, and ideally prevent infestation by parasites such as arthropods, helminths, or protozoa and the consequences associated with them [3, 4]. From an animal health perspective, acarid and insect ectoparasites are particularly key target species, with ticks, fleas, and flies affecting cattle as well as cats and dogs severely. Prominent species are *Ctenocephalides felis* (flea) and *Dermacentor variablis* (tick) in cats and dogs, respectively, and *Rhipicephalus microplus* (tick) in cattle. Many of them are primarily blood feeders; however, the immediate and more apparent pathology linked with the initial bite of the parasites should not deflect from their role

*Corresponding author.

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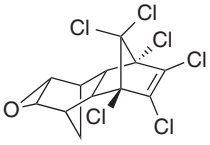
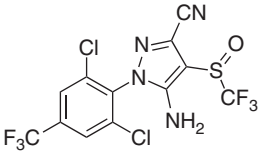
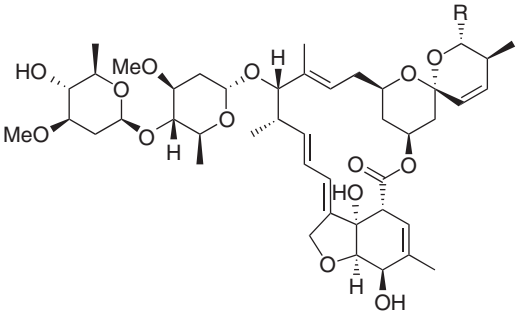
as vectors for parasitic microorganisms and as a source of infections. Extensive research has consequently gone into the development of efficient and selective veterinary ectoparasiticides [5]. As humans are also heavily burdened by ectoparasites, they might also benefit from these developments.

Ligand-Gated Chloride Channels as Suitable Targets for Ectoparasiticides

Considering the mode of action, a significant number of active compounds target ligand-gated ion channels [6, 7]. Three representatives of the major structural insecticide classes that act on those targets are depicted in Table 4.1.

Dieldrin belongs to the organochlorides, more specifically to the cyclodienes, which were extensively used in the agricultural sector for over three decades since the 1940s [8]. Long persistence in the environment, however, proved detrimental, as bioaccumulation of dieldrin gave rise to toxic effects in species not initially targeted, including humans, along with the development of resistance. A more recent

Table 4.1 Ectoparasiticides acting on ligand-gated chloride channels.

Structure	Name	References
	Dieldrin	[8]
	Fipronil	[9, 10]
	Ivermectin (R = *i*Pr/*s*Bu)	[11]

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alternative is fipronil, a phenylpyrazole with contact as well as systemic activity that was introduced in the 1990s [9, 10]. The broad-spectrum ectoparasiticides are distributed as the racemate and are used in plant protection schemes as well as in the veterinary sector. Structurally more complex are naturally occurring macrocyclic lactones and derivatives thereof, such as ivermectin [11–15]. The potent parasiticides exhibit better safety and selectivity profiles with greater affinity for invertebrate receptors than mammalian analogs [16].

Mode of Action

The three parasiticide classes described herein act by disrupting the signaling ability of the synapses between the neurons within the central nervous system (CNS), but also at the neuromuscular junctions [17]. In this process, a nerve impulse or action potential typically triggers axon uptake of calcium ions via specific Ca^{2+} channels. The increase in Ca^{2+} concentration influences the synaptic vesicles that contain neurotransmitters, causing them to fuse with the presynaptic cell membrane and to open up into the extracellular space between an axon and a dendrite: the synaptic cleft. The released neurotransmitters then bind and modulate receptors such as ligand-gated ion channels in the postsynaptic cell, subsequently shifting the local transmembrane potential and thus allowing transmission of an electrical pulse or signal. In this cascade, the ion channels are of particular interest as parasiticide targets, as disruption of their regular function by blockage or prolonged activation may decisively interfere in the signaling process. Characteristic features in arthropod ion channel sequences are envisaged as strategic points for selectivity.

Dieldrin and fipronil have been identified as antagonists for γ-aminobutyric-acid-gated chloride channels (GABACls), essentially blocking the influx of chloride ions and consequently counteracting the inhibitory function of the natural neurotransmitter [8, 9, 18]. As a result, depolarization and ensuing hyperexcitation occur, leading to tremors, convulsion, and death. GABACls are essential in both the mammalian and invertebrate CNS, which makes parasiticide selectivity a prerequisite to ensure a relevant therapeutic window [19]. In contrast, glutamate-gated chloride channels (GluCls) are specific to invertebrates and are predominantly located in the skeletal muscle tissue and the CNS. They have recently been associated with the same superfamily of cysteine-loop ligand-gated chloride channels (CysLGCCs) as GABACls, with the added advantage of being species-specific [20, 21]. Notably, ivermectin has been shown to activate glutamate receptors and intensify the chloride influx, giving rise to hyperpolarization followed by ataxia and coma at higher concentrations [7, 20–22]. The sedative effect has also been partially attributed to the activation of GABACls, albeit to a lower degree [23].

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Isoxazolines: Novel Ectoparasiticides Acting on GABACIs and GluCIs

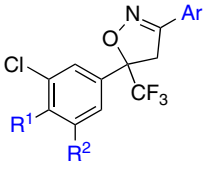
Twenty years since the introduction of the phenylpyrazole fipronil as the most recent addition to the existing portfolio of ectoparasiticides, the demand for new molecular entities addressing the CysLGCCs in a different manner has grown steadily. A clear distinction from known commercially available antagonists is essential, with a novel mode of action or binding site to circumvent cross-resistance concerns. In this regard, structural diversity is key. Previously investigated as highly selective modulators of arthropod ryanodine receptors [24, 25], derivatives of phthalic and anthranilic diamides came into focus when the incorporation of isoxazoline moieties changed the activity profile toward ligand-gated chloride channels [26, 27]. Extensive research on this novel compound class led to the discovery of potent ectoparasiticides by researchers at Du Pont [28, 29] and Nissan [26, 27]. Active ingredients with the international nonproprietary names (INNs) such as afoxolaner and fluralaner were later approved by the US Food and Drug Administration (FDA) in short succession for Merial's NexGard (September 2013) [30, 31] and MSD-AH's Bravecto (May 2014) [26, 32–34], respectively (Table 4.2). Structurally related to fipronil to a certain degree with its chloride-substituted phenyl heterocyclic core, the novel compound class contains a nonaromatic isoxazoline and an extended side chain that indicates its link to the preceding diamides. The aryl amide side chain and the substitution pattern on the terminal aromatic unit have consequently been the central areas for further derivatization. With regard to the chiral center within the isoxazoline ring, studies have revealed only the *S* enantiomer of fluralaner to be active [27], with no adverse effects of the respective *R* enantiomer known to date. In this regard, the ectoparasiticides afoxolaner and fluralaner both are distributed as racemic mixtures [27, 31].

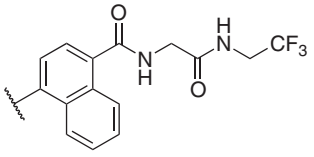
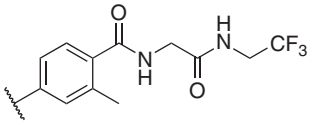
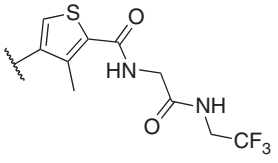
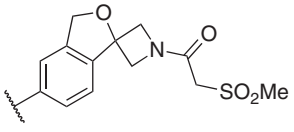
Structure and Active Sites of Chloride Channels

As a family, CysLGCCs differ noticeably in complexity in terms of their subunits' composition; however, the homo- or heteromeric ion channels show common structural and functional features and can be generalized to some degree [20, 21]. Embedded in the postsynaptic cell membrane, each unit of the pentameric ion pore that forms the CysLGCCs consists of four distinct helical transmembrane regions (M1–M4) and a characteristic cysteine loop located in the large extracellular N-terminal domain. A schematic representation of the ion channel's α-helical domain is depicted in Figure 4.1, with M2 creating the inner lining of the pore while the five independent helix clusters are linked between M1 and M3 of adjacent subunits. Accordingly, the amino acid sequence in M2 has a pronounced influence on the ion selectivity of the respective receptor. With the emergence of parasiticide resistance, especially against cyclodiene derivatives, research has

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Table 4.2 Isoxazoline-derived parasiticides.



R^1	R^2	Ar	Name	References
H	CF_3		Afoxolaner	[29, 31]
H	Cl		Fluralaner	[26, 33, 34]
Cl	Cl		Lotilaner	[35]
F	Cl		Sarolaner	[36]

focused on locating the most conspicuous modifications in the relevant genes that code for the ion channels.

An amino acid exchange of alanine to serine or glycine at position 302 in the M2 helix was observed in a dieldrin-resistant (dlr) GABA subunit gene of *Drosophila melanogaster*, and the single point mutation at this position was later identified as a central cause for resistance development across a number of different species [37–39]. Additionally, cross-resistance to fipronil and other GABAergic parasiticides has been observed to varying degrees of intensity, suggesting a high congruency of the binding sites for the different potent GABACl blockers and linking the similarities in mode of action to a common, but not necessarily identical, allosteric-binding site in the channel lumen [23, 40]. For ways of better illustration, a homology model of a pentameric GABACl of *C. felis* was generated based on crystallographic data for the respective *Erwinia chrysanthemi* analog

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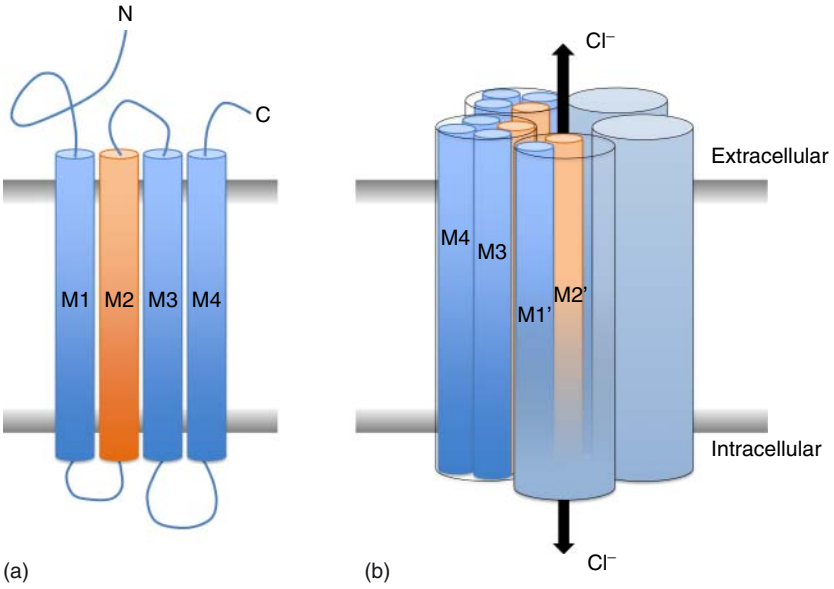


Figure 4.1 Schematic representation of the transmembrane domain of a ligand-gated chloride channel. (a) Four transmembrane helices (M1–M4) that make up one subunit are depicted with their connecting loops. (b) Arrangement of the transmembrane subunits to form a pentameric ion channel with M2 helices (orange) lining the pore, M1 and M3 linking the adjacent subunits, and M4 facing the outside of the pore. Helices M3′ and M4′ of the foremost subunit have been omitted for clarity. Only three of the five subunits reveal their composition of helices.

(PDB ID: 2VL0; Figure 4.2) [41–43]. The crucial resistance-inducing point mutation located in the transmembrane region is highlighted in dark green with the amino acid residue as a red sphere in Figure 4.2c, the characteristic Cys loop in the extracellular region in light green. In a view along the channel pore axis (Figure 4.2b,c), the channel-pore-lining M2 helices are visible.

Besides voltage clamp electrophysiology studies for investigations on the parasiticides' influence on the chloride ion flux, a multitude of target site binding assays have been used to evaluate their inhibition and modulation capacity [44, 45]. The frequently used radiolabeled receptor channel blocker 4′-ethynyl-4-*n*-propylbicycloorthocarboxylate ([^{3}H]EBOB) shows good correlation between its displacement and the toxicity of GABACl antagonists such as cyclodienes and fipronil, thus further supporting the close link between the parasiticides' binding sites [44, 45]. Noncompetitive and incomplete binding was observed for avermectins, however, emphasizing the distinction in pathology and their conserved sensitivity even in dlr-GABACls [45]. Analogous studies on GluCls suggest an ivermectin-stimulated activation of the ion channel by insertion

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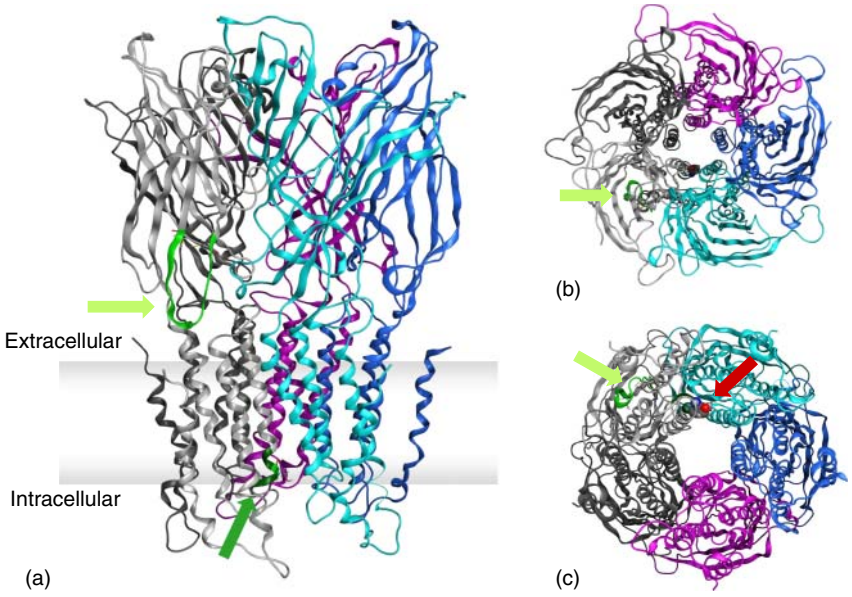


Figure 4.2 Homology model of GABACl in a proposed closed form. Sequences of *Erwinia chrysanthemi* GABACl and dlr-GABACl of *Ctenocephalides felis* were aligned using the BLAST algorithm, and final modeling was performed with the MOE software package [41]. The five individual subunits are shaded in different colors. The Cys loop of one subunit is highlighted in light green (light-green arrows), the resistance-inducing mutation in dark green (dark-green arrow). (a) Protein shown parallel to membrane; horizontal gray bars indicate membrane boundaries. (b) View along the channel pore axis from the extracellular side, with M2 helices visible as the inner pore lining. (c) View along the channel pore axis from the intracellular side, with resistance-inducing residue (red dot) highlighted with a red arrow.

between the linking M3 and M1′ helices of adjacent subunits in a transmembrane region closer to the N-terminal extracellular domain [46–48]. Crystallization of a GluCl of *Caenorhabditis elegans* in complex with Fab molecules and ivermectin has been reported by Hibbs and Gouaux, defining a binding site and activation mechanism of the avermectin potentiators (PDB ID: 3RHW) [49]. A representation of the ion channel in its proposed open conformation is shown in Figure 4.3, with one ivermectin-binding pocket highlighted in yellow.

Despite the favorable distinction and high parasiticide selectivity for invertebrates, the complex scaffold of the macrocyclic lactones constrains further extensive and profitable derivatization and optimization of this compound class. Coupled with the emerging resistance toward GABA blockers, the development of novel modulators of ligand-gated chloride channels is pressing. However, a new innovative substrate class has been developed over recent years with an intriguing activity profile.

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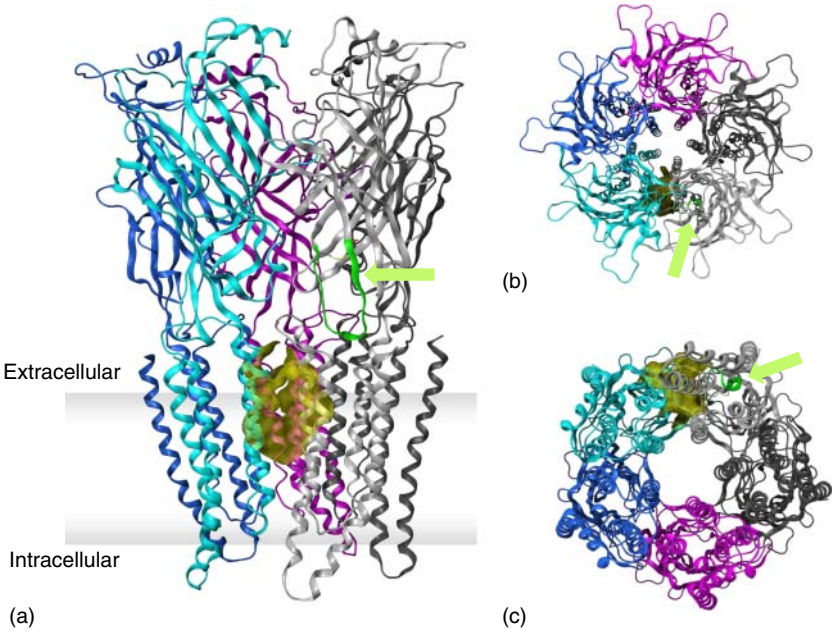


Figure 4.3 GluCl crystal structure of *C. elegans* represented in an activated, open-channel state. These images are based on an original X-ray crystallographic structure (PDB ID: 3RHW) [49] and were produced with the MOE software package [41]. Fab molecules are omitted for clarity. The five individual subunits are shaded in different colors. The surface of one ivermectin-binding pocket is highlighted in yellow. The Cys loop of one subunit is highlighted in light green (light-green arrows). (a) Protein parallel to membrane, horizontal gray bars indicate membrane boundaries. (b) View along the channel pore axis from the extracellular side, with M2 helices visible as the inner pore lining. (c) View along channel pore axis from the intracellular side.

Isoxazoline Mode of Action and Binding Site

A dose-dependent inhibition of GABACls in *D. melanogaster* by afoxolaner was observed in electrophysiology studies with an IC_{50} value of 3.7 nM and no detectable cross-resistance effect [29]. Similarly, electrophysiology studies on CysLGCCs of *Musca domestica* confirmed the efficient inhibition of agonist-induced currents in GABACls and GluCls by fluralaner, with IC_{50} values of 5 and 80 nM, respectively, identifying the GABA receptor as the more sensitive of the two [27]. Additionally, it retains its potency even in the dlr mutant of the *M. domestica* chloride channel (A299S). In comparison with fipronil, both isoxazoline derivatives exhibit markedly higher potency toward GABACls, emphasizing

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Table 4.3 Ectoparasiticide activity (IC_{50} (nM)) in binding assays on *M. domestica* head membrane.[a)]

Radioligand	Fluralaner	AVE[b)]	Fipronil	References
[^{3}H]fluralaner	0.4	3	PI	[50]
[^{3}H]EBOB[b)]	~40	I	~40	[51]
[^{3}H]AVE[c)]	PI	20	NI	[51]

a) I, inhibition; PI, poor inhibition; and NI, no inhibition.
b) 4′-Ethynyl-4-*n*-propylbicycloorthocarboxylate. Fipronil showed no significant association to the isoxazoline- or avermectin-binding sites, setting it apart from the other two insecticide classes. AVE seems to have a modulating effect on the isoxazoline-binding site, as [^{3}H]fluralaner is displaced; however, fluralaner has only poor activity on [^{3}H]AVE. All three ectoparasiticides displace the more general [^{3}H]EBOB ligand to various degrees, with AVE showing the least activity. The overview is compiled from studies reported by Casida and coworkers [50, 51].
c) Avermectin B_{1a}.

the crucial advancement to the aforementioned parasiticides achieved and reviving the susceptibility of CysLGCCs as valid drug targets.

In initial assays on housefly head membrane to elucidate the binding site of isoxazoline derivatives, fluralaner successfully impeded [^{3}H]EBOB binding at subnanomolar concentrations in a similar manner to known antagonists [27]. Ensuing comprehensive evaluation of the specific binding site in housefly head membrane revealed avermectin analogs as effective inhibitors of radiolabeled [^{3}H]fluralaner binding; however, poor displacement by antagonists such as fipronil was detected [50]. The isoxazoline consequently addresses a binding site reasonably distinct from that of CysLGCCl blockers and is closely associated with that of activators, despite being a potent chloride channel blocker. Casida *et al.* summarized this unique sensitivity profile in a comparative binding site study with various radiolabeled ligands including [^{3}H]fluralaner, [^{3}H]EBOB, and avermectin B_{1a} ([^{3}H]AVE) (Table 4.3) [51]. It highlights the exceptional activity of isoxazolines and their distinct target site. Notably, this also implies the absence of common features that might lead to the appearance of cross-resistance and supports the previously reported observations in dlr mutants. No clear assignment of an isoxazoline-binding pocket by X-ray crystallography or other methods has yet been reported. However, based on the evidence provided by the aforementioned binding studies, an interaction with the helical subunits of the transmembrane domain in CysLGCCs between the identified agonist and antagonist sites is conceivable. In comparison, parasiticide-binding regions of GABA blockers and ivermectin relative to each other are depicted in a schematic illustration of a CysLGCC (Figure 4.4). Further in-depth research is required to fully elucidate the inhibition site and mechanism of the novel isoxazoline compound class.

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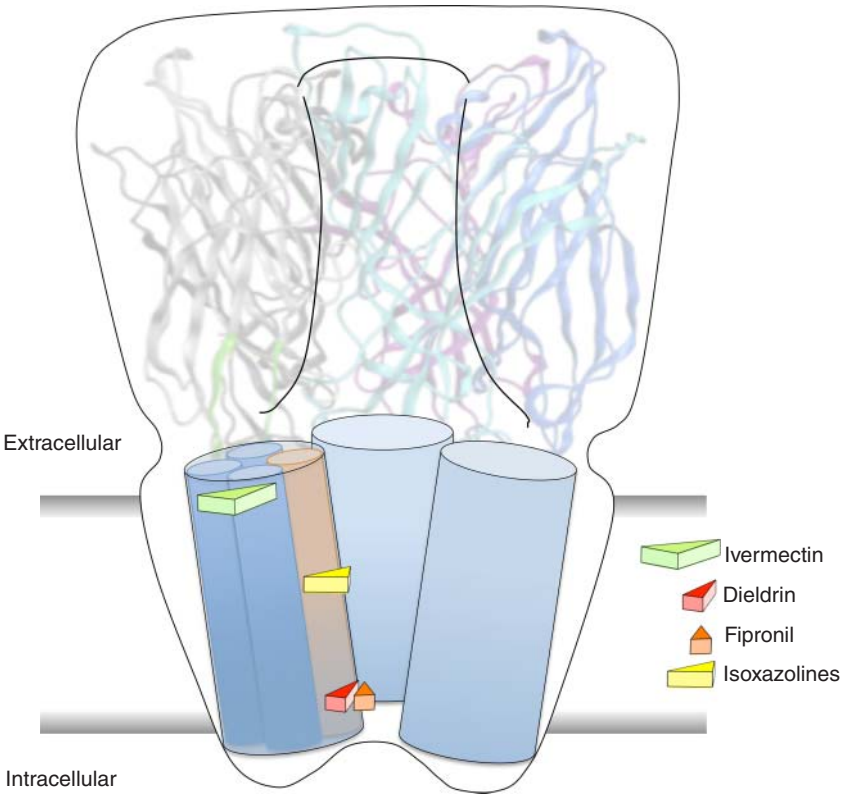


Figure 4.4 Schematic representation of CysLGCCs in a sectional view with only three units of the pentameric transmembrane region depicted for clarity. Positioning of isoxazolines is putative.

Selectivity and Safety Profile

The ectoparasitic activity of the novel isoxazoline chemotype was evaluated in a series of studies against prominent flea and tick species, such as *C. felis*, *D. variablis*, and *R. microplus*, as well as other pests including an assortment of flies and mosquitoes [27, 33, 52, 53]. Identification, cloning, and functional expression of GABA- and glutamate-receptor genes of *R. microplus* in addition to the expression of dlr and susceptible *C. felis* and *D. melanogaster* GABA receptors have sparked the development of on-target assays, and a comprehensive exploration of the chloride channel inhibiting qualities relative to relevant representative parasiticides ensued [33]. Accordingly, a plethora of studies verify the superiority of the new chemotype, emphasizing the advanced potency over fipronil, dieldrin, and other GABACl antagonists in arthropods. In a direct comparison, the corresponding GluCls are effectively impeded in their activity, but show less sensitivity than GABACls, inferring a clear preference in the targeted receptors. Mammalian

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chloride channels, as in rat brain membrane, showed no significant response to isoxazoline ectoparasiticides in binding assays [27].

Critically, [^{3}H]EBOB binding in recombinant human GABACl β3 homopentamers, which represent an appropriate model for the analysis of toxicity by displaying generally high sensitivity toward standard GABAergic antiparasitic agent, remained equally unaffected [51]. In this regard, a high level of specificity for arthropods had been accomplished, justifying the renewed interest in CysLGCCs as antiparasitic drug targets. Fipronil, on the other hand, exhibits less pronounced selectivity against the mammalian GABACls, with IC_{50} values of 800 and 3.4 nM in rat brain membrane and the human β3 homopentamer, respectively [51].

Isoxazoline Derivatives: Continuous Exploration of the Novel Chemotype

The introduction of isoxazoline derivatives has shown impressive results concerning the selective inhibition of insect and acarid GABACls and GluCls. Moreover, a novel binding site is key to the innovative activity profile, which bypasses the critical cross-resistance observed in other noncompetitive antagonists [51]. With regard to the disclosed isoxazoline selectivity and activity qualities, research is continuously striving to explore the full potential. As such, Du Pont researchers reported on isoxazoline derivatives for agricultural applications with small *N*-heterocycles as substitutes for the carboxamide side chain [28]. Structurally reminiscent of the phenylpyrazole fipronil, a range of thus generated compounds exhibit parasiticidal activity against a variety of pests. Additionally, one representative was found to efficiently block GABA-induced currents in both wild-type and dlr mutant receptors of *D. melanogaster* [28].

Two other isoxazoline-related compounds have been registered by leading pharmaceutical companies as antiparasitic agents, with the INNs lotilaner [35] by Novartis AH, now Elanco, and sarolaner [36] by Zoetis (Table 4.2); however, no full data have been released yet. Both introduce heterocycles as modifications to the phenyl isoxazoline core and adjust the substitution pattern on the terminal phenyl moiety. On September 11, 2015, Zoetis Belgium SA received a positive opinion from the European Medicines Agency and its Committee for Medicinal Products for Veterinary Use, recommending the granting of a marketing authorization for the veterinary medicinal product Simparica chewable tablets – active ingredient sarolaner – intended for the treatment of tick, flea, and mange mite infestations in dogs [54]. In contrast to NexGard and Bravecto, which represent racemates of afoxolaner and fluralaner, respectively, Simparica represents the biologically active *S* enantiomer of sarolaner only [55, 56]. Searching the patent literature revealed numerous additional isoxazoline-related data and filings coming out of crop science and animal health [57, 58]. The interest in and research on isoxazolines as effective ectoparasiticides are therefore still unbroken and the high potency in GABACl and GluCls coupled with the encouraging safety profile is an attractive portfolio to justify further investigations.

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Conclusions

Acarid and insect ectoparasites such as ticks and fleas pose a serious problem in the agricultural and veterinary sector, as they elicit a severe derogatory effect on crop yield and animal health and welfare. The development of potent antiparasiticides is therefore not only essential but also urgent.

Ligand-gated chloride channels have been prominent targets in the battle against these parasitic species; however, emerging resistance development across various chemotypes, subsequent potency depletion, and slow advancement in this research area have raised concerns over the sustainability of those targets. The outlined recent examples of novel isoxazoline derivatives as potent insecticides mark an inflection point in this development, resurrecting GABA and GluCls as valid drug targets and reviving research interest. Notably, comprehensive exploration and evaluation have led to the development of noncompetitive antagonists with activities as low as subnanomolar against a range of insect and acarid pests while exhibiting low inhibition toward the mammalian GABACl analogs. Unprecedentedly, the isoxazoline blockers were shown to circumvent cross-resistance by addressing a distinct new binding pocket in the chloride channels, setting the stage for an innovative new approach to tackling ectoparasitic infestations. Until now, this progress has culminated in the approval of three orally administered veterinary products: NexGard, Bravecto, and Simparica against ectoparasites on dogs [26, 31, 36]. Additionally, combination products such as NexGard Spectra, which combines the ectoparasiticide afoxolaner and the endoparasiticide milbemycin oxime, have been launched [54]. In view of these developments, further derivatives and combinations are certainly in the pipeline to fully explore the isoxazoline scaffold's potential. Notably, although this compound class originates from research in crop science, it was first marketed in the veterinary sector, and no product for the crop science market has been released yet. Similar to other compounds that were initially launched with a veterinary parasitology application, for example, benzimidazoles, praziquantel, ivermectin [59], isoxazolines seem to have the potential to enter the human-host parasitology applications as well, preventing extensive feeding or blood meals by insects, mites, lice, and ticks. If so, isoxazolines might be able to impede arthropod-borne diseases such as Lyme disease, tick-borne encephalitis, or even neglected tropical diseases such as human African trypanosomiasis (sleeping sickness), leishmaniasis, and malaria, for which respective claims have already been made in the patent literature [26, 31, 35, 36, 57, 58]. However, for the time being, the question remains open as to how fast systemic isoxazolines act on the different feeding parasites. It would require the ectoparasite to fall off the host before the disease-causing agent is transmitted, which might be, depending on the parasite and disease, a challenging goal to achieve.

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Trypanosomal Cysteine Peptidases: Target Validation and Drug Design Strategies

*Elany Barbosa da Silva, Glaécia Aparecida do Nascimento Pereira, and Rafaela Salgado Ferreira**

Abstract

Parasitic cysteine peptidases (CPs) have been intensely studied and validated as therapeutic targets for several diseases. They perform diverse functions in the life cycle of parasites, being important for their nutrition, host cell invasion, differentiation, and evasion of the host immune system [1, 2]. Experiments with synthetic and endogenous inhibitors have allowed a better understanding of their role in disease pathogenesis. This chapter focuses on trypanosomal CPs, especially cruzain, from *Trypanosoma cruzi*, and rhodesain and TbCatB, from *Trypanosoma brucei*, describing their characterization and functions for the respective parasites and disease pathogenesis. Based on these studies, these enzymes have been validated as therapeutic targets for the neglected diseases Chagas disease and African sleeping sickness. The available chemotherapies for these diseases have limited efficacy and high toxicity, therefore new drugs are needed, and cysteine peptidase inhibitors have been exploited toward this goal. The final section of this chapter describes such efforts.

Cysteine Peptidases from *Trypanosoma cruzi*

Identification and Validation of Cruzain as a Therapeutic Target for Chagas Disease

Cruzain (also referred to as *cruzipain* or *GP57/51*)[1] is the major and best characterized cysteine peptidase (CP) from *Trypanosoma cruzi*, and it is a therapeutic target for Chagas disease. Despite description of a cathepsin-B-like CP from *T. cruzi* [3, 4] and of cruzain isoforms [5], this chapter focuses on cruzain itself, given its potential as a therapeutic target.

* Corresponding author.

[1] Some authors refer to the native enzyme as cruzipain and to the recombinant form as cruzain. Throughout the chapter this distinction will not be made.

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Reports in the late 1970s indicated proteolytic activity in *T. cruzi* extracts, suggesting that it might be due to an unknown CP. A few years later, successive efforts reported the purification of a CP from *T. cruzi* epimastigote extracts, which was most active in acidic pH [6]. This enzyme was described as a glycoprotein and its partial sequence was reported, leading to its classification into the papain family, with high similarity to a *Trypanosoma brucei* enzyme, later named rhodesain [7, 8]. In the early 1990s, initial studies on synthetic substrate specificity were described, indicating similarities to cathepsin L, but also the peculiarity of cleaving a substrate containing Arg in the P2 position, a known characteristic of cathepsin B [9]. The enzyme is encoded by multiple genes [10], organized in tandem in different chromosomes [11], and expressed in all life-cycle stages of the parasite, as judged based both on mRNA [10] and on protein presence using specific antibodies [12]. It was subsequently cloned and recombinantly expressed in bacteria. Kinetic parameters and substrate specificity for the recombinant protein, called *cruzain*, were consistent with those previously reported for the endogenous peptidase [10]. The complete sequence indicated a C-terminal extension of 130 amino acids, a specific feature of trypanosomal and other parasitic CPs [10]. This domain is autocatalyticaly removed [13]. Engineering of a truncated cruzain-Δ*c* allowed preparation of homogeneous samples, which was crystallized [14]. This was closely followed by the publication of the first cruzain crystal structure, obtained in complex with a fluoromethylketone (FMK) [15]. Initial validation of cruzain as a drug target was by experiments in which FMK inhibitors arrested intracellular amastigote replication and differentiation to trypomastigotes [16, 17]. Additionally, the growth of *T. cruzi* overexpressing cruzain is less affected by the cysteine peptidase inhibitor (CPI) diazomethane Cbz-Phe-Phe-CHN_2, when compared to wild-type parasites [18]. The efficacy of FMK and vinyl sulfone inhibitors was also demonstrated on mouse models of the disease, both nonimmunodeficient [19] and immunodeficient [20] and on dogs [21]. At a cellular level, these inhibitors produced abnormalities in the endoplasmic reticulum and in the Golgi complex, due to accumulation of unprocessed enzyme, causing expansion of these compartments both in epimastigotes [22] and in amastigotes [19].

Cruzain has different localizations in the parasite, depending on the life stage and related to the distinct functions it performs. Through investigation of peptidase activity on fractions obtained by ultracentrifugation of epimastigotes, it was found to localize in the lysosomes [23]. This was later confirmed by immunoelectron microscopy, which also demonstrated its presence in the surface of epimastigotes and amastigotes. In trypomastigotes, it is localized in lysosomes and in the flagellar pocket, suggesting a mechanism for protein secretion at this life-cycle stage [24]. Mutagenesis studies of the cruzain prodomain indicate that this region is important for protein trafficking, directing the enzyme to lysosomes [25]. Activation of cruzain is also essential for membrane localization, since treatment with a vinyl sulfone inhibitor, which leads to accumulation of unprocessed

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protein in the Golgi complex, reduces enzyme concentration in membranes of amastigote forms of the parasites [19].

Cruzain Is Important for Metacyclogenesis and Host Cell Invasion

Several studies indicate the importance of cruzain for host cell invasion and metacyclogenesis, based on the effects of synthetic [16, 17, 26] and endogenous [27] inhibitors or on phenotypes expressed by genetically modified parasites, which express different levels of cruzain [18, 28, 29] or of its endogenous inhibitor, chagasin [27]. A comparison between three pairs of virulent *T. cruzi* strains and their attenuated counterparts, which show reduced infectivity in animal models, revealed lower proteolytic activity in the latter [29].

Metacyclogenesis is blocked by incubation of epimastigotes with synthetic CPIs [26]. In accordance to these results, transfection of epimastigotes with cosmids containing multiple cruzain copies increases enzyme activity and enhances metacyclogenesis [18]. Overexpression of chagasin in *T. cruzi* epimastigotes resulted in impaired metacyclogenesis and reduced sensitivity to a vinyl sulfone cruzain inhibitor. Moreover, studies with trypomastigote cultures show that addition of chagasin or its overexpression impaired invasion of smooth muscle cells by the parasites. This is consistent with the lower infectivity of the *T. cruzi* G strain, which naturally has a higher chagasin:cruzain ratio [27].

Two pathways for cruzain-mediated cell invasion have been characterized, differing in their kinin dependence. Cruzain generates bradykinin through kininogen cleavage, and this process is inhibited by the CPI E-64 [30]. Assays with cells that express high levels of bradykinin-2 receptors (B_2Rs) showed that *T. cruzi* could induce Ca^{2+} transients, and this effect was blocked by B_2R antagonists. Additional evidence for the importance of this pathway was given by the observation that captopril, a drug that prevents bradykinin degradation, potentiated cell invasion by *T. cruzi in vitro*. Native cruzain also induces Ca^{2+} transients and increases cell invasion, in a manner dependent on its catalytic activity [31]. Bradykinin-mediated pathways, through B_1R and B_2R, are related to the development of an edematogenic response, contributing to the Chagas disease pathology [32]. The inflammatory response generated leads to plasma leakage and kininogen accumulation, therefore facilitating cell invasion and proliferation of *T. cruzi* [33]. Furthermore, kinin release by cruzain is modulated by heparin sulfate (HS). In the presence of HS, k_{cat} is increased and K_m is decreased, enhancing enzymatic activity (Figure 5.1) [34].

A kinin-independent invasion pathway, which is not affected by either B_2R or B_1R antagonists, has also been described. Several *T. cruzi* strains were studied, and their infectivity was associated with cruzain secretion levels. A vinyl sulfone inhibitor decreased host cell invasion, while cruzain-rich supernatants from trypomastigotes were shown to increase invasion by the less infective strains. Additional experiments indicated that cruzain processes a substrate released

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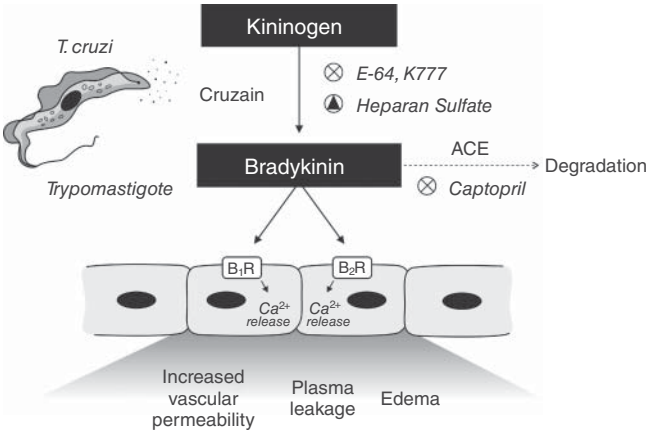


Figure 5.1 Cruzain promotes cell invasion through a kinin-mediated pathway. Cruzain generates bradykinin from kininogen, a cysteine peptidase activity inhibited by E-64 and K777 (⊗) and enhanced by heparan sulfate (▲). Bradykinin acts on bradykinin receptors type 1 and 2 (B_1R and B_2R), leading to Ca^{2+} release and increasing vascular permeability, edema, and plasma leakage. Captopril, an angiotensin-converting enzyme (ACE) inhibitor, reduces bradykinin degradation and potentiates *T. cruzi* invasion.

by trypomastigotes, forming the substance actually responsible for invasion. Similarly to previously described mechanisms, this also involved Ca^{2+} mobilization [28].

Another invasion mechanism promoted by cruzain is TGF-β activation. Anti-TGF-β neutralizing antibodies inhibit Vero cell invasion by *T. cruzi*. Both experiments with an FMK inhibitor and with parasites overexpressing chagasin indicated inhibition of TGF-β activation [35]. Altogether, these studies show cruzain's role in several invasion mechanisms, contributing to disease pathogenesis of the *T. cruzi* infection.

Cruzain as an Antigen and Immunomodulator

Originally described as GP57/51, cruzain has been identified as an important antigen and evaluated for diagnostic and vaccine applications. Studies conducted which Chagas disease patients either in the cardiac or in the undetermined stage of the disease confirmed an important role for cruzain on the induction of T-cell responses. Sera from all the patients evaluated responded to this enzyme, regardless of the clinical form of the disease observed [36]. Interestingly, while several *T. cruzi* antigens show cross-reactivity against leishmaniasis [37], cruzain has been shown to be a specific antigen for Chagas disease, being promising for diagnosis [38].

Humoral response to cruzain is related with the severity of the disease, as patients with a severe form of the disease (myocardial dysfunction) show high antibody titers directed against this enzyme, when compared to those with a milder form of the disease [39]. Antigens against either the full-length, mature

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cruzain or the C-terminal domain were observed in sera from chronic-stage Chagas disease patients [40]. However, further studies indicated that most antibodies were directed to the C-terminal, suggesting independent catalytic and immunogenic domains [41]. Furthermore, C-terminal domain sulfates are essential for antibody production, especially IgG2b, and involved in heart tissue damage [42]. The C-terminal targeted response may constitute a mechanism to protect the catalytic domain. A possible strategy to overcome this is the immunization with the N-terminal domain, which redirects the immune response and more effectively reduces parasitemia and tissue damage [43].

To further evaluate cruzain as a candidate for vaccine development, effects of cruzain injection in mice have been evaluated. Skin tests performed 2 weeks after injections revealed hypersensitivity responses and IgE and IgG1 antibodies were detected in the sera. Mice immunized showed higher survival rates 2 months after a challenge *T. cruzi* infection [44]. Several responses to cruzain were investigated, such as antibody production in response to mucosal infections and indications of B- and T-cell responses. IFN-γ responses are induced, indicating T_h1 responses. Immunized animals also induced lysis of *T. cruzi*-infected cells [45]. Cruzain-specific T_h1 cells protect against *T. cruzi* intracellular replication, reducing both the number of infected host cells and the number of amastigotes per infected host cell [46]. Considering these promising results, different regimens for immunization with recombinant cruzain or DNA vaccines were taken forward, demonstrating successful protection of mice from *T. cruzi* infections, decreasing parasitemia and reducing tissue damage and death rates [46–48]. A recent study in which a DNA vaccine encoded cruzain and two additional antigens, the thioltransferase (Tc52) and a flagellar calcium-binding protein (Tc24), showed better results than the immunization for each individual antigen [49]. Regarding the toxicity of immunization with cruzain, contradictory effects have been reported, and one study reports no adverse effects [46], while other studies report the generation of myosin-directed antibodies and transitory splenomegaly [50].

The generated T_h1 response is dependent on the activation of B_2R, which, as described previously, are activated by cruzain generated kinins, and on Toll-like receptor 2 (TLR2), which induces plasma leakage and kininogen accumulation at the site of infection [51]. In accordance to the importance of B_2R for maturation of dendritic cells and the immune response, $B_2R^{-/-}$ were shown to be more susceptible to *T. cruzi* infection [52].

An induction of T_h2 response by cruzain immunizations has also been reported. Considering that this profile favors susceptibility to infection, it was suggested that cruzain supports the persistence of the parasite in the host. This T_h2 response may also favor the activation of the alternative macrophage pathway [50].

Cruzain's role in the evasion of the immune system has also been investigated. Degradation of all IgG classes by this enzyme *in vitro* provides evidence that the immune response is indeed affected by the protein [53]. Comparing wild-type and cruzain-deficient *T. cruzi* strains, it was shown that cruzain is important to avoid NF-κb production, degrade NF-κb, and prevent macrophage activation. Additionally, cruzain-deficient parasites are not lethal to their host even at much

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higher concentrations than the wild-type counterparts [54]. Interestingly, cruzain can also induce cardioprotective effects, which may be important for long-term infection. Inactivated cruzain activates TRL2 and induces IL-6, leading to an antiapoptotic effect, while active cruzain abrogates this effect. Active cruzain is able to cleave gp130, possibly playing a central role on immune evasion mediated by this cytokine [55].

Cysteine Peptidases from *Trypanosoma brucei*

Identification of Cysteine Peptidase Activity and Essentiality for *T. brucei*

CP activity was identified in *T. brucei* cultures in the 1980s. Partial sequences and cloning revealed the presence of cathepsin-L-like peptidases [8, 56], which, however, contained an addition C-terminal domain [57], later identified in other trypanosomal CPs. More than 20 CP coding genes were identified in tandem [57]. Several CPIs, such as vinyl sulfones and methylketones, are trypanocidal against bloodstream forms of *T. brucei in vitro* [58, 59]. Biotinylated inhibitors indicated rhodesain as the most likely intracellular target [59, 60]. Further evidence of the essentiality of this enzyme was provided by *in vivo* studies in mouse models, in which treatment with Z-Phe-Ala-diazo methylketone decreased parasitemia and increased mice survival to about twice the time observed for animals treated with placebo [61]. To allow further enzyme characterization, rhodesain has been recombinantly expressed in *Escherichia coli* and [62] and later in *Pichia pastoris*, as a mature and active protein, allowing biochemical characterization and inhibitor studies with several vinyl sulfones and epoxide inhibitors. Rhodesain was shown to be the major CP in *T. brucei* and to be localized in the lysosomes using immunoelectron microscopy [63]. The ortholog enzymes from *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei brucei*, which share 98.4% residue identity, were respectively named rhodesain and brucipain [63]. Later, a lysosomal cathepsin-B-like enzyme was identified and named TbCatB [64, 65]. This discovery led to the investigation of roles for each of these CPs, as described as follows.

Dissecting the Relative Importance of TbCatL and TbCatB Enzymes

Given that both CatL-like (known as *rhodesain*, *brucipain*, or *TbCatL*) and CatB-like (TbCatB) CPs are present in *T. brucei*, RNAi studies were conducted to investigate which of these enzymes would be a suitable target for chemotherapy. While rhodesain knockdown did not result in any detectable phenotypic modifications, even partial TbCatB knockdown led to parasite death, leading to the conclusion that the latter was the most appropriate target for drug discovery. It is important to note, however, that rhodesain was only partially knocked down in this study [64].

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Later, RNAi studies in mice confirmed the essentiality of TbCatB, as its knockdown led to clearance of parasitemia and prevented splenomegaly [66].

In accordance with the *in vitro* studies [64], RNAi rhodesain knockdown (by 60%) neither induced parasite death nor prevented splenomegaly in mice. However, it increased mice survival, therefore suggesting a key role in disease development [66]. Moreover, *in vitro* and *in vivo* RNAi studies only led to partial rhodesain knockdown proposing that a complete inactivation of the protein may have profound effects on parasite survival. This was addressed by Steverding, who investigated the importance of this enzyme through chemical inhibition. By employing inhibitors with different specificities against *T. brucei* CPs, this work led to conclusions opposed to the previous RNAi studies. Inhibition of greater than 95% of TbCatB activity, by the CatB specific inhibitor CA-074, did not cause parasite death. It was argued that the RNAi data previously reported were due to off-target effects of the RNAi. On the other hand, the authors concluded that rhodesain is a target for drug intervention of African sleeping sickness because trypanocidal activity was observed by compounds that inhibited this enzyme greater than 99% and TbCatB by 70%, while compounds specific for TbCatB were not trypanocidal [67]. Another investigation reported trypanocidal activity by nitriles, which were potent rhodesain inhibitors and exhibited modest TbCatB inhibition [68].

Investigating the Role of Cysteine Peptidases for *T. brucei*

Encouraged by the RNAi study results [64, 66], which indicated the importance of TbCatB, the role of this peptidase has been further investigated with a single allele knockout. The mutant parasites had enlarged lysosomes upon treatment with the diazomethyl ketone CPI, Z-Phe-Ala-CHN_2. Abnormal protein localization was also observed: whereas the peptidase is usually lysosomal, in the knockout it was found in a region proximal to the lysosome. Previous work showed transferrin accumulation upon TbCatB knockdown, indicating this protein, which is essential for iron acquisition in the parasite, as a possible substrate. Substrate profiling with combinatorial libraries allowed for determining the specificity of recombinant TbCatB and indicated possible cleavage sites in transferrin. The degradation of transferrin was confirmed *in vitro*, and the subsequent upregulation of transferrin receptors, which occurs as a consequence of iron starvation, was also verified. This study also suggested rhodesain and variant surface glycoprotein as possible substrates for TbCatB [65].

On the other hand, rhodesain is involved in crossing the blood–brain barrier (BBB) by *T. brucei*. It is believed that most likely BBB transmigration occurs through a paracellular mechanism. However, experiments employing fluorescent parasites and confocal microscopic showed that *T. brucei* also enter brain microvascular endothelial cells (BMECs) [69]. Initial studies indicated that *Trypanosoma brucei gambiense* was more effective in crossing the BBB than *T. b. brucei* [70]. The difference among *T. brucei* species to cross the BBB was later linked to rhodesain levels [71]. It was also shown that BBB crossing was inhibited

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by the addition of K777 in a dose-dependent manner, but not by CA-074. Additionally, the ability of *T. b. brucei* to cross the BBB was increased upon addition of a medium enriched with rhodesain [71]. Parallel evidence was provided by RNAi studies, in which rhodesain knockdown reduced the ability of *T. b. brucei* to cross a BBB model by 50% [66]. The mechanism by which penetration of the BBB is facilitated is related to triggering of Ca^{2+} fluxes in BMECs. This was supported by the observation that rhodesain inhibitors abolish parasite-induced calcium oscillations and that calcium chelators inhibit BBB crossing by *T. b. gambiense.* Additionally, pretreating human BMECs with PLC-β or PKC inhibitors prevented parasite crossing, providing further evidence of a Ca^{2+}-dependent mechanism [71]. Rhodesain has also been implicated in cardiac dysfunction, increasing cardiomyocyte contractions, leading to Ca^{2+} release in these cells and to arrhythmic events in the whole heart. These effects were inhibited by K777 or by rhodesain RNAi [72].

Similarly to cruzain, *T. brucei* CPs are also endogenously regulated by an ICP inhibitor. Experiments with Δicp parasites indicate a role for CPs in surface coat exchange, necessary for parasite differentiation, and also in anti-VSG IgG degradation and parasite infectivity and that these functions are regulated by ICP [73].

Development of Antitrypanosomal Cysteine Peptidase Inhibitors

In recent decades, different chemotypes were identified as trypanosomal CPIs, including peptidic and nonpeptidic small molecules that interact with the enzymes in a reversible or irreversible manner [74, 75]. Among the peptidic inhibitors, usually there is a di- or tripeptide connected to an electrophilic warhead, which reacts with the active-site cysteine residue [74, 76, 77]. Despite the high affinity these compounds usually display, they are more susceptible to enzymatic or chemical hydrolysis; thus, special emphasis has been applied to identifying nonpeptidic inhibitors. Based on the bioisosteric relation between peptide bonds and ureas, aryl ureas and thioureas, competitive and reversible nonpeptidic cruzain and rhodesain inhibitors were identified [78]. In addition, thiosemicarbazones were also shown to be potent inhibitors [75, 79], and further searches for other classes of nonpeptidic CPIs led to the discovery of hydrazones [80, 81], triazines [68], *N*-acylhydrazones [82, 83], and vinyl sulfones [76] as structural prototypes (Figure 5.2, Table 5.1). Vinyl sulfones are a widely studied class for which both peptidic [93, 106] and nonpeptidic [76] derivatives have been described. More details concerning each of these classes are described as follows. We focus on the known structure–activity relationships (SARs) obtained against the enzymes and how well these compounds were further validated, including determination of crystallographic complexes and evaluation against the parasites and animal models of the respective diseases.

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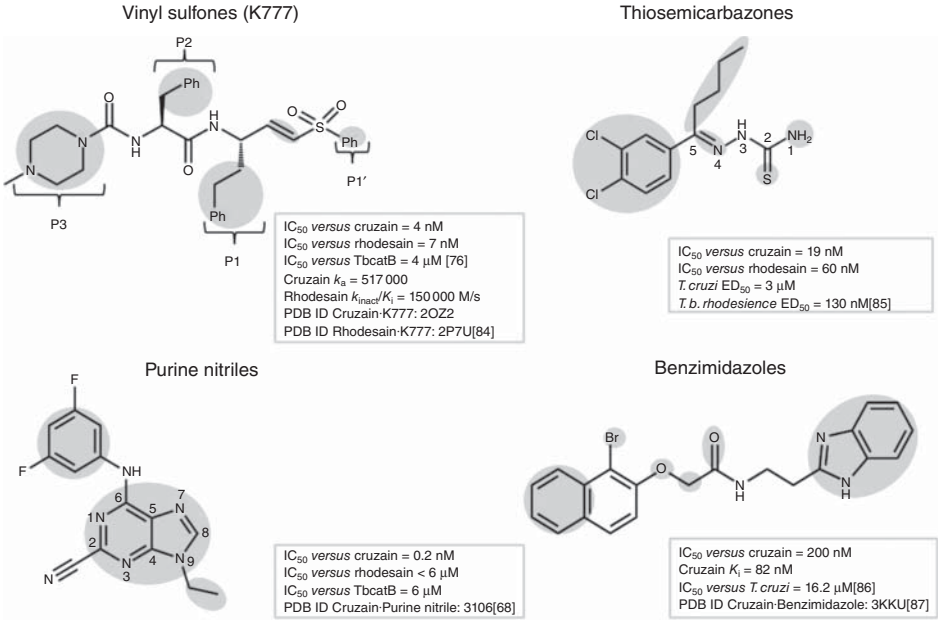


Figure 5.2 Different classes of potent cysteine peptidase inhibitors. Regions shaded in gray represent exploited portions in SAR studies.

Peptidic Derivatives

Peptide analogs are among the first CPIs explored [107]. Their development took advantage of the known substrate specificities for the respective peptidases. The most studied classes of peptidic derivatives are peptidyl chloromethyl [108], fluoromethyl [19], epoxy ketones [88], and α-ketoesters [74]. These inhibitors usually exploit the S1′, S1, S2, and S3 subsites, through interactions with moieties located at P1′, P1, P2, and P3, respectively. Several modifications were performed to obtain SAR trends for this inhibitor class. The carboxybenzyl (Cbz) moiety in P3 is present in a number of known potent irreversible inhibitors of cruzain, and its removal caused dramatic loss of activity. Similarly, its substitution by an acetyl [74] or by a morpholine was not beneficial, whereas the replacement by 3-pyridinyl increased activity [89]. Besides, Cbz peptide derivatives inhibited the growth of *T. cruzi* amastigotes and were not toxic against J774 mouse macrophages (Table 5.1) [74]. Due to the large and predominantly hydrophobic nature of the S2 pocket in cruzain and rhodesain, the P2 substituent usually presents these features. The presence of L-Phe in P2 subunit, and to a lesser extent L-Leu, L-Val, and L-Met, is favorable for activity [74, 89]. However, it is important to note that cruzain also accepts positively charged groups in P2 [109]. SAR trends observed for the P1 moiety indicate preference for a center with *S*-configuration

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Table 5.1 Reported properties for several classes of trypanosomal cysteine peptidase inhibitors.

Compound class	Most potent inhibitors			Activity against *T. cruzi*	*T. brucei* ED_{50} [a]	Crystal structure PDB IDs	Cytotoxicity (nontoxic against)	Enzyme selectivity
	Cruzain IC_{50}	Rhodesain IC_{50}	TbCatB IC_{50}					
Peptidic derivatives	5.5 nM [88] See also: [88–90]	3.5 nM [88]	400 nM [88]	Inhibition of amastigotes growth at 30 μM [74]	Low activity [88]	Cruzain: 1U9Q [74] 1ME3 and 1ME4 [91]	J774 macrophages [74]	Cruzain over Cat B and L [89]
Vinyl sulfones	<1 nM [92] See also: [76, 77, 93]	40 nM [93] See also: [76]	16 μM [76]	Inhibition of amastigotes growth at 10 μM [77]	0.1 μM [59] See also: [76]	Rhodesain: 2P7U Cruzain: 2OZ2 [84] 3HD3 [76]; 1F29, 1F2A, 1F2B, and 1F2C [92]	Jurkat cells [76] J774 macrophages [77]	NT
Thiosemicarbazones	8 nM [94] See also: [75, 85, 95]	7 nM [96] See also: [79, 85, 97]	150 nM [96]	Inhibition of amastigotes growth at 10 μM [75, 79] See also: [85, 94]	130 nM [85] See also: [96]	NT	J774 macrophages [75], BJ, Raji, HEK 293, and HEP G2 cells [96]	CatL-like enzymes [96]
Hydrazones	10 nM [81] See also: [80, 82, 98]	10 μM [80]	NT	IC_{50} for trypomastigotes = 2.3 μM [82] See also: [80, 81, 98]	NT	NT	Mice splenocytes [80, 81, 96, 98] HEP G2 [81]	Cruzain over Cat S and L [98]
Nitriles	0.2 nM [99] See also: [68, 100]	60 pM [100] See also: [68, 101, 102]	240 nM [102] See also: [68, 103]	IC_{50} for amastigotes = 18 nM [68]	0.9 μM [100] See also: [68, 102]	Cruzain: 3I06[68]	BJ, HEP G2,[102] L-6 myoblasts [101], HEK 293, Raji cells [103]	Cruzain over Cat L, B, S, and F [99] See also: [99, 101, 102]
Other nonpeptidic inhibitors	0.21 μM K_i = 80 nM [86] See also: [87]	44 nM [104]	270 nM [104]	IC_{50} for amastigotes = 1.6 μM [86] See also: [105]	2 nM [104]	Cruzain: 3KKU [87] 4KLB [105]	Mice spleen cells [105]	NT

a) Activity against bloodstream-form trypanosomes.

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(or L-amino acids) [74, 89, 107] and for β-branched alkyl substituents, especially L-Val [74]. Some inhibitors with a homophenylalanine side chain at P1, which tend to be more resistant to metabolism, showed high potency [89].

Lastly, (S)epoxy [107], epoxyesters [88], and *p*-substituted benzylamines [74] are very potent compounds. Dipeptidyl epoxyesters also inhibited rhodesain at very low concentrations [88]. It was also observed that the insertion of large bulky substituents or long hydrophobic chains, such as *tert*-butyl mercaptan and 3-phenyl propyl mercaptan, results in nanomolar cruzain inhibitors. In addition, some of these compounds were selective over human cathepsins B and L (Table 5.1) [89]. In addition to variations in the P1′ – P3 substituents, modifications on the Michael acceptors have also been evaluated, leading to potent rhodesain inhibitors [110].

Several structures of cruzain covalently bound to peptidic inhibitors have been obtained, including complexes with an α-ketoester (Table 5.1) [74], vinyl sulfones [76, 84, 92, 111], and FMKs [109, 112]. Covalent binding between cruzain and a peptidyl halomethyl ketone has been recently investigated by hybrid quantum mechanics/molecular mechanics and molecular dynamics studies, allowing evaluation of the most likely binding mode [108]. Additionally, high-resolution complexes of noncovalently bound peptidyl ketones with cruzain have also been obtained, providing further basis for structure-based drug design efforts (Table 5.1) [91].

Vinyl and Allyl Sulfones

Vinyl sulfones are an important CPI class, especially due to their high potency and selectivity for CPs over serine peptidases [113]. These irreversible inhibitors contain a Michael acceptor and react with the active-site cysteine [77] of the peptidases. Since the validation of vinyl sulfone K777 (Figure 5.2), one of the earliest potent cruzain inhibitors identified, a lot of work has been performed to explore this promising class, including validation against trypanosomes [111], in animal models of Chagas disease [19, 21, 111] and African sleeping sickness [114].

Several optimization efforts were based on modifications at P1, P1′, P2, and P3. Attempting to form π-stacking interactions with Trp184 at S1′, the linker between the vinyl sulfone and the aromatic ring at P1′ was modified. This resulted in potent *N*-alkoxy vinyl sulfanilamides acting against cruzain [77]. Indeed, compounds containing longer links were more potent cruzain inhibitors; however, structural analyses revealed that the inhibitors did not interact with Trp184. Due to the difference in linker length, the phenyl ring in this region of the inhibitors can interact with different amino acids in each case, and no consistent binding mode is observed [92]. A subset of P1′ modifications were also evaluated against rhodesain, and similar SAR trends were observed for this enzyme and cruzain [63]. Concerning P3, substituting Cbz by morpholinyl urea results in increased potency against cruzain and replacement by *N*-methylpiperazinyl is beneficial and results in trypanocidal activity [77]. Taking K777 as a starting

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point, modifications at P2 and P3 were evaluated against cruzain, rhodesain, and TbCatB. Replacement of the P2 Phe by a 3- or 4-methylphenyl moiety slightly reduced potency against cruzain, while improving potency against rhodesain and TbCatB. Replacement at P3 – aiming to reduce basicity at this position – by 3,5-difluorinephenyl, 4-trifluorophenyl, 2-pyridyl, or 1,4-benzodioxan-6-yl usually reduced activity against all enzymes, but was more detrimental to cruzain inhibition, therefore enhancing selectivity for rhodesain over cruzain [115].

Owing to the limitations of peptidic derivatives, nonpeptidic vinyl sulfones were developed. Modifications in the P2/P3 moieties of the nonpeptidic fragments into isoxazole or pyrazole rings linked to a hydrophobic moiety at the ortho-position reduced enzyme inhibition, while isoxazole-bonded pyridines as well as analogs containing a thiazole ring connected to a phenyl, 3-pyridine, or pyrimidine inhibited cruzain and rhodesain at low micromolar concentrations. The insertion of 2-(4-chlorobenzoyl)cyclohexane-1-carboxylic acid enhances the potency against both enzymes [76]. On the other hand, insertion of a 10- or 11-membered macrocycle at P2/P1 moieties drastically reduces the activity of the compounds [106]. Some nonpeptidic vinyl sulfones inhibited growth of *T. brucei* and were not toxic to Jurkat cells (Table 5.1) [76]. The binding mode of several vinyl sulfones was determined by crystallography, and these molecules were shown to occupy the S1′–S3 subsites of cruzain [76, 84, 92, 111] and rhodesain (Figure 5.2; Table 5.1) [84].

The importance of the vinyl radical was exploited and derivatives containing an allyl radical were very active against cruzain and rhodesain, demonstrating greater potency than their vinyl sulfone analogs, being up to 30-fold more potent. Variations at P2 were also assessed, and replacement of Phe by Leu was beneficial (Table 5.1), while replacement for Ala reduced activity and substitution with Val did not affect the activity against both enzymes [93]. Later, modifications on P3 and P1 and the *E* and *Z* configurations were also exploited. The most potent compound had an *E* configuration, and replacing homophenylalanine by Phe decreased the potency by approximately 100-fold [116].

Nonpeptidic Derivatives

Thiosemicarbazones (TS)

Thiosemicarbazones are compounds with known activity against cruzain and rhodesain; they also exhibit trypanocidal activity *in vitro* (Table 5.1) [79]. Due to their low molecular weight, low cost of synthesis, and nonpeptidic nature, this class of reversible covalent inhibitors has been intensively studied [75]. Several modifications have been made in the thiosemicarbazones scaffold to optimize this promising class (Figure 5.2). The NH_2 moiety at N1 was explored, and it was found that the insertion of alkyl groups, phenyl, methylfuran, and ethylmorpholine led to reduced activity against cruzain [75]. Similarly, inclusion of the SMe in the same position resulted in poor activity against both cruzain and

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rhodesain. Nevertheless, the substitution for piperidyl, *N*-methylpiperazinyl, and diethylamine did not reduce the inhibitory activity against these two enzymes [79], whereas the presence of the chlorophenyl, trifluoromethylphenyl, and hexyl groups resulted in moderate activity against cruzain. Another SAR trend observed was that replacement of thione group to carbonyl at C2, as well as the reduction of the imine, caused dramatic loss of activity against cruzain [75, 97]. However, the insertion of the substituted aryl group in the imine position leading to a T-shape conformation increases activity against cruzain and *T. cruzi in vitro* (Table 5.1) [94]. Variations in the C5 position have great impact on potency, since ethyl- and butyl-groups produce potent cruzain and rhodesain inhibitors (Figure 5.2) [75, 85]. Similarly, 2-thiophene, 3-trifluoromethyl phenyl, and 4-methyl phenyl groups produce the same effect against rhodesain and TbCatB (Table 5.1) [96]. Their replacement by hydrogen, methyl [75], and phenylalanine side chains reduces activity against cruzain, and bulkier branched substituents drastically decrease the potency against cruzain and rhodesain. Furthermore, homophenylalanine, phenyl ether, and phenylethene did not affect inhibitory activity against both enzymes [85]. Substituted aromatic rings linked to the C5 of thiosemicarbazones have also been explored. Monosubstitution with bromine, trifluoromethyl, chlorine [75], phenyl [79, 94], methoxyphenyl [79], *p*-dimethylamine [97], α-tetralone, benzophenone, and 4-chromanone [95] and even disubstitution with 3,4-dichloro [75, 94], 3,5-di-trifluoromethyl substituents [75] were reported as potent cruzain inhibitors, and 4,5-dimethoxy-indanones were also active [117]. In another series, 3,5-dichloro, 3,5-di-trifluoromethyl, and 4-methyl phenyl groups were able to inhibit rhodesain and TbCatB at submicromolar concentration (Table 5.1) [96]. However, *ortho-* and *para*-substitutions in the aromatic ring are not favorable [75]. Iodine and methyl isatins [118] as well as peracetylated galactoside [97] provided potent compounds, especially against rhodesain. Several thiosemicarbazones were able to inhibit growth of *T. cruzi* in human macrophages (Figure 5.2; Table 5.1) [75, 79, 85], and proliferation of epimastigotes [117]. *T. brucei* growth was also inhibited, and selectivity for rhodesain and TbCatB over homologous human cathepsins was observed (Table 5.1) [96]. In addition, some thiosemicarbazones are not toxic against different cell types, such as J774 macrophages [75], human erythrocytes [117], human embryonic kidney 293, human Hepg2 cells (Table 5.1) [96], and in *in vivo* assays [79]. Docking studies against cruzain suggest that thiosemicarbazone scaffold is oriented close to Cys25 and His162 [75], allowing the formation of a covalent bond. Additional predicted interactions include hydrogen bonds with Trp184, Gln19, and His162 residues at the S1′ cruzain subsite [117].

Hydrazones

Hydrazones have been exploited to identify nonpeptidic CPIs due to their structural similarity with amides and important features such as lower reactivity and higher stability [80]. Hydrazone derivatives such as *N*-acylhydrazones and

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thiazolylhydrazones have shown different activities according to the substituent profiles. *N*-Acylhydrazones replacement for halogenated oxadiazoles at C1 is beneficial for inhibitory activity against cruzain at nanomolar concentrations [82] while methyl and phenyl do not affect potency against the enzyme [83], and the presence of *p*-hydroxyphenyl oxadiazoles in the same position abolishes activity. The insertion of 4-hydroxy-3-methoxyphenyl [82] and *p*-nitrophenyl oxadiazole [83] at C5 results in compounds with similar potency. Docking studies suggest that the *N*-acylhydrazone portion is accommodated on the S2 pocket of cruzain, while the phenyl moiety connected to the oxadiazole ring is accommodated in the S1 pocket, close to Gln19 [119]. In addition, *N*-acylhydrazones were not toxic to mouse splenocytes [82]. Thiazolylhydrazones have also been exploited, and the 3-methylthiazolyl [80], insertion of a *p*-substituted aromatic ring connected to the C4 thiazolyl [81] and replacement of the thiazolyl by *N*-phenyl 2-thiazolidin-4-one increase potency, while 2-iminothiazolidin-4-thiones maintain inhibitory activity [98]. The 2-pyridine group and methyl connected to the imine carbon are also beneficial for activity against cruzain (Table 5.1) [81]. On the other hand, the presence of a phenol group linked in thiazolylhydrazones does not increase inhibitory activity [98]. Thiazolylhydrazones also inhibit *T. cruzi* trypomastigotes *in vitro* and are toxic neither to human Hepg2 cells [81] nor to mouse splenocytes (Table 5.1) [80, 98].

Nitriles

Nitriles are electrophilic compounds that form a covalent thioimidate bond with the active-site cysteine [101]. A nitrile containing cathepsin K inhibitor, odanacatib, showed efficacy and safety for postmenopausal osteoporosis treatment, and phase III clinical trials for this compound were recently completed [120]. Odanacatib analogs are also potent cruzain inhibitors. The insertion of L-Phe in P1, *p*-substituted biphenyl in P3, and replacement of fluoroleucine by Val or Leu in P2 led to subnanomolar potency and selectivity for cruzain over human cathepsins L, B, F, S, K, and V (Table 5.1). The presence of heterocycles did not affect the inhibitory activity, while Phe removal or replacement by a smaller group in P1 reduced activity [99]. Rats infected with *T. cruzi* when treated orally with 3 mg/kg doses of odanacatib analogs showed high cure rate [121]. Azadipeptide nitriles showed potent activity inhibitor against cruzain and rhodesain at picomolar concentration especially when containing Phe or Leu at P2, methyl at P1, and *tert*-butyloxycarbonyl (Boc) at P3. These compounds also inhibited *T. brucei* replication (Table 5.1) [100].

The electrophilicity of nitriles is highly influenced by nearby substituents, impacting their potency [101]. Triazines increased the potency of nitriles against rhodesain and cruzain to low nanomolar concentrations [68, 101], while pyrimidine and quinazoline analogs are high-nanomolar rhodesain inhibitors and pyrazine, pyridine, thiazole, and oxazole are not active against the enzyme. Moreover, aryl nitriles were not toxic in rat skeletal myoblast (Table 5.1) [101]. Aryl

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substituted 6-aminophenyl purine nitriles with 3,4-dichlorine phenyl [103], small heterocycles, substituted phenyl groups, and larger fused rings systems [102] lead to potent activity against TbCatB, displaying submicromolar IC_{50} (Table 5.1). However, the insertion of hexyl, α-methylbenzyl [103], 1-phenylsulfonylindol-3-yl, or 1-phenylsulfonylindol-2-yl [102] is not beneficial. Apart from these features, purine nitriles do not induce toxicity (Table 5.1), promote good selectivity for TbCatB over cathepsins B and L, and show trypanocidal activity against *T. brucei* [102, 103]. Purine nitriles were optimized, and an orally available inhibitor with a good pharmacokinetic profile was developed. It penetrates the central nervous system and is not toxic at 200 mg/kg. Survival time of infected mice with *T. b. brucei* increased, but unfortunately, this positive effect was not observed in mice infected with *T. b. rhodesiense* [122]. A crystal structure of cruzain in complex with a purine nitrile was obtained (Table 5.1) [68].

Additional Classes of Nonpeptidic Inhibitors

Several other efforts have been made to find diverse classes of nonpeptidic CPIs. Natural products screening and synthesis of derivatives resulted in cruzain and rhodesain inhibitors. Diketopiperazines and semisynthetic derivatives [104] and tetromycins isolated from marine sponges [123] were discovered as new rhodesain inhibitor scaffolds (Table 5.1). Cryptolepine derivatives were also shown to be cruzain and rhodesain inhibitors, with IC_{50}s in the low micromolar range, and to be potent against *T. brucei* [124].

A few strategies employing virtual screening of large compound libraries have also been reported. All efforts discussed here target cruzain, and most were structure-based [60, 87, 125] while a study included ligand- and structure-based techniques [105]. A virtual screening effort led to discovery of a lead-like molecule with K_i of 32 μM. A series of analogs was synthesized and an apparent SAR was obtained. However, careful validation revealed that the most potent compounds were aggregators [125], a common source of artifacts in *in vitro* screens [126]. Another virtual screening study considered protein flexibility through ensemble docking on structures observed by molecular dynamic simulations, leading to the discovery of three cruzain inhibitors with mid-micromolar IC_{50}s [60].

A combination of virtual and experimental high-throughput screening led to the discovery of five new classes of competitive cruzain inhibitors, with K_i values in the low micromolar range. Among these classes, one was characterized as a substrate [87]. A crystal structure with a benzimidazole was obtained, [87] and synthesis of analogs led to SAR determination and potency optimization, generating a compound with K_i of 80 nM and trypanocidal activity (Figure 5.2; Table 5.1) [86].

Through a consensus score strategy, employing docking, shape-based ligand similarity and HQSAR (Hologram QSAR), 12 inhibitors with low- to mid-micromolar IC_{50}s were discovered, and the most active compounds were

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also evaluated against cathepsin L and *T. cruzi*. In one case, initial SAR based on commercially available analogs was obtained and a crystal structure was reported, revealing binding to the S2 and S3 subsites (Table 5.1) [105].

Conclusion

Over the past three decades, trypanosomal CPs have been widely studied and validated as therapeutic targets for Chagas disease and African sleeping sickness. Their roles for cell invasion, nutrition, and evasion of the immune system have been described, and essentiality for parasite survival has been demonstrated. These results motivated many medicinal chemistry efforts, leading to potent CPIs with trypanocidal activity *in vitro* and in animal models of the diseases. However, so far, few compounds were evaluated in additional preclinical stages and there are still no approved drugs that act through this mechanism. Hopefully, the leads discovered so far or new CPI scaffolds can be optimized and provide new efficient treatments for these neglected tropical diseases.

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6
Potential of Pyrimidine Metabolism for Antitrypanosomal Drug Discovery

*María Valente, Antonio E. Vidal, and Dolores González Pacanowska**

Abstract

Parasitic diseases, and particularly those caused by kinetoplastid parasites, are a major public health problem especially in poor and nonindustrialized countries. Since currently available drugs are of limited efficacy and present numerous side effects, significant effort is being directed toward the identification of new drugs and new drug targets. Here, the potential for therapeutic intervention at the level of pyrimidine metabolism is reviewed. Pyrimidines perform a diverse set of vital cellular functions ranging from synthesis of DNA and RNA to the production of lipids and carbohydrates. The pyrimidine metabolic pathway involves not only *de novo* synthesis but also the salvage and interconversion of intermediates. Similarly, pyrimidine requirements may vary depending on the species and life stage of the parasite. The efficiency of drug therapy will be therefore influenced by metabolic redundancies, parasite-specific pyrimidine needs, and accessibility to suitable intermediates in their hosts. We summarize the information available on pyrimidine metabolism inhibitors that show antiparasitic activity in kinetoplastid parasites and pinpoint several essential enzymes that constitute potential targets amenable to drug design. Distinct enzymes of the pyrimidine pathway that perform nonredundant functions such as deoxyuridine triphosphate nucleotidohydrolase (dUTPase), dihydrofolate reductase–thymidylate synthase, and thymidine kinase exhibit unique features that could be exploited in the design of selective compounds.

Introduction

Pyrimidines are essential components of nucleic acids, providing both structural and genetic information. In addition, they play important roles as signaling molecules and in the synthesis of phospholipids, complex lipids, and glycoconjugates. Trypanosomatid active DNA synthesis prior to cell division demands a

*Corresponding author.

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strong pyrimidine supply. *Leishmania* and *Trypanosoma* possess the metabolic machinery for *de novo* synthesis of pyrimidine nucleotides but also have the capacity to obtain them from their hosts by the salvage pathway. In the past, the apparent redundancy between these two strategies to provide the cell with pyrimidine nucleotides was seen as a drawback for the therapeutic targeting of these metabolic pathways. However, in highly proliferating parasite cells, pyrimidine salvage may be insufficient to overcome defects in the *de novo* biosynthesis pathway and *vice versa*. Moreover, salvage and *de novo* synthesis routes converge

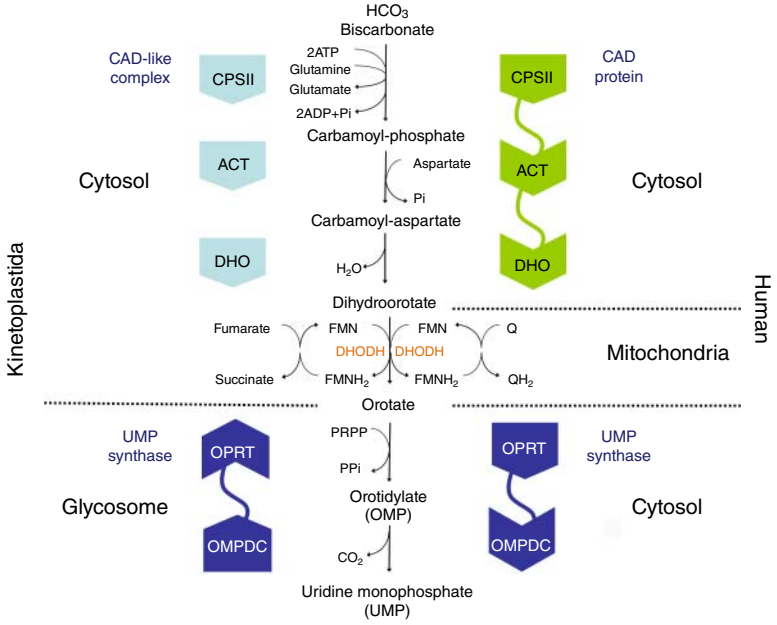


Figure 6.1 UMP synthesis routes in humans and kinetoplastida. In humans, the first three activities of *de novo* synthesis of pyrimidines are expressed as a single polypeptide (CAD protein) while kinetoplastids express CPSII, ACT, and DHO as individual proteins but with the capacity to establish intermolecular interactions and to form a CAD-like enzyme complex [1]. The subcellular distribution of the enzymes involved in *de novo* biosynthesis also differs between humans and parasites. In humans, CAD is cytosolic but DHODH is localized in the inner membrane of mitochondria. In contrast, protozoan CPSII, ACT, DHO, and DHODH are all cytosolic enzymes [2]. Human DHODH is directly linked to the mitochondrial respiratory chain using ubiquinone as electron acceptor while parasite DHODHs catalyze the electron transfer from dihydroorotate to fumarate to generate succinate [3–5]. The final reaction steps that lead to UMP biosynthesis are catalyzed by a single bifunctional protein referred to as *UMP synthase*. In kinetoplastids, the functional domains are reversed with respect to mammals, with the N-terminal domain corresponding to OMPDC and the C-terminal to OPRT [6]. These final two enzymatic reactions take place in kinetoplastid-specific organelles called glycosomes [2]. Human UMPs are localized in the cytosol around and outside the mitochondria [7]. Enzyme abbreviations: carbamoyl phosphate synthetase (CPSII), aspartate carbamoyltransferase (ACT), dihydroorotase (DHO), dihydroorotate dehydrogenase (DHODH), orotidine 5′-monophosphate decarboxylase (OMPDC), and orotate phosphoribosyltransferase (OPRT).

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in the synthesis of uridine 5′-monophosphate (UMP) and the enzymes acting in subsequent steps carry out nonredundant metabolic functions that become essential for parasite survival. Experimental studies in different forms of these parasites have recently contributed not only to define the activities involved in *de novo* and salvage pathways but also to generate compelling evidences that validate the selective inhibition of pyrimidine metabolism as a potential therapeutic strategy.

De novo Biosynthesis of Pyrimidines

In trypanosomatids, as in humans, *de novo* pyrimidine synthesis entails a six-step enzymatic process that results in the production of UMP (Figure 6.1). The first enzyme and the rate-limiting activity of the pathway is a glutamine-dependent carbamoyl phosphate synthetase II (CPSII, EC 6.3.5.5) that catalyzes the synthesis of carbamoyl phosphate from L-glutamine, bicarbonate, and two ATP molecules. Aspartate carbamoyltransferase (ACT, EC 2.1.3.2), the second enzyme of the pathway, generates carbamoyl aspartate from carbamoyl phosphate and L-aspartate. Next, dihydroorotase (DHO, EC 3.5.2.3) catalyzes the reversible cyclization of *N*-carbamoyl L-aspartate to form dihydroorotate, which is oxidized to orotate by the flavoenzyme dihydroorotate dehydrogenase (DHODH, EC 1.3.3.1). The final reaction steps that lead to UMP biosynthesis are the conversion of orotate and 5-phosphoribosyl 1-pyrophosphate (PRPP) into orotidine 5′-monophosphate (OMP) by orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and the decarboxylation of OMP by orotidine 5′-monophosphate decarboxylase (OMPDC, EC 4.1.1.23). In humans and trypanosomatids, these two enzymatic activities reside in a single bifunctional protein referred to as uridine 5′-monophosphate synthase (UMPS).

An open question is whether *de novo* biosynthesis is essential in the presence of a functional salvage pathway. In kinetoplastids, inactivation of the enzymes involved in the synthesis of UMP can be mostly overcome *in vitro* by the uptake of uracil, thus suggesting a nonessential function [3, 8–10]. There are, however, notable exceptions reported in the literature that encourage further validation of these enzymatic targets: (i) proliferation of intracellular CPSII-deficient *Trypanosoma cruzi* amastigotes is severely impaired in host cells [10]; (ii) a DHODH knockout could not be generated in *T. cruzi* even though parasites were supplemented with an external source of pyrimidines [11]; and (iii) inactivation of *Trypanosoma brucei* UMPS by double gene replacement led to avirulent parasites incapable of infecting mice [12].

CAD-Like Complex

In spite of the high level of conservation of the pyrimidine *de novo* biosynthesis pathway, *Trypanosoma* and *Leishmania* show distinct features not present in other organisms, notably in humans. One of the most striking differences involves the structural organization of the first three enzymatic activities. Thus,

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while mammalian cells express these activities as a single polypeptide (CAD protein), in the kinetoplastid genomes, they are encoded by individual genes that express separate proteins [6, 13] (Figure 6.1). Even though CPSII, ACT, and DHO are individual enzymes, in *T. cruzi*, the recombinant proteins can establish intermolecular interactions suggesting that they may act as a protein heterotrimer forming a CAD-like complex with similar properties to the fusion enzyme [1]. Structure-based drug design for targeting the intermolecular interactions among these three proteins has been suggested as a strategy to inhibit the synthesis of dihydroorotate but no studies are available at the moment to validate this approach. Nonetheless, the biochemical properties of the individual enzymes support their role as valid targets and the feasibility of the design of specific inhibitors.

Carbamoyl Phosphate Synthetase II (CPSII)

Protozoan CPSII is an individual gene product composed of both a glutamine amidotransferase (GAT) and a carbamoyl-phosphate synthetase (CPS) domain. Its biochemical characterization revealed significant differences with other prokaryotic and eukaryotic enzymes [14]. Human, trypanosomal, and bacterial CPSIIs are all allosterically regulated but in different manners: mammalian CPSII is activated by PRPP and inhibited by UTP while protozoan CPSII activity is unaffected by PRPP and inhibited by nucleoside diphosphates, especially UDP, but not by nucleoside mono- or triphosphates [15]. On the other hand, *Escherichia coli* CPSII is allosterically activated by IMP and inhibited by UMP [16]. Crystallographic and site-directed mutagenesis analysis of the bacterial CPSII indicates that Lys^{993} is involved in the interaction with UMP [17]. The replacement of this residue by tryptophan in mammalian CPSIIs has been linked to the inhibition by UTP. In kinetoplastids, the residues Lys or Trp are substituted by a hydrophilic glutamine, which could be responsible for the enzyme sensitivity to UDP but not to UMP or UTP [14].

Acivicin ((α*S*,5*S*)-alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; AT-125) is a heterocyclic analog of glutamine with antitumor activity probably mediated through the inhibition of enzymes catalyzing amido transfer from L-glutamine including CPSII. Acivicin inhibits *T. cruzi* CPSII more pronouncedly than the mammalian enzyme ($K_i = 0.2\,\mu M$ vs. $7.0\,\mu M$, respectively). Indeed, an analysis of the amino acid sequence of the *T. cruzi* enzyme revealed specific residues in the sequence surrounding the active site of the GAT domain that may account for this greater susceptibility. Acivicin induces proliferation arrest of *Leishmania donovani* promastigotes *in vitro* and also inhibits the growth of the pathogenic amastigote form in mouse peritoneal macrophages ($ED_{50} = 50\,nM$) [18]. The antiproliferative activity correlates with a significant inhibition of CPSII activity present in cell extracts from parasites treated with the chlorinated glutamine analog. Inhibition promoted by 0.55 μM acivicin in promastigote parasites could not be effectively reversed by the addition of an excess of glutamine (5 mM), an amount that exceeds the concentration available in mammalian tissues. The irreversible nature of CPSII inhibition was also confirmed with partially purified

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enzyme in *in vitro* experiments. Nevertheless, acivicin is a glutamine antagonist that inactivates many of the GATs, and hence, it cannot be ruled out that other potential targets including CTP synthetase may be responsible for the antiparasitic effect [19]. In fact, several studies on its antiparasitic action conducted on other related trypanosomatids, including African trypanosomes, have shown that in these organisms, the main target is indeed CTP synthetase [20]. These observations will be discussed in the next section.

Aspartate Carbamoyltransferase (ACT)

Similarly to acivicin, *N*-(phosphonoacety1)-L-aspartic acid (PALA), the transition state analog for the enzymatic formation of carbamoyl aspartate has been proposed as an antiparasitic agent against *L. donovani* [18]. Notably, PALA was found to be a strong competitive inhibitor of ACT *in vitro* ($K_i = 0.5\,\mu M$). Despite the very potent inhibition of ACT, PALA has a moderate effect on parasite growth in both amastigote and promastigote forms of the parasite, with an ED_{50} of approximately 20 μM. The discrepancy between enzymatic and cellular sensitivity has been attributed to an inefficient uptake of the drug by the parasite [21]. The inhibition of growth by PALA can be, however, partially reverted by the supplementation of the growth medium with 100 μM of either uracil or uridine, which probably contributes to bypass the defect in the *de novo* synthesis by inducing UMP formation via salvage. In *T. cruzi*, PALA exhibits only a weak inhibitory effect over ACT [22] even though the 12 residues participating in the binding of this substrate analog are fully conserved [6]. However, no successful chemotherapy targeting parasitic ACTs has been developed to date, mostly because PALA and other inhibitors of pyrimidine *de novo* biosynthesis equally inhibit the protozoan and human enzymes [23].

Dihydroorotase (DHO)

Contrary to *Plasmodium* species, which totally depend on *de novo* pyrimidine biosynthesis, the essentiality of the DHO enzyme in kinetoplastida has not been fully examined to date, although significant loss of fitness was observed in a *T. brucei* bloodstream RNAi screen [24]. Kinetoplastid DHO is a zinc metalloenzyme with a high homology to the DHO domain of mammalian CAD. In *T. cruzi*, the amino acid sequence contains the conserved substrate-binding residues and the five histidines presumably involved in Zn binding as well as the DxHxHxR motif that contains two of the metal–ligand histidines and an aspartate residue essential for catalysis [6]. In contrast to the first two enzymes of the pathway, no inhibitors have been reported to date for any kinetoplastid DHO, even though a great number of inhibitor molecules have been designed against *Plasmodium*, *Toxoplasma*, and *E. coli* DHOs [25, 26]. 5-Substituted orotate derivatives, L-6-thiodihydroorotate (TDHO), and several di- and tetrahydropyrimidine analogs have been shown to exhibit different degrees of inhibitory activity against *Plasmodium*, *E. coli,* or mammalian DHOs [27]. The active site architecture and the presence of Zn atoms are elements required for effective inhibition. The conservation of these elements in kinetoplastid DHOs is still uncertain since the

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atomic structure is yet to be solved, but the compounds described may well serve as leads for the development of kinetoplastid-specific inhibitors.

Dihydroorotate Dehydrogenase (DHODH)

Kinetoplastid DHODH presents unique biochemical and structural properties that make the enzyme a promising target for drug design. *Trypanosoma* DHODHs are homodimers that belong to the group of Class 1A enzymes that catalyze the electron transfer from dihydroorotate to fumarate [3–5]. In contrast, human DHODH is a Class 2 monomeric enzyme with ubiquinone as electron acceptor [28, 29]. Both human and protozoan enzymes contain prosthetic flavin mononucleotide (FMN) groups as internal electron acceptors. Class 1 and Class 2 DHODHs active sites differ at the catalytic base that promotes the abstraction of a proton from the C5 of orotate. In Class 2 enzymes, the catalytic serine is surrounded by a more hydrophobic environment than that of the equivalent catalytic cysteine of Class 1 enzymes [30]. Inhibitors of the Class 2 DHODHs belonging to humans [31], *Helicobacter pylori* [32], or *Plasmodium falciparum* [33] were designed to target the ubiquinone-binding site located at the Class 2-specific N-terminal domain, and therefore, they are not effective against the Class 1 enzymes such as the trypanosomal proteins. On the contrary, orotate analogs such as hydroxybenzoates are effective against Class-1 *Tb*DHODH but not against Class 2 DHODHs since in trypanosomes, the orotate binding site is more accessible than in humans due to the absence of the N-terminal domain [3]. In addition to the differences in the orotate binding site, the FMN binding site of *Tb*DHODH contains specific residues that are absent in the human protein, and these subtle divergences might also be exploited in the design of drugs to disrupt the interaction with the FMN cofactor.

A series of novel orotate analogs with capacity to inhibit *Tc*DHODH and *Lm*DHODH in the low micromolar range were identified by combining X-ray crystallography information and virtual screening techniques [34]. Structure–activity relationship (SAR) data obtained in this initial study also provided rational guidelines for further structure-based optimization of these orotate derivatives. Furthermore, a detailed topological analysis of the structures of *T. cruzi* and *Leishmania major* DHODHs indicates the existence of other distinctive protein regions that could be successfully exploited for multitarget-guided design of selective ligands of trypanosomatid DHODHs [34, 35].

UMP Synthase (UMPS)

Human and kinetoplastid UMPSs are bifunctional enzymes that consist of an OPRT and an OMPDC domain. However, while in mammals, the OPRT and OMPDC activities are located at the N- and C-terminus of the protein, respectively, in kinetoplastids, the functional domains are reversed with the

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N-terminal domain corresponding to OMPDC and the C-terminal to OPRT [6] (Figure 6.1).

The OMPDC domain has been shown in mammalian cells to be the target of pyrazofurin, a *C*-riboside antibiotic that acts as a potent inhibitor of pyrimidine *de novo* synthesis [36]. In *T. cruzi* epimastigotes, pyrazofurin seems to be active against OMPDC and inhibits UMP formation in glycosome extracts supplemented with ATP [37]. In a different study, exposure to pyrazofurin impaired the growth of *T. brucei* bloodstream parasites with an EC_{50} of 0.55 μM [12]. However, pyrazofurin potency remained practically unaltered in parasites lacking one or both UMPS alleles, indicating that at least in *T. brucei*, OMPDC is not the physiological target of pyrazofurin. Inactivation of UMPS, on the contrary, conferred resistance to 5-fluoroorotate, as this prodrug needs to be converted by UMPS into 5-FUMP and eventually into 5-FdUMP in order to inhibit the bifunctional enzyme dihydrofolate reductase/thymidylate synthase (DHFR-TS) [12].

Several transition-state analogs designed to target *Plasmodium* OPRT exhibited K_i values in the low nanomolar range but failed to kill the parasite in culture [38]. The activity of these potent OPRT inhibitors against the *Leishmania* and *Trypanosoma* remains to be established.

Pyrimidine Salvage

Leishmania and *Trypanosoma* can also produce pyrimidine nucleotides by salvage of preformed pyrimidine nucleobases or nucleosides, which are actively internalized by specialized transporters from the host environment or the culture medium (Figure 6.2). Uracil is efficiently translocated into both procyclic and bloodstream forms of *T. brucei* by the *Tb*U1 and *Tb*U3 transporters, respectively [39, 40], and into *L. major* by *Lm*U1 [41]. With the exception of procyclic *T. brucei* cells that express a uridine-specific (*Tb*U2) and a cytosine-efficient transporter (*Tb*C1), internalization of other nucleobases and nucleosides is generally much less efficient and requires high concentrations to be significant. This is the case of thymidine, whose uptake is mediated by a P1-type purine nucleoside transporter (*Tb*T1) with a low translocation efficiency [40] (Figure 6.2).

Since uracil is the necessary precursor in the formation of UMP, cytosine and cytidine nucleosides should be previously deaminated to enter the pathway. Uracil-containing nucleosides or deoxynucleosides can also be broken down to uracil and α-ribose-1-phosphate by the action of an uridine phosphorylase (UPase, EC 2.4.2.3). Whatever the source, uracil is eventually converted into UMP through its phosphoribosylation by uracil phosphoribosyltransferase (UPRT, EC 2.4.2.9). The reaction catalyzed by UPRT is the unique way to synthesize UMP in these organisms since a uridine kinase activity that converts uridine into UMP is apparently absent [2, 13].

Even though the uptake of thymidine under physiological conditions is unlikely to be relevant, kinetoplastids are endowed with thymidine kinase (TK, EC 2.7.1.21)

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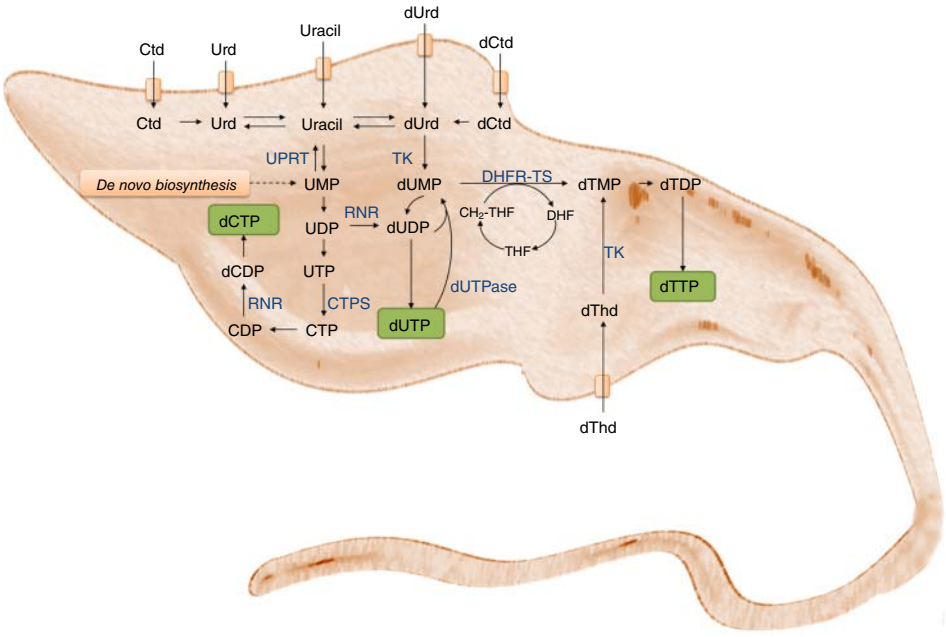


Figure 6.2 Integrated view of the pathways that lead to the synthesis of pyrimidine nucleotides. Kinetoplastids salvage preformed pyrimidine nucleobases or nucleosides from the host's fluids to obtain pyrimidine nucleotides. After the entrance into the parasite through specific transporters, all pyrimidines, except thymidine, are converted into uracil, which is then phosphorylated to UMP by UPRT. Thymidine is phosphorylated via TK to produce dTMP, which is subsequently phosphorylated to dTTP. UMP is a common intermediate of *de novo* and salvage pathways and all the reactions downstream UMP synthesis are catalyzed by enzymes performing nonredundant functions. Kinetoplastid CTPS, RNR, DHFR-TS, and dUTPase are included in this group. Enzyme abbreviations: uracil phosphoribosyltransferase (UPRT), thymidine kinase (TK), cytidine triphosphate synthetase (CTPS), ribonucleotide reductase (RNR), dihydrofolate reductase–thymidylate synthase (DHFR-TS), and deoxyuridine triphosphate nucleotidohydrolase (dUTPase).

activity. The parasite TK enzyme catalyzes the ATP-dependent phosphorylation of thymidine, thus initiating the route that leads to dTTP synthesis [42].

No inhibitors with drug-like properties targeting enzymes of the pyrimidine salvage pathway have been described to date in kinetoplastid parasites. However, two enzymes of the pyrimidine salvage deserve to be discussed in relation to their role in drug activation and drug resistance pathways: uracil phosphoribosyltransferase (UPRT) and TK.

Uracil Phosphoribosyltransferase (UPRT)

Although UMP is a key metabolite in kinetoplastid protozoa, UMP formation via salvage does not appear to be an essential process in these parasites. Indeed,

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both *Leishmania* and *T. brucei* UPRT null mutants proliferate normally [8, 9]. However, UPRT is pivotal in the pyrimidine salvage pathway since all incorporated pyrimidine precursors, except thymidine, are metabolized into uracil prior to conversion to UMP [8]. This makes UPRT the ideal candidate in a potential combined therapy directed against both the salvage and *de novo* biosynthesis routes of these parasites. In agreement with this notion, it has been reported that *L. donovani* CPSII/UPRT double mutant amastigotes, lacking both *de novo* and salvage pathways, are completely avirulent *in vivo* [8]. Moreover, uracil salvage reverts growth defects caused by inactivation of *de novo* biosynthesis enzymes such as CPSII in *T. cruzi* epimastigotes [10] or UMPS in *T. brucei* and *L. donovani* [9, 43] and counteracts the inhibitory effect of PALA on the ACT enzyme of *Leishmania* [18].

UPRT also plays an important role in the activation and selectivity of toxic pyrimidine analogs. An example of this is the pyrimidine analog 5-fluorouracil (5-FU), which is cytotoxic for both kinetoplastid parasites and humans after conversion into 5F-UMP [40, 41, 44]. However, this conversion appears to occur by different organism-specific metabolic reactions: while in kinetoplastids, 5-FU is activated via UPRT [40, 45], human cells lack this enzyme and incorporate the fluorinated prodrug into the nucleotide pool through activation by OPRT, uridine phosphorylase, or thymidine phosphorylase [46]. Therefore, the design of toxic uracil analogs that are selectively activated by parasitic UPRT could be exploited as a potential strategy against trypanosomiasis. On the other side, mutations that inactivate UPRT would be a potential cause for the emergence of drug resistance phenotypes and should be taken into consideration.

Thymidine Kinase (TK)

TK is another key enzyme in pyrimidine salvage that plays a major role in the phosphorylation of endogenous and exogenous thymidine to produce dTTP. In contrast to human TK1, *T. brucei* TK is a tandem protein consisting of two TK domains where only domain 2 is active. Another unique feature is that it is less discriminative against purines than human TK1 [42]. No TK-selective inhibitors have been tested to date against kinetoplastids. It has been described, however, that suramin, the antimicrobial drug used in the treatment of human African trypanosomiasis, inhibits TK activity present in partly purified preparations from *T. brucei* by 60% at the concentration of 0.5 mM [47], although the relevance of this observation remains to be established.

More recently, a characterization of TK from *L. major* has shown that the enzyme is strongly inhibited by the bisubstrate analog Ap5dT (P^1-[5′-Adenosyl]-P^5-[5′-deoxythymidyl]-pentaphosphate) *in vitro* with an IC_{50} of 1.1 μM [48]. The crystal structure of the catalytic core of *Lm*TK in complex with Ap5dT was also determined, providing an insight into the structural basis for inhibition that might guide future rational drug design.

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Kinetoplastid TKs have also been involved in the metabolic activation of nucleoside analogs with cytotoxic activity. *L. major* and *T. brucei* TK can efficiently phosphorylate 5-fluorodeoxyuridine (5-FdUrd) [42, 48]. Consistently with the kinetic data, it has been reported that supplementation with thymidine (100 μM) reduces *T. brucei* sensitivity to 5-FdUrd by 24-fold [40]. In addition to 5-FdUrd, LmTK also exhibits catalytic activity on the antiretroviral prodrug zidovudine (3′-azido-3′-deoxythymidine, AZT) *in vitro* with a K_m of 3.0 μM [48]. Phosphorylation of this substrate by other parasite TKs has not been established, but inhibition of *T. cruzi* growth by AZT suggests the functional conservation of the activities involved in metabolic activation [49].

TK may also play a critical role in protection against antifolates or thymidylate synthase (TS) inhibitors. In *T. brucei* and *L. major*, the generation of *DHFR-TS* double knockouts required thymidine supplementation to be viable [50, 51], indicating that TK activity is involved in the metabolic bypass of defective dTMP *de novo* synthesis.

UMP Downstream Enzymes

This denomination comprises a group of enzymes that operate at biochemical steps following the synthesis of UMP and contribute to both salvage and pyrimidine *de novo* biosynthesis pathways. We have included in this category cytidine triphosphate synthetase (CTPS, EC 6.3.4.2), ribonucleotide reductase (RNR, EC 1.17.4.1), deoxyuridine triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23), DHFR (EC 1.5.1.3), and TS (EC 2.1.1.45). These enzymes are involved in nonredundant metabolic reactions that are crucial for pyrimidine synthesis and other metabolic routes such as lipid biosynthesis and house-cleaning functions. The specific inhibition of such reactions has been reported to impair parasite growth and survival, which has led to propose these enzymes as attractive targets for chemotherapy [20, 50, 52, 53]. The enzymatic steps involved in pyrimidine biosynthesis following the production of UMP are depicted in Figure 6.2.

Cytidine Triphosphate Synthetase (CTPS)

CTP is an essential nucleotide required for nucleic acid and phospholipid biosynthesis. *Trypanosoma* CTPS is a glutamine amidotransferase (GAT) that catalyzes the conversion of UTP to CTP in the presence of ATP, ammonia or glutamine, and magnesium [54]. In contrast to mammalian cells, where salvaged cytosine-derived nucleosides can be converted into CTP, CTPS is the exclusive source of CTP in kinetoplastids. Therefore, CTPS has been the focus of extensive characterization and a preferred target for inhibitor design.

A group of glutamine analogs extensively used in cancer clinical trials have been described to irreversibly inhibit GATs including CTPS. Some of them have been

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tested against *T. brucei* and *Tb*CTPS: 6-diazo-5-oxo-L-norleucine (DON), acivicin and derivatives. DON treatment reduced the CTP pool in *T. brucei* parasites and inhibited the proliferation of cultured cells [20]. DON also inhibited the growth of human pathogenic *Trypanosoma brucei rhodesiense* with an IC_{50} of 0.36 μM and exhibited a strong trypanocidal effect on infected mice at the dose of 25 mg/kg, but the parasitemia was eventually restored after treatment interruption. DON chemotherapy against trypanosomal infections is, however, hampered by its low stability in the host bloodstream and the inconvenient inhibition of purine synthesis in mammalian cells. Supplementation with hypoxanthine, which diminishes the toxic effect of the drug on human cells, has been proposed as an alternative chemotherapy.

In contrast to DON, acivicin penetrates the blood–brain barrier and has a long *in vivo* half-life [55]. *In vitro*, acivicin inhibits purified *Tb*CTPS with a K_i of 2.3 μM [56] and impairs trypanosomal proliferation [20]. *T. brucei*-infected mice treated with a daily dose of 5 mg/kg exhibited reduced parasitemia below detection level [56]. However, as in the case of DON, trypanosomes were detected again in the animals upon drug withdrawal.

The interaction of the enzyme with acivicin generates a covalent adduct produced by the nucleophilic attack by the thiol group of a cysteine residue to the C3 of the acivicin ring, with chlorine as the leaving group. Conti *et al.* [57] considered that the nature of the leaving group would affect the reaction between enzyme and inhibitor and designed and synthesized an analog characterized by an increased affinity for the target. Substitution of the chlorine by a bromine atom at the C3 position yielded the 3-bromoacivicin derivative. In comparison to acivicin, 3-bromoacivicin is a more potent inhibitor of *Tb*CTPS enzymatic activity and of trypanosome proliferation in cell culture. Disappointingly, treatment of trypanosome-infected mice with the maximum tolerable dose of 3-bromoacivicin (50 mg/kg) did not result in parasite clearance from the blood [57]. Other acivicin derivatives with higher affinity for *Tb*CTPS have been synthesized but failed to suppress trypanosome proliferation *in vitro* [58].

The inhibitory properties of purine heterocycles and the requirement of oligomerization for activity have suggested novel approaches for CTPS inhibition. The biochemical characterization of *Tb*CTPS showed that glutamine-dependent CTP synthesis is activated by GTP at concentrations below 0.2 mM and inhibited by the same nucleotide at concentrations above 0.2 mM [59]. Other purine derivatives such as guanosine, caffeine, and uric acid also inhibit *Tb*CTPS with IC_{50} values in the high micromolar range, indicating that the purine base moiety is the sole requirement for the inhibitory effect. These observations have led to propose the use of the purine ring as scaffold for the design of trypanosomal CTP synthetase inhibitors [59]. A different strategy to inhibit *Tb*CTPS would consist of interfering with the nucleotide-dependent conversion of the inactive monomeric form into the active tetramer [56]. A mutational analysis in *E. coli* CTPS revealed that residue Gly142, located at the UTP-binding site, is essential

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for tetramerization and, consequently, for activity [60]. The equivalent Gly residues are present in kinetoplastid CTPSs (Gly173 in *T. brucei* and Gly176 in *L. major*), but their participation in tetramerization of the protozoan enzymes remains to be determined.

Ribonucleotide Reductase (RNR)

A key regulatory step in the pathway of synthesis of deoxyribonucleotides is the reduction of the C2′–OH bond of the ribose moiety of ribonucleoside triphosphates (NDPs) to generate their corresponding dNDPs. In trypanosomes, this reaction is carried out by a Class I RNR (EC 1.17.4.1), which is a heterodimer formed by two large (R1) and two small (R2) subunits. The trypanosomal RNR has been characterized in detail [52, 61] and as its mammalian counterpart, the parasite enzyme exhibits a complex allosteric regulation but with specific properties with regard to human RNR that may be exploited for the design of selective inhibitors.

The R1 subunit contains the allosteric binding sites and the substrate-binding site [62]. While the R1 subunit binds the substrate, R2 initiates the reduction of ribonucleotides by generating an iron–tyrosyl free radical [63]. There are two different allosteric effector-binding sites in the R1 subunit [64]: the activity site, which determines the overall activity of the enzyme by binding either ATP or dATP, positive and negative effectors, respectively; and the specificity site, which determines which of the four nucleotides is reduced, either ADP or GDP with dGTP and dTTP as effectors, respectively, or CDP and UDP with ATP or dATP (at a very low concentration) as regulators. Unlike other class I RNRs, *T. brucei* RNR reduces CDP more efficiently in the presence of dTTP or dGTP than in the presence of ATP or dATP [61]. Allosteric RNR inhibitors have been produced and are widely used in cancer treatment, and precisely because of this, none of them are suitable for specific antitrypanosomal chemotherapy. The existence of a specific allosteric regulation of CDP reduction in kinetoplastids may have a special relevance in the design of allosteric RNR inhibitors with specificity for these pathogen parasites where RNR is the bottleneck in the synthesis of dCTP.

Class I RNRs are inhibited by hydroxyurea, which specifically scavenges the iron–tyrosyl free radical of the R2 subunit, thus inactivating the enzyme [65]. In *T. brucei*, hydroxyurea causes a rapid decrease of the deoxyribonucleotide pools within the first 5 min of exposure [52] and a reversible inhibition of cell division at concentrations above 50 μg/ml [66] with an IC_{50} comparable to the IC_{50} reported for mammalian cells [52]. Other chelating agents' potential inhibitors of RNR have been tested on trypanosomatids with different outcomes. Thus, *T. brucei* parasites are 10-fold more sensitive to deferoxamine treatment *in vitro* than mammalian cells [67] and undergo significant growth inhibition along with a decrease in the rate of DNA synthesis and oxygen consumption after

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exposure indicating that deferoxamine may affect RNR and alternative oxidase. The compound does not inhibit the holoenzymes directly but probably acts by chelating cellular iron, thus preventing its incorporation into the newly synthesized apoproteins. However, deferoxamine is not so effective *in vivo* and failed to clear parasitemia in *T. brucei* infected mice at a daily dosage of 300 mg/kg [67]. This is probably due to its short half-life in body fluids, which in human blood is 5–10 min, and its poor lipid solubility, which limits membrane permeability [68]. Quercetin (3,30,40,5,7-pentahydroxyflavone), on the other hand, is a lipophilic natural flavonoid with capacity to chelate metal ions. Hamsters infected with *L. donovani* and treated with quercetin – for 30 days exhibited reduction of parasite proliferation [69]. The combination with serum albumin, which facilitates the bioavailability of the flavonoid, increased its antiproliferative activity and reduced the splenic parasite load in infected mice.

An interesting class of RNR inhibitors, the so-called dimerization inhibitors, have been considered promising alternatives for the cure of infectious diseases. These inhibitors are usually small peptides or peptidomimetics with similar sequences to the C-terminus of the R2 subunit responsible for the interaction with the R1 subunit. They bind to the R1 subunit and block the R1-R2 complex, thus precluding the formation of an active RNR [70]. Because of the differences existing between R2 subunits from different classes, these molecules are very specific for the target organism. Supporting this notion, *in vitro* and *in vivo* studies on herpes simplex virus revealed the potency of these compounds as antiviral drugs without any interaction with the infected host cell [71]. However, this approach presents limitations in kinetoplastida since the C-terminus of the trypanosomal R2 proteins is very similar to the mammalian subunit, making the design of dimerization inhibitors to specifically inhibit nucleotide reduction in trypanosomatid protozoa unfeasible.

Dihydrofolate Reductase–Thymidylate Synthase (DHFR-TS)

Trypanosomatids present the dihydrofolate reductase and TS activities on a single polypeptide [72], thus exhibiting a unique bifunctional dimeric DHFR-TS. The TS domain catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate (CH_2-H_4folate) yielding dTMP and dihydrofolate (TS domain). The dihydrofolate is then reduced by NADPH to produce tetrahydrofolate (THF) in a reaction catalyzed by the DHFR domain. THF is next recycled into CH_2–H_4folate by serine hydroxymethyltransferase (absent in *T. brucei*) or the glycine cleavage complex [73]. DHFR-TS has been cloned and characterized in all the major kinetoplastids: *L. major* [74], *T. cruzi* [75], and *T. brucei* [76]. *Leishmania* and *T. brucei* null mutants are thymidine auxotrophs [50, 51], while in *T. cruzi*, attempts to eliminate the *DHFR-TS* gene completely were unsuccessful, suggesting that it is essential for parasite survival [77]. The native

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enzymes are homodimers with biochemical properties very similar to those of the bacterial, yeast, or mammalian monofunctional enzymes [72].

Due to their critical cellular functions, DHFR and TS activities have been the focus of major interest as potential drug targets against cancer and a variety of infectious diseases caused by bacteria, fungi, and protozoa. Methotrexate (MTX), trimethoprim (TMP), and pyrimethamine (PYR) are competitive inhibitors of DHFR that initially came into use as anticancer drugs [78] and antibacterials [79]. MTX has been reported to be a potent inhibitor of *Tc*DHFR ($K_i = 0.038$ nM) but a weak inhibitor of amastigote proliferation [80]. Other classical antimicrobial DHFR inhibitors such as TMP, cycloguanil, and PYR generally showed weak activity against purified trypanosomal enzymes and low selectivity [81, 82], with the exception of *Tb*DHFR, which is inhibited by TMP and PYR with K_i values of 0.2 nM and high selectivity [76].

The lack of selective inhibitors for *Leishmania* prompted the determination of the crystal structure of *L. major* DHFR in 1994 [83]. The structural information together with the homology models for *Tb*DHFR and *Tc*DHFR revealed differences in the binding pocket of the parasite proteins compared to the human enzyme that encouraged the rational design of inhibitors with the aim of improving selectivity.

By an *in silico* approach, Chowdhury *et al.* screened around 25 000 compounds from the Cambridge Structural Database (CSD). Drug selection was made by the steric fit within the active site (contact score), by the selectivity index (comparison between contact scores with *Lm*DHFR and human DHFR) and by protein–ligand interactions (visual screening and force field scoring) [84]. At the end of the process, five compounds were selected for biological assay. *In vitro*, they exhibited moderate inhibition over *Lm*DHFR enzyme and weak antiparasitic activity with the exception of one of the compounds that was very active against *T. cruzi* amastigotes ($IC_{50} = 8.6$ μM) with low cytotoxicity in rat L6 host cells. A similar screen was carried out using a *Tc*DHFR homology model as molecular target [85]. More than 50 000 compounds were docked into the active site of the *Tc*DHFR model and *h*DHFR with the selectivity index being the leading selection criterion [85]. Two of the molecules selected, a diltiazem analog and benzodiazepine derivative, showed moderate enzyme inhibition and *in vitro* activity against *T. cruzi* amastigotes (with IC_{50}s in the medium micromolar range). Disappointingly, further optimization of these two lead compounds did not contribute to generate novel molecules with improved antiprotozoal activity.

A different approach in the search for selective inhibitors was used by Atreya *et al.* [86] that performed molecular docking-based virtual screening to identify inhibitors that specifically target the shallow groove connecting the TS and DHFR active sites of the parasite enzymes. As a result of this screen, eosin B was predicted to bind the bifunctional DHFR-TS enzyme. Indeed, eosin B specifically inhibits *Lm*DHFR-TS with an IC_{50} of 100 μM as determined by steady-state time course assays. Further analysis revealed that eosin B binds outside of both the TS and DHFR folate-binding pockets [86]. However, eosin B was a weak inhibitor of

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L. major growth *in vitro*, although a reduced drug uptake or stability may also account for the poor inhibitory activity.

The lipophilic antifolate trimetrexate (TMQ), an FDA-approved drug for the treatment of *Pneumocystis carinii* infection in AIDS patients, has been shown to be a potent inhibitor of the *Tc*DHFR-TS enzyme ($K_i = 6.6$ nM) and a strong antiparasitic agent against *T. cruzi* trypomastigotes ($LD_{50} = 19$ nM) and amastigotes ($LD_{50} = 26$ nM) *in vitro* [87], much more effective than the currently used drugs for Chagas disease, benznidazole and nifurtimox. However, its activity has not been tested to date in an animal model of Chagas disease. Another important limitation to its use as antitrypanosomal drug is its high inhibitory activity on *h*DHFR. In the case of *P. carinii*, TMQ is coadministered with leucovorin in order to minimize the toxicity associated with inhibition of *h*DHFR [88]. Unfortunately, the costs and complications associated with leucovorin make this combined treatment impracticable in Chagas disease treatment.

Following up on their previous work, Schorman *et al.* obtained the crystal structures of *T. cruzi* DHFR-TS in a folate-free state or in complex with the antifolates TMQ and MTX [89, 90]. A detailed analysis revealed structural similarities among kinetoplastid enzymes but molecular and mechanistic divergences with human or *Plasmodium* DHFRs. The structural information resulting from X-ray crystallography of these complexes and docking alignment of all possible bioactive conformations of the compounds was used as input for the generation of a three-dimensional quantitative structure–activity relationship (3D-QSAR) model. The goal of the study was the identification of inhibitors of *Tc*DHFR that showed increased selectivity ($SI > 100$) and could be used as scaffold for the synthesis of new highly selective drugs. This strategy led to the synthesis and biological characterization of six compounds containing a 2,4-diaminoquinazoline scaffold similar to TMQ as potent *Tc*DHFR inhibitors but equally active against the human enzyme [91].

One of the major mechanisms of laboratory-induced resistance to classical inhibitors of folate biosynthesis in *Leishmania* involves overexpression of pteridine reductase 1 (PTR1) that reduces not only biopterin and dihydrobiopterin but also other pterins and folates including dihydrofolate [92, 93], thus providing a bypass for DHFR-TS inhibition [94]. In spite of sequence and structural identities between the *Leishmania* and *T. brucei* enzymes, *Tb*PTR1 displays lower capacity to reduce folate and dihydrofolate [95] and may not be sufficient to compensate a defect in DHFR activity [50]. Nevertheless, PTR1 is an essential enzyme in itself in *T. brucei* [96], and simultaneous inhibition of PTR1 and DHFR elicits a synergistic effect on the inhibition of growth of bloodstream forms of *T. brucei in vitro* [97]. Therefore, the combination of antifolates with specific inhibitors of PTR1 is now considered a valid strategy to improve antiparasitic efficacy.

Different approaches have been employed in the search for PTR1 inhibitors: enzyme inhibition screenings of series of pteridine and quinoxaline derivatives [98, 99], docking modeling starting from scaffolds known to inhibit

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DHFR [97, 100–102] and virtual screening of nonpteridine derivatives to selectively inhibit PTR1 over the human enzyme [103, 104].

It is generally accepted that a successful antifolate chemotherapy in trypanosomatids will require the simultaneous inhibition of both DHFR and PTR1 in order to deprive the cell of its pool of reduced folates. However, the identification of a compound with high affinity for both enzymes is extremely difficult to envisage considering the structural differences between both proteins. In addition, it is probable that this type of molecules will inhibit other folate-metabolizing enzymes including human DHFR [105].

Deoxyuridine Triphosphate Nucleotidohydrolase (dUTPase)

dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate (PPi). Thus, dUTPase plays a dual function, and it provides the dUMP precursor for the synthesis of dTTP and at the same time keeps a low dUTP/dTTP ratio and prevents the misincorporation of dUTP by the DNA polymerase during replication. *Leishmania* and *Trypanosoma* possess an all-α homodimeric dUTPase with two active sites [106–110] that differs structurally from the trimeric dUTPases expressed by bacteria, yeast, and humans, which are mainly composed of β-sheet structures. Kinetoplastid dUTPases have five highly conserved sequence motifs that contribute to the formation of the active sites and are involved in substrate binding and/or catalysis. Kinetic studies showed that the uracil moiety and the lack of a hydroxyl group at the 2′ position are crucial for enzyme activity and that, unlike trimeric dUTPases, the dimeric enzymes hydrolyze dUDP as well as dUTP, yielding dUMP in both cases [111]. The structural basis for uracil selectivity and the catalytic mechanism have been further elucidated from the crystal structure of trypanosomal dUTPases [108, 110]. Interestingly, dimeric dUTPases are strongly inhibited by dUMP in a competitive manner ($K_i = 13\ \mu M$) and by α,β-imido-dUTP at a much higher extent ($K_i = 0.89\ \mu M$), indicating that the triphosphate moiety improves the inhibitory activity [112].

The phenomenon of product inhibition provided the molecular basis for the initial design of inhibitor molecules. Nguyen *et al.* synthesized several dUMP analogs, with substituents at the 5′- and 3′-positions of 2′-deoxyuridine, and tested them against *L. donovani* and *P. falciparum* dUTPases and also against the erythrocytic stages of the chloroquine-resistant K1 strain of *P. falciparum*, the amastigote form of *L. donovani*, the trypomastigote form of *T. b. rhodesiense*, and the amastigote form of *T. cruzi* [113]. However, while this study led to the discovery of novel and selective antimalarial molecules, no effective dUTPase inhibitors were identified for *Leishmania* and *Trypanosoma* parasites. As alternative approach, a docking study on *T. cruzi* dUTPase was carried out using the complexed form of the enzyme. The ligands with best superimpositions and favorable binding energies were synthesized and tested for enzyme and parasite growth inhibition *in vitro.* Unfortunately, none of the compounds tested inhibited purified *Tc*dUTPase or parasite proliferation [114]. Indeed, the presence of negative charge has been identified as a major requirement for enzyme closure

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and for the formation of a high-affinity enzyme–ligand complex in dimeric dUTPases [109], thus hindering the design of druggable molecules.

Despite the fact that initial attempts have been unsuccessful, the structural differences between trimeric and dimeric dUTPases and their essential role for parasite survival still make these enzymes a valid drug target for chemotherapy. Therefore, the search for inhibitors of these essential and structurally singular dUTPases warrants further investigation. Novel structural data arising from the *L. major* enzyme in complex with substrate analogs, dUMP and deoxyuridine, may guide the development of a potent and specific new class of dUTPase inhibitors [109].

Final Remarks

In conclusion, although parasites of the Trypanosomatidae family have both the *de novo* biosynthetic and salvage pathways for providing pyrimidines and therefore would present an apparent redundancy in satisfying their cellular needs, there are several enzymes of these pathways that are necessary for normal cell growth and infectivity. A number of pyrimidine metabolism enzyme null cell lines are auxotrophs for pyrimidines, yet the requirements exceed physiological levels present in the host and thus do not grow in animal models. On the other hand, many of these enzymes present unique characteristics that are being exploited in target-based drug design and thus constitute highly druggable targets for further optimization studies. Of major interest are the enzymes involved in thymidine salvage together with those responsible for the synthesis of dTMP from dUMP and further conversion to dTTP. Future studies aimed at exploiting this information will aid in the identification of novel avenues for drug design at the level of a pathway that has shown enormous therapeutic potential in other organisms.

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7
Phosphatidylcholine and Phosphatidylethanolamine Biosynthesis Pathways in *Plasmodium*

Ewelina Guca[1], *Alicia Contet*[1], *Henri J. Vial, Kai Wengelnik, and Rachel Cerdan*[*]

Abstract

During their life cycle *Plasmodium* species rely on phospholipids to build the membranes necessary for daughter cell development. Phosphatidylcholine and phosphatidylethanolamine together represent about 85% of total membrane lipids, and inhibition of their biosynthesis leads to parasite death. Both phospholipids are synthesized by the parasite's own machinery mainly through the two parallel *de novo* Kennedy pathways. Besides, the parasite exhibits additional reactions that bridge these routes. We summarize the current findings on the essential roles, the mechanisms of action, and the structures of the enzymes involved. Particular emphasis is given to their potential use as drug targets in antimalarial therapy by structure-based drug design approaches.

Introduction

There were an estimated 214 million malaria cases and 438 000 deaths in 2015, most of them children under the age of 5 [1]. The infectious agent of this disease is a protozoan parasite *Plasmodium*, adapted to live in the red blood cells of humans and transmitted by female *Anopheles* mosquitoes. Among the five species that infect humans, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi*, and *Plasmodium falciparum*, the last one is the most virulent, causing the vast majority of deaths. The *Plasmodium* life cycle is complex with several developmental stages in humans and mosquitoes, but all the symptoms of malaria are caused during the massive replication in the red blood cells. Parasite membrane lipids are mainly composed of phospholipids (PLs) [2, 3], and thus, production or scavenging of these molecules is required at every stage of parasite development. The host cells infected by *Plasmodium* differ considerably in their capacity for PL synthesis. Recently, it has been shown that during

[*] Corresponding author.
[1] These authors contributed equally to this review.

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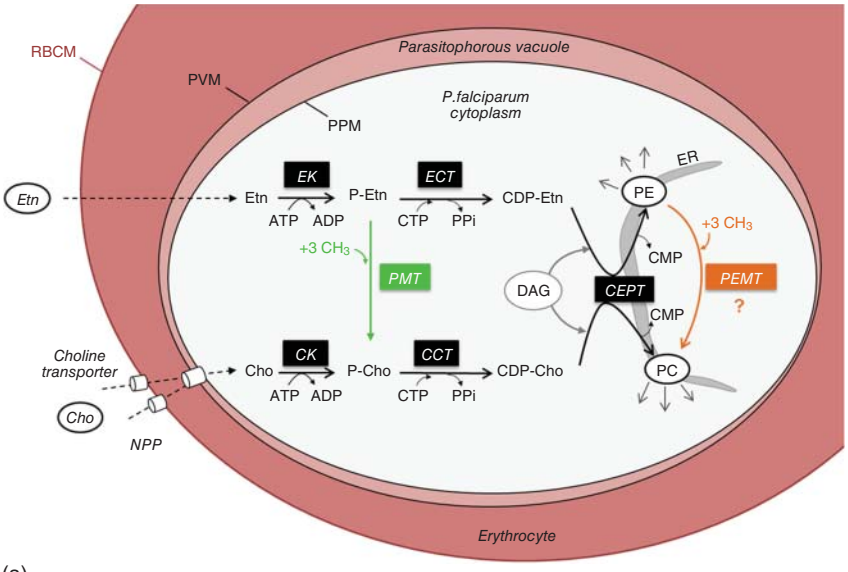
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the hepatic stage, the parasite depends on the phosphatidylcholine (PC) production of the host cell to multiply efficiently, indicating that at the hepatic stage, the parasite is unable to synthesize sufficient PC to allow for the production of thousands of daughter merozoites [4]. In contrast to hepatocytes, mature erythrocytes, the host cells of *Plasmodium* blood stages, do not synthesize PLs at all [5]. Even though *P. falciparum* establishes exchanges with high- and low-density lipoproteins, the exogenous lipids from the host serum contribute only marginally to the total amount of PLs in the parasite [6]. The PL composition of uninfected erythrocytes is 30% PC, 30% phosphatidylethanolamine (PE), 25% sphingomyelin, and 15% phosphatidylserine. Other PLs, such as phosphatidylinositol, phosphatidic acid, and lysophosphatidylcholine, represent only about 1% of total PL [5, 7]. After infection by *P. knowlesi* (a monkey parasite that also infects humans), a sixfold increase in PL content is observed in erythrocytes infected with trophozoites [3]. Again, the major PLs are PC and PE accounting for 40–50% and 35–45% of the total PL content, respectively [2, 8, 9].

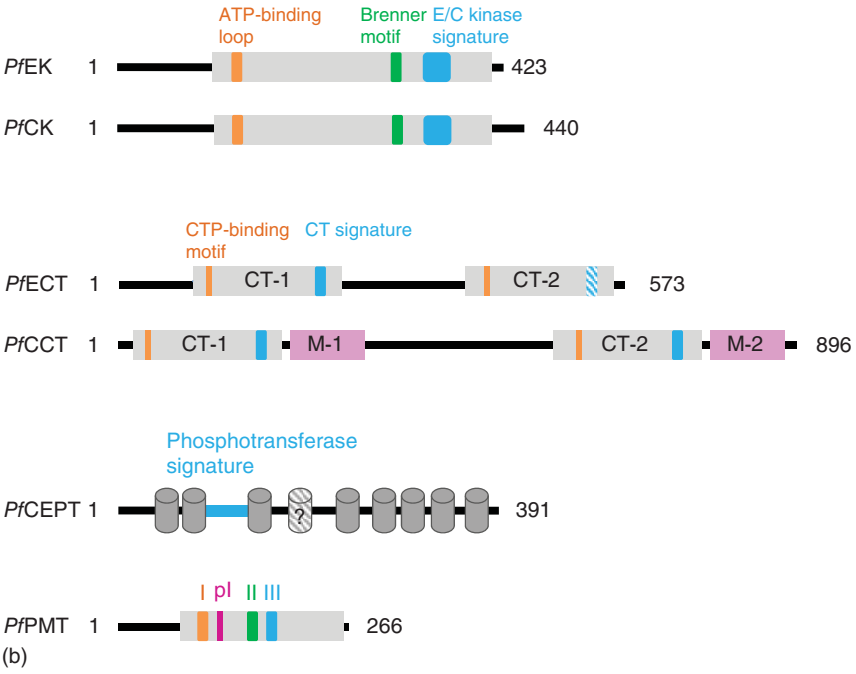
Similarly to other eukaryotic cells, *Plasmodium* synthesizes PC and PE via the *de novo* CDP–choline (CDP-Cho) and CDP–ethanolamine (CDP-Etn) pathways (Figure 7.1a). These two pathways are also named "Kennedy pathways" after their discovery in rat cells by Eugene Kennedy almost 60 years ago [10, 11]. The Kennedy pathways consist of three enzymatic reactions for the synthesis of PC from choline (Cho) and of PE from ethanolamine (Etn). These polar heads are taken from the host plasma. Once in the parasite, Cho and Etn are phosphorylated by choline kinase (CK) and ethanolamine kinase (EK), respectively (Figure 7.1a). In the second step, the products phosphocholine (P-Cho) and phosphoethanolamine (P-Etn) are coupled with CTP to form CDP-Cho and CDP-Etn, respectively. CTP: phosphocholine cytidylyltransferase (CCT) and CTP: phosphoethanolamine cytidylyltransferase (ECT) catalyze these reactions and have been described as the rate-limiting steps of the Kennedy pathways, both in mammals [12] and in *Plasmodium* [13, 14]. The last step involves the transfer

Figure 7.1 Enzymes of the *de novo* phosphatidylcholine (PC) and phosphatidylethanolamine (PE) biosynthesis pathways in *P. falciparum* blood stage parasites. (a) PC and PE *de novo* biosynthesis pathways. Choline (Cho) and ethanolamine (Etn) are scavenged from the host. Shown in black are the two parallel *de novo* Kennedy pathways of PC and PE synthesis, in green the transversal PMT pathway, and in orange the transversal PE methylation pathway. Enzymes are boxed and the question mark corresponds to enzyme activity for which the corresponding gene has not been identified yet. RBCM: red blood cell membrane, PPM: parasite plasma membrane, PVM: parasitophorous vacuole membrane, NPP: new permeation pathways, and DAG: diacylglycerol. Details concerning enzymes, substrates, and products are given in the text. (b) Schematic representation of the *P. falciparum* enzymes of the PC and PE biosynthesis pathways. For *Pf*CK, *Pf*EK, *Pf*CCT, and *Pf*ECT, catalytic domains are represented by gray boxes and membrane-binding domains of *Pf*CCT by pink boxes. Conserved motifs and family signatures are indicated by colored rectangles and described in the text. Cylinders in the *Pf*CEPT representation correspond to predicted transmembrane domains. CT: cytidylyltransferase domain (catalytic domain) and M: membrane binding domain.

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(a)



(b)

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of CDP-Cho and CDP-Etn to diacylglycerol (DAG). In *Plasmodium*, this reaction is catalyzed by a single enzyme, Cho/Etn phosphotransferase (CEPT), and generates the final products PC and PE [15] (Figure 7.1a). In addition, both Kennedy pathways are linked by two transversal pathways. The phosphoethanolamine methyltransferase (PMT) converts P-Etn into P-Cho by *S*-adenosylmethionine (SAM)-dependent triple methylation (Figure 7.1a). Finally, synthesis of PC could also occur by triple methylation of PE by phosphatidylethanolamine N-methyltransferase (PEMT). The *de novo* Kennedy pathway is the main route for the PC synthesis in *P. falciparum*, accounting for approximately 80% of the produced PC at the trophozoite stage [16]. These results are corroborated by quantitative kinetic modeling in *P. knowlesi* [17], identifying the PC Kennedy pathway as the main route of PC synthesis also in the simian parasite.

The Kennedy pathways are crucial for sustaining parasite growth and proliferation during blood stage development. Therefore, they have been proposed as pharmacological targets for the treatment of malaria. The most advanced pharmacological approach is based on the use of Cho analogs that have been designed to inhibit the biosynthesis of PC, the major structural phospholipid, leading to the development of a new class of antimalarial drugs. The strategy was to block Cho import and/or to inhibit the enzymes involved in PC synthesis in blood stage parasites [18]. Structure–activity relationship studies led to a first generation of quaternary bis-ammonium compounds [19] whose lead compound, G25, had potent antimalarial activity *in vitro* and *in vivo* both in mice and in monkeys [20]. Several generations of quaternary ammoniums were subsequently developed and led to the synthesis of bis-thiazolium. This compound family has provided the clinical candidate albitiazolium (also named T3 or SAR97276), developed in partnership with Sanofi for the treatment of severe malaria by parenteral route. The antimalarial activity of albitiazolium is strongly antagonized by Cho, and inhibition of Cho entry into the parasite appears to be the primary mechanism by which albitiazolium exerts its potent antimalarial effect [21, 22]. Most interestingly, this compound accumulates specifically and to a high extent (>400-fold) in the infected red blood cell [20, 23, 24]. It is likely that in this way, albitiazolium reaches the concentration required to inhibit the three enzymes of the PC Kennedy pathway [21]. A radiolabeled bis-thiazolium derivative was also found to accumulate in the food vacuole of the parasite and to interact with α-hematin, which contributes to the antiparasitic activity [25, 26]. This dual antimalarial mode of action on PC synthesis and on α-hematin is a substantial advantage as it could prevent the emergence of drug resistance. In clinical trials, albitiazolium showed a confirmed antimalarial activity in adult patients with a low dose of 0.18 mg/kg/day during 3 days. For children, the dose had to be optimized due to a more rapid clearance of the drug. The major drawback of albitiazolium is its low oral bioavailability (<5%). This hampers clinical development for the treatment of noncomplicated malaria that covers most of the market needs. Therefore, today, the clinical development of albitiazolium is on hold. Nonetheless, Cho analogs have demonstrated their importance for new approaches to develop antimalarials as they have been shown to target PC synthesis with an innovative mechanism of action.

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In a more general context, the PE and PC biosynthesis pathways offer potential therapeutic targets and strategies aiming at interfering with the enzymatic steps of PL biosynthesis appear attractive. The full biochemical and structural characterization of targets is a prerequisite for target-based therapeutic approaches and the rational design of new drugs. In this chapter, we provide a detailed account of the enzymes involved in PE and PC biosynthesis pathways in *Plasmodium*. We focus on their mechanisms of catalysis as well as the in-depth characterization of the enzyme's structural features.

Kinases

CK [EC 2.7.1.32] and EK [EC 2.7.1.82] catalyze the first steps of the Kennedy pathways. The enzymatic reaction is the addition of the γ-phosphate from an ATP molecule to Cho or Etn (Figure 7.1a). The essential nature has only been studied for CK of the rodent malaria parasite *Plasmodium berghei* [27]. While *Pb*CK was successfully GFP-tagged, neither deletion nor disruption of the coding sequence by double or single homologous recombination was obtained. These results strongly suggest that *ck* mutant *P. berghei* are not viable and therefore argue for an essential role of CK in *P. berghei* asexual development.

The CK and EK endogenous activities have been identified and quantified in *P. falciparum* [28]. *Pf*CK is the most efficient enzyme in its Kennedy pathway when comparing the kinetic values of the different steps of the endogenous *Plasmodium* enzymes (Table 7.1). *Pf*CK shows an affinity (K_m) for Cho of 31 μM and a maximum velocity (V_{max}) of 63 pmol/min for 10^7 parasites while a K_m value of 71 μM and a V_{max} of 39 pmol/min for 10^7 parasites are found for *Pf*EK, the counterpart PE pathway enzyme [21, 29]. Recombinant *Pf*CK and *Pf*EK have been characterized *in vitro*, and each *Plasmodium* enzyme is selective for its respective substrate [29, 30]. *Pf*CK and *Pf*EK are expressed mainly during the trophozoite and schizont stages and are localized in the cytoplasm of the parasite [29, 30].

Table 7.1 Kinetic parameters of the endogenous enzymes of the PC and PE biosynthesis pathways.

Endogenous enzyme	Substrate	K_m (μM)	V_{max} (pmol/min/10^7 parasites)	References
*Pf*EK	Ethanolamine	71.3 ± 4.6	39.6 ± 0.1	[29]
*Pf*ECT	Phosphoethanolamine	373 ± 123	4.0 ± 0.3	[14]
*Pk*CEPT	CDP–ethanolamine	18 ± 3	12.6 ± 5.0	[15]
*Pf*CK	Choline	31.2 ± 4.1	63.3 ± 11.6	[21]
*Pf*CCT	Phosphocholine	511 ± 24	5.2 ± 1.4	[21]
*Pf*CEPT	CDP–choline	13.2 ± 0.7	17.0 ± 1.5	[21]

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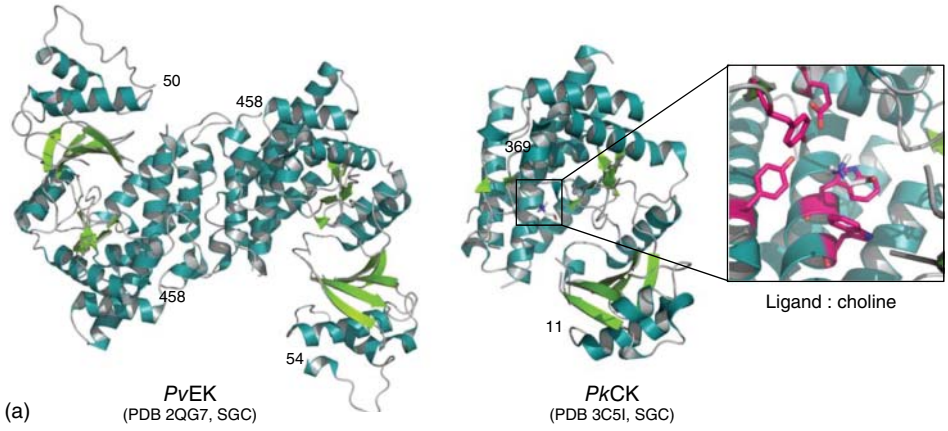
Both enzymes share the common signature motifs of eukaryotic CK and EK (Figure 7.1b): (i) the ATP-binding loop, (ii) the Brenner's phosphotransferase motif (CS)HNDhX_3N (h denotes a large hydrophobic residue), and (iii) the Cho/Etn kinase motif (ILV)X_2ID(FWY)E(YF)X_3NX_3(FYW)DX_6E.

The usual catalytic mechanism for kinases involves the sequential (random or ordered) binding of both substrates to form a ternary complex. However, recent kinetic and mechanistic studies of human CK (CK-α) suggest a double-displacement (ping-pong) mechanism for the enzymatic reaction [31]. The phosphate transfer from ATP to Cho occurs in two steps. First, the formation of a phosphoenzyme intermediate occurs and then the phosphate is transferred from the enzyme to Cho. In this process, the conserved aspartate residue of the Brenner's motif (Asp306 in human CK-α) plays a pivotal role by hydrogen bonding with the phosphate anion and with P-Cho.

Crystal structures of CK have been solved for the human and other eukaryotic and prokaryotic *Streptococcus pneumoniae* enzymes [32–34]. Concerning *Plasmodium* species, the structural parasitology program of the Structural Genomics Consortium (SGC-Toronto) has deposited the 3D structures of *Pf*CK in the presence of ADP (PDB 3FI8) and *P. knowlesi* CK cocrystallized with Cho (PDB 3C5I) in the Protein Data Bank (PDB). Both *Plasmodium* CK structures are very similar with a root-mean-square deviation of 0.99 Å for the backbone atoms. A *P. vivax* EK structure (PDB 2QG7), also solved by the SGC-Toronto, is the only EK structure determined to date. *Plasmodium* CK structures are monomeric while the majority of eukaryotic CK and *Pv*EK are dimeric. The overall fold of CK/EK is conserved. *Pf*CK, *Pk*CK, and *Pv*EK contain an N-terminal domain of approximately 100 amino acids consisting of a five-strand antiparallel β-sheet and three α-helices and a C-terminal domain (~250 residues) with eight α-helices and two β-sheets of two and three short antiparallel β-strands (Figure 7.2a). The catalytic pocket is located between both domains. Site-directed mutagenesis studies combined with structural analyses allowed the identification of residues involved in the substrate-binding site. The Cho-binding site is composed of a deep hydrophobic groove with a rim of negatively charged residues. The patch of negative residues directs Cho in the hydrophobic pocket, and it has been recently shown that both

Figure 7.2 Structures of the enzymes of the *de novo* PC and PE biosynthesis pathways in *Plasmodium*. (a) X-ray crystal structures of *P. vivax* EK (left) and *P. knowlesi* CK (right) with Cho bound in the binding site (inset). Cho (gray) and residues participating in its binding (composite aromatic box) are in pink sticks. (b) 3D comparative models of the domains CT of *P. falciparum* ECT (left) and CCT (right) with CDP–choline in the binding site (inset). CDP-Cho (gray) and residues participating in the coordination of CDP-Cho are represented as sticks. Yellow: HxGH motif, purple: RTEGVSTT motif, pink: composite aromatic box. (c) X-ray crystal structure of *P. falciparum* PMT with P-Etn and SAH bound in the active site (inset). Important residues binding to the ligands are indicated as sticks. Orange: residues involved in catalysis, light pink: tyrosine residues participating in the coordination of the phosphate moiety of P-Etn. All figures have been generated using PyMol [35].

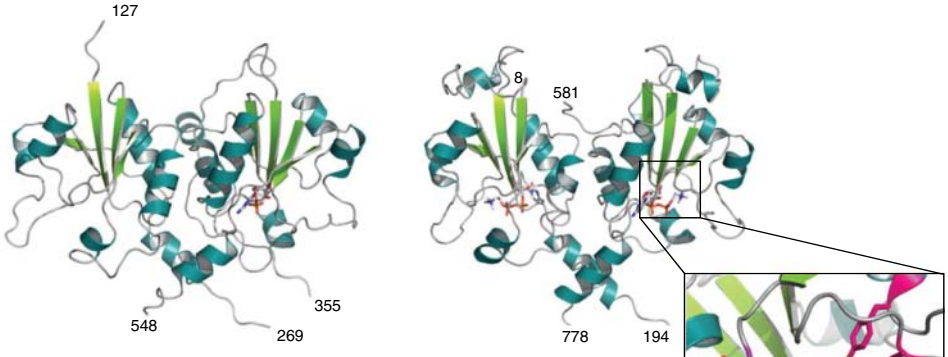
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(a)

*Pv*EK
(PDB 2QG7, SGC)

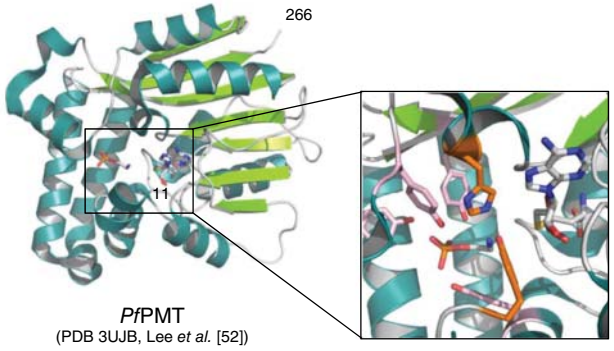
*Pk*CK
(PDB 3C5I, SGC)



(b)

*Pf*ECT CT domains
(model, Maheshwari *et al.* [14])

*Pf*CCT CT domains
(model, Contet *et al.* [41])



(c)

*Pf*PMT
(PDB 3UJB, Lee *et al.* [52])

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cation-π and electrostatic interactions govern the binding of the Cho moiety in *Pf*CK (Figure 7.2a). More generally, quaternary ammonium containing molecules such as Cho have been described to be recognized by a "composite aromatic box" in enzymes and by an "aromatic box" in receptors, both boxes differing in their composition [36]. A glutamate (Glu324 in *Pf*CK) conserved in all CKs might stabilize the positive charge of Cho. One interesting difference appears when comparing the *Pf*CK and *Pv*EK crystal structures. A tyrosine residue (Tyr329 in *Pf*CK), located at the end of the Cho/Etn kinase motif participating in the formation of the hydrophobic pocket and necessary for the stabilization of the Cho quaternary ammonium, is not conserved in *Pv*EK. This difference could contribute to the observed substrate specificity of the parasite CK and EK. Concerning the ATP-binding site, hydrophobic interactions involving the adenine ring and a network of hydrogen bonds made by the ribose moiety and the phosphate both stabilize the nucleotide triphosphate.

The interest regarding CK goes far beyond research on malaria. For years, human CK-α has extensively been investigated, since this enzyme is involved in several cancers and connects PL metabolism with cell-cycle regulation [37]. Increased CK-α activity has been detected in several human cancers and in many tumor-derived cell lines [38]. Target-based antitumoral strategies have been efficiently developed and an inhibitor of human CK-α has recently entered phase I clinical trials [39]. Interestingly, this antitumoral agent has been shown to exert *in vitro* inhibitory activity on *P. falciparum* in the low nanomolar range by significantly reducing parasitemia and blocking the intraerythrocytic development [40].

Cytidylyltransferases

CCT [EC 2.7.7.15] and ECT [EC 2.7.7.14] catalyze the second step of the Kennedy pathways. They allow the transfer of a cytidylyl group from CTP to either P-Cho or P-Etn, thereby leading to the formation of the high-energy intermediates CDP-Cho or CDP-Etn (Figure 7.1a). In *P. berghei*, as it was shown for CK, unsuccessful attempts to disrupt the genes encoding CCT and ECT suggested that both activities are essential for parasite survival [27]. Immunofluorescence studies using anti-*Pf*CCT and anti-*Pf*ECT antibodies localized the two enzymes in the parasite cytoplasm with the presence of some regions of higher concentration that could not be attributed to any specific organelle [14, 41]. Further experimental data are therefore needed to clearly describe the localization of both *Plasmodium* cytidylyltranferases. On the other hand, it is now well established that CCTs are amphitropic enzymes switching between a soluble inactive form and an active membrane-bound form [42–44]. In mammals, CCT localizes to the membrane of endoplasmic reticulum (ER) [45]. It could thus be hypothesized that *Pf*CCT and *Pf*ECT localize near the ER in close proximity to CEPT, the following enzyme of the Kennedy pathway (Figure 7.1a).

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Cytidylyltransferases (CTs) are the central players of the Kennedy pathways as they catalyze the rate-limiting steps of their respective branch and thus regulate the flux of PC and PE synthesis. Forty years ago, first evidence of this role was provided by a study performed in rat hepatocytes, where increasing concentrations of radiolabeled Etn yielded to the accumulation of the ECT substrate P-Etn, while the level of CDP-Etn and the rate of PE synthesis remained unchanged [12]. Similarly, experiments performed in *P. falciparum* [14] and *P. knowlesi* [13] infected erythrocytes showed that the steps catalyzed by *Pf*ECT and *Pk*CCT are rate-limiting for the *Plasmodium* Kennedy pathways. *P. falciparum* ECT and CCT activities have been extensively studied using both endogenous and recombinant enzymes [14, 21, 41, 43, 46]. Kinetic parameters of the endogenous proteins revealed that CTs are the least efficient enzymes of their respective pathway with high micro molar K_m for their phosphomonoester substrates as well as a 3- to 15-fold lower V_{max} value compared to the other enzymes (Table 7.1). These data are in agreement with the rate-limiting role of these enzymes. This was corroborated by a recent metabolomic study performed in *P. falciparum*. Indeed, P-Cho and P-Etn concentrations were found to be 15- and 10-fold higher than the respective CDP-ester concentration in the parasite [47].

In *Plasmodium*, both CCT and ECT contain two CT catalytic cores (domains CT) separated by a long linker (Figure 7.1b). Interestingly for CCT, this duplication feature is found only in three organisms, all from the Apicomplexa phylum: *Babesia*, *Theileria*, and *Plasmodium*. On the other hand, the presence of two domains CT is a general characteristic in all ECT enzymes known so far. CCT, but not ECT, also contains membrane-binding domains (domains M) flanking each catalytic core (Figure 7.1b). The catalytic core of CTs including all CCT, ECT, and the prokaryotic GCT (CTP: phosphoglycerol cytidylyltransferase) possess two canonical motifs: the "cytidylyltransferase family signature" RTxGVST(T/S) and the "CTP-binding motif" HxGH (Figures 7.1b and 7.2b), also found in class I aminoacyl tRNA synthetase. In *Pf*ECT, as well as in ECT homologs, the conservation of the RTxGVST(T/S) motif is only partial in the second-domain cytidylyltransferase (CT-2). For the *Plasmodium* enzyme, inactivation of the first-domain cytidylyltransferase (CT-1) by site-directed mutagenesis led to a complete loss of activity indicating that the domain CT-2 does not contribute to the catalysis [14]. Similar studies performed on human ECT also identified the domain CT-1 as the only active domain, suggesting that the loss of function of the domain CT-2 might be a general feature of ECT enzymes [48]. Unlike *Pf*ECT, kinetic characterization of recombinant *Pf*CCT showed that both domains CT are enzymatically active and display similar kinetic parameters highlighting important differences between CCT and ECT in *Plasmodium* [41].

The CT-catalyzed transfer of the CMP moiety from CTP to a specific phosphomonoester proceeds via a mechanism involving the sequential binding of both substrates to form a ternary complex [46, 49–51]. The formation of the CDP ester involves the nucleophilic attack of the phosphomonoester on the α-phosphate of CTP and the release of the second product pyrophosphate (PPi) [51, 52]. However, even if this general mechanism is apparently conserved across the family,

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the order of substrate binding appears to differ between known CTs. In *Bacillus subtilis* GCT, rat CCT, and *Pf*CCT, product inhibition studies suggest a random-ordered mechanism of substrate binding [46, 50, 51], while an ordered mechanism was proposed for the human ECT with CTP binding prior to P-Etn [49].

Unlike CK and EK, no crystal structure of a *Plasmodium* CCT or ECT is currently available in the PDB. Therefore, we proposed comparative 3D models of *P. falciparum* CCT and ECT based on experimental structures of homologous enzymes (Figure 7.2b). A comparative model of the two domains CT of *Pf*ECT was built based on the human ECT crystal structure in complex with CMP (PDB 3ELB) [14]. Human ECT folds as an intramolecular dimer, with both domains CT being in contact. Concerning *Pf*CCT, two structural models of the domains CT have been proposed [41, 46] based on the crystal structure of the domain CT of rat CCT solved in complex with CDP-Cho (PDB 3HL4) [52]. Domains CT of *Pf*ECT and *Pf*CCT show a similar global folding. Each domain CT adopts an α/β-topology organized in a Rossmann fold consisting of five parallel β-strands flanked by either five or six α-helices (Figure 7.2b). Both *Pf*ECT and *Pf*CCT 3D models were built as intramolecular dimers.

Together, structural and site-directed mutagenesis data allowed the molecular description of the CCT catalytic sites. Many of the conserved residues have been mutated to decipher their role in substrate binding, catalysis, or structural integrity. In rat CCT, mutation of the arginine to lysine in the RTxGVST(T/S) motif drastically impaired CTP-binding capability, unveiling a direct role of this motif in the accommodation of CTP [53]. Similarly, the substitution of Gly → Ser in the HxGH motif led to a 25-fold increase of the K_m value for CTP, suggesting that this motif is also directly involved in CTP binding [54]. However, replacement of both histidine residues of the motif by alanine residues in either *Bs*GCT [55] or rat CCT [51] led to a decrease of the k_{cat} value by approximately 4 orders of magnitude without affecting the affinity for the substrates. Therefore, it has been proposed that the histidine residues of the HxGH motif are directly involved in catalysis and are responsible for the stabilization of the pentacovalent transition state. Both histidine residues surround the electronegative oxygens of the α-phosphate of CTP with imidazole NH groups [51, 52]. How CCTs and ECTs coordinate their respective phosphomonoester substrate allowing binding specificity is diversely understood. In the absence of any Etn-, P-Etn-, or CDP-Etn-bound structure of ECT, there is currently no structural description of the Etn-binding cleft in ECT enzymes. The binding mode of the Cho moiety in CCT is far better known, both at the enzymatic and at the atomic level. The Cho-binding cleft of CCT is made up of both aromatic and charged residues [41, 46, 52] (Figure 7.2b). As described for *Pf*CK, a composite aromatic box allows the coordination of the quaternary ammonium of the Cho moiety in *P. falciparum* CCT [36] (Figure 7.2b).

The key point of CCT regulation is its translocation to membranes mediated by the domain M [56]. This has been mainly studied for rat CCT. The domain M varies between a mostly disordered conformation in the soluble form and an amphipathic helix in the membrane-bound form [57, 58]. The interaction of the

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domain M with the membrane responds to a lack of PC content characterized by an increase in the negative charge (anionic lipids) density and a negative membrane curvature induced by lipids with small polar head groups [59, 60]. Consequently, lipids promoting CCT translocation and therefore activation include anionic PLs, fatty acids, type II lipids, and neutral lipids in rat [61]. In *P. falciparum*, CCT is activated in the presence of PC vesicles containing the anionic oleic acid or anionic PLs but not by PC vesicles alone or vesicles containing neutral lipids [43]. The predicted C-terminal domain M (domain M-2) of *Pf*CCT has been studied by using synthetic peptides. Interestingly, while the amphipathic helix of rat CCT consists of one long unbroken α-helix, the domain M-2 of *Pf*CCT could contain two distinct α-helices behaving differently upon lipid binding [62]. The domain M acts not only as a lipid composition sensor but also as a silencer in the soluble form of CCT. Indeed, CCT constructs encompassing the domain(s) CT and lacking domain(s) M are constitutively active [41, 46, 63]. An autoinhibitory (AI) segment of 22 residues present in the domain M has been recently identified as the major element responsible for silencing of the soluble form of rat CCT [64]. The crystal structure of the domain CT of rat CCT in the presence of the AI segment (PDB 4MVC) revealed that the segment folds as an α-helix and docks at the base of the catalytic pocket, partially occluding its entrance [65]. A sequence of the *Pf*CCT AI segment (domain M-2) has been proposed based on the partial conservation with the rat AI sequence [66]. However, experimental validation is still needed to confirm its role as a silencer.

In the absence of membrane translocation in ECT, the regulation of the rate-limiting enzyme of the PE pathway is still enigmatic and is poorly understood at the protein level in comparison with CCTs. Recently, several phosphorylated residues have been localized in the linker but also in the CT-1 RTxGVST(T/S) motif of a mouse ECT isoform under serum deficient conditions promoting ECT activity [67]. Nevertheless, how phosphorylation positively regulates mammalian ECT activity remains unknown.

So far, only a couple of moderately efficient inhibitors of *Pf*CCT have been identified [41, 68]. To our knowledge, there is currently no inhibitor of other CCT that could be used as starting point for the development of additional *Plasmodium* CCT and ECT inhibitors. A phospholipid analog, initially identified from a drug screen of *P. falciparum* intraerythrocytic growth *in vitro*, was found to inhibit PC synthesis by blocking recombinant *Pf*CCT activity with an IC_{50} (concentration to inhibit 50% of the enzyme activity) of 28 μM [68]. Recently, the druggability of *Pf*CCT has been investigated using a structure-based virtual screening approach of CDP-Cho analogs followed by biochemical validation [41]. One compound, characterized as a competitive inhibitor but exerting moderate inhibition of the recombinant *Pf*CCT, was shown to specifically inhibit PC synthesis (IC_{50} = 187 μM). Beyond the "classical approach" consisting of targeting the active site of enzymes, other approaches could be also developed in the case of *Pf*CCT. For instance, due to its specific regulatory mechanism, *Pf*CCT could also be targeted by interfering with its membrane translocation to prevent its activation and/or by developing compounds that mimic the role of the AI

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segment. Besides, the two domains M are much less conserved between human and *Plasmodium* CCT than their catalytic sites, possibly allowing the design of chemicals that specifically inhibit the parasite enzyme. However, such approaches rely on a better understanding of the mechanism of regulation of *Pf* CCT, both at the biochemical and at the structural level. It is clear that the resolution of *Plasmodium* CCT crystal structures is now an important prerequisite for further optimization of the moderately effective existing compounds as well as for the identification of new scaffolds.

Phosphotransferase

Choline phosphotransferase (CPT) [EC 2.7.8.2] and ethanolamine phosphotransferase (EPT) [EC 2.7.8.1] catalyze the last steps of the Kennedy pathways by the transfer of P-Cho from CDP-Cho, or P-Etn from CDP-Etn, to DAG. Unlike mammalian cells that contain distinct CPT and EPT enzymes [69, 70], *Plasmodium* appears to possess only one enzyme for the catalysis of the last steps of both Kennedy pathways. This was suggested by studies performed in *P. knowlesi* that showed that CPT and EPT activities may be shared by a single enzyme (CEPT) as the reactions are supported by a similar pattern of heat inactivation and similar dependence on Mn^{2+} and Mg^{2+} cations [15]. Accordingly, only one gene has been identified in all *Plasmodium* genome sequences. As for CK, CCT, and ECT, attempts to disrupt the *cept* gene in *P. berghei* were unsuccessful, suggesting that CEPT is also essential for parasite survival during blood stage development [27]. In *P. falciparum*, the *cept* gene encodes a 391-amino-acid protein containing eight or nine putative transmembrane domains [71] (Figure 7.1b). GFP-tagged CEPT was localized to the membrane of the ER in *P. berghei*-infected erythrocytes [27] in agreement with its localization in mammalian cells [72]. Kinetic parameters of *Plasmodium* CEPT were determined for CDP-Cho in *P. falciparum* [21] and for CDP-Cho and CDP-Etn in *P. knowlesi* [15] (Table 7.1). *Pk*CEPT was found to exhibit similar affinity for its two CDP esters with K_m values of 13 and 18 μM for CDP-Cho and CDP-Etn, respectively. The specificity of the enzyme for various molecular species of DAG was also investigated with respect to acyl chain length and level of unsaturation. Both CPT and EPT activities showed certain preferences for DAG species containing at least one mono-unsaturated acyl chain. Lipidomic analyses of *P. falciparum* identified stearic (18 : 0), oleic (18 : 1), and palmitic (16 : 0) acids as the main fatty acid species present in the parasite membranes [9]. Consistently, *Pk*CEPT activities were found to be highly stimulated in the presence of DAG species containing these three fatty acids [15].

In the context of drug development targeting PE and PC biosynthesis pathways in *Plasmodium*, CEPT would be of much interest as its inhibition is likely to disrupt both PE and PC biosynthesis pathways. Recent photo-cross-linking and click chemistry studies identified CEPT as one of the targets of the highly potent Cho analog albitiazolium in *P. falciparum*-infected erythrocytes [73]. However, despite its crucial function for PE and PC biosynthesis and its interest as a drug target,

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CEPT is by far the least characterized enzyme of the Kennedy pathways. Biochemical studies and drug screening are still difficult due to both the inability to produce recombinant enzyme and the absence of an *in vitro* enzymatic assay. To date, there is no crystal structure of a CEPT enzyme available in the PDB, preventing any structure-based identification of inhibitors.

Transversal Pathways

The transversal pathways link both the Kennedy pathways either from P-Etn to P-Cho with the PMT [EC 2.1.1.103] or from PE to PC with the PEMT [2.1.1.17] (Figure 7.1a).

PMT catalyzes the triple methylation of P-Etn to P-Cho with SAM as cofactor [74, 75]. The deletion of the gene encoding the PMT is not lethal for *P. falciparum* at the asexual blood stages, but causes a decrease in parasite growth and defects in parasite development [76]. However, *Pf* PMT appears essential for the maturation of gametocytes, the parasite forms responsible for the mosquito infection [77]. Using GFP-transgenic parasites and immunoelectron microscopy, it was shown that *Pf* PMT localizes to the Golgi apparatus [78].

PMT activity had first been identified *in vivo* by incorporation of radioactive Etn into P-Cho and PC in *P. falciparum*-infected erythrocytes [74]. In contrast to plant PMT orthologs with two catalytic domains [79, 80], *Pf* PMT is a 266-amino-acid protein and contains only a single SAM-binding domain for all three methylation reactions [74]. *Pf* PMT possesses four catalytic motifs: (i) KVLDxGxGxG (motif I), (ii) xGID (motif p-I), (iii) NxxDxIYS (motif II), and (iv) WLKPxGxxLI (motif III) (Figure 7.1b). The *in vitro* biochemical and *in vivo* genetic studies using yeast as heterologous system showed the P-Etn substrate specificity of the enzyme [74, 75]. The recombinant enzyme was characterized biochemically and revealed affinities (K_m) of 79 and 135 μM for P-Etn and SAM, respectively [74].

The mechanism of catalysis of PMT involves three rounds of methylation of P-Etn by three SAM molecules. *N*-Methylethanolamine phosphate and *N*,*N*-dimethylethanolamine phosphate are released as the intermediate products and the third reaction of methylation results in synthesis of P-Cho. One *S*-adenosyl-L-homocysteine (SAH) molecule is released at each step as an additional product [74]. It has been shown that PMT follows a random sequential mechanism of catalysis implying the formation of a ternary complex [81, 82]. Crystal structures and site-directed mutagenesis of *Pf* PMT revealed that Tyr19 and His132 are crucial for the enzyme activity and form a catalytic dyad [83] (Figure 7.2c). In the proposed mechanism of phosphobase methylation, His132 abstracts a proton from the hydroxyl group of Tyr19. The negatively charged oxygen of Tyr19 interacts with a hydrogen of the P-Etn amine in order to facilitate the transfer of a methyl group of SAM to the phosphobase [83].

The X-ray crystal structures of the PMT enzymes from *P. falciparum*, *P. vivax*, and *P. knowlesi* with their ligands have been published [83, 84]. The *Pf* PMT structure contains 10 α-helices and 7 β-strands that are arranged in 2

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domains: an α-helical "lid" domain and a Rossmann-fold-binding domain consisting of 7 β-strands surrounded by 5 helices [83] (Figure 7.2c). The sequences of PMT from *P. vivax* and *P. knowlesi* share high sequence identities with *Pf* PMT (65% and 62%, respectively) as well as structural similarity with root-mean-square deviations of backbone atoms of 0.45 Å for *Pv*PMT and 0.80 Å for *Pk*PMT, respectively [84]. The P-Etn-binding pocket involves four tyrosine residues forming hydrogen bonds with the phosphate group and two positively charged residues (Figure 7.2b). The SAM-binding pocket is located at the interface of the two enzyme domains and involves several residues of the conserved motifs. More specifically, the substrate interacts with an aspartate (Asp85) and an isoleucine (Ile86) residue of *Pf* PMT since the mutation of these residues abrogated the enzymatic activity [83].

Because of the importance of *Pf* PMT in parasite growth and the absence of PMT orthologs in mammals, this enzyme could offer interesting features for future drug development. Amodiaquine (AQ), which is a commercially used antimalarial drug, was found to inhibit *Pf* PMT activity *in vitro* (60% at 5 μM) [85]. However, other studies showed a millimolar range inhibition of the recombinant *Pf* PMT by AQ [86]. A crystal structure of *Pf* PMT in complex with AQ is available in the PDB (PDB 4FGZ). A screening of a National Cancer Institute (NCI) library was performed and identified another compound (NSC158011) with an IC_{50} value of 9.2 ± 3.5 μM against the enzyme activity *in vitro* and of 5.2 μM against *P. falciparum* intraerythrocytic growth [77]. This compound also inhibits *Pv*PMT and *Pk*PMT with IC_{50} values of approximately 99 and 50 μM [84] and is able to block gametocytogenesis [77]. It thus shows interesting and promising features for future structure-based lead development.

A PMT gene has been identified in the genome sequences of *Plasmodium* species that infect humans, monkeys, and birds, but the gene is absent from all three sequenced rodent malaria species [87]. From phylogenetic analyses, it was suggested that the absence of a rodent malaria *pmt* gene was caused by an early deletion correlated with the separation of the *Plasmodium* rodent and nonrodent branches [87–90]. As already stated, genetic experiments have indicated that the gene coding for *Pb*CK appears essential for the rodent *P. berghei* parasite survival at its blood stage. Due to the absence of PMT, it is likely that PC synthesis could not be rescued in rodent parasites after CK deletion. In human malarial parasites that possess PMT, PC synthesis could theoretically occur by this transversal pathway and thus circumvent the CK step (Figure 7.1a). Hence, the question arises: How essential and/or complementary are these metabolic pathways? Exogenous Cho produced a dose-dependent repression of *Pf* PMT transcription and also induced proteasomal degradation of the enzyme [91]. However, metabolic flux measurements failed to reveal any significant interaction between Etn level and the choline-dependent PC biosynthesis or between the Cho level and the PE biosynthesis (H. Vial, unpublished data). So far, results suggest that both transversal and *de novo* CDP-Cho pathways are not or are poorly redundant. The limited cross-pathway regulatory mechanisms suggest that both pathways have distinct roles in *P. falciparum*.

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The other enzyme that could connect both Kennedy pathways is PEMT [2.1.1.17]. PEMT activity has originally been detected in *P. falciparum* and *P. knowlesi* by incorporation assays with radiolabeled soluble precursors or PE [92, 93]. However, no corresponding gene has been identified yet in any *Plasmodium* genome sequence. The existence of the enzymatic activity allowing the conversion of PE to PC remains to be clarified in particular after the discovery of PMT activity in *Plasmodium* species allowing the synthesis of P-Cho from P-Etn (Figure 7.1a). Indeed, it has been reported that the deletion of the gene encoding the *Pf* PMT completely abolished the incorporation of Etn into PC [76].

Conclusion

The PC and PE biosynthesis pathways are crucial for the survival and development of *Plasmodium*. To target the enzymes of these routes by structure-based approaches, the knowledge of their biochemistry and their detailed structural description is of utmost importance. The data summarized here present our current biochemical and structural knowledge of the seven enzymes involved in these pathways in *Plasmodium*. Five of them (EK, CK, ECT, CCT, and PMT) are biochemically well characterized *in vitro* and *in vivo*. For three of them (EK, CK, and PMT), X-ray crystal structures are available and for two (CK and PMT), potent inhibitors have been identified. The example of the bis-thiazolium class of compounds that targets PC biosynthesis proves that the development of Kennedy pathway inhibitors can be a powerful strategy in the battle against malaria. Detailed knowledge of the biochemical properties of the targets and their structures should then also help to design compounds that specifically inhibit *Plasmodium* enzymes with little effect on their human homologs.

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8
Immunophilins as Possible Drug Targets in Apicomplexan Parasites

Alessandra Bianchin, Anthony J. Chubb, and Angus Bell*

Abstract

Immunophilins is the collective name given to two structurally unrelated protein families, the cyclophilins (CYPs) and FK506-binding proteins (FKBPs). Proteins of these two families, which are found throughout biology, tend to have certain common features, although these are not conserved in all members: (i) ability to bind immunosuppressive ligands and related small molecules, particularly cyclosporins in the case of the CYPs and FK506 (also known as *tacrolimus*, Figure 8.1) for the FKBPs; (ii) peptidyl-prolyl *cis–trans* isomerase (PPIase) activity, an enzymic activity relevant to protein folding (see in the following); and (iii) ability to act as molecular chaperones. Since their discovery in the 1980s, understanding of the importance of immunophilins has expanded and modulation of their activities is being explored as a therapeutic approach to disease areas as diverse as viral infection and neurodegeneration. The reasons for including these proteins – among so many possibilities – in a book about antiparasitic drug discovery are discussed in detail as follows. In a nutshell, they derive from observations of the potent and selective activity of nonimmunosuppressive immunophilin ligands on a number of parasites, particularly Apicomplexa, on which we shall focus here. The emphasis will be on the potential of immunophilins as drug targets or mediators of drug action, but there is also some interesting biology to be found in the study of parasite immunophilins, and we shall touch on some of it in this chapter.

Immunophilins

Correct protein folding is sometimes dependent on the *cis–trans* isomerization of one or more X–P bonds, where X is any amino acid on the N-terminal side of a proline residue [1]. Among proteinogenic amino acids, the peculiarity of proline is having low difference in free energy between the *cis*- and *trans*-conformations of the peptide bond preceding it. Therefore, X–P bonds (which are all initially *trans*-) must reach their intended conformations, and this can limit the rate of protein folding [2]. It is this reaction that is catalyzed by peptidyl-prolyl *cis–trans*

*Corresponding author.

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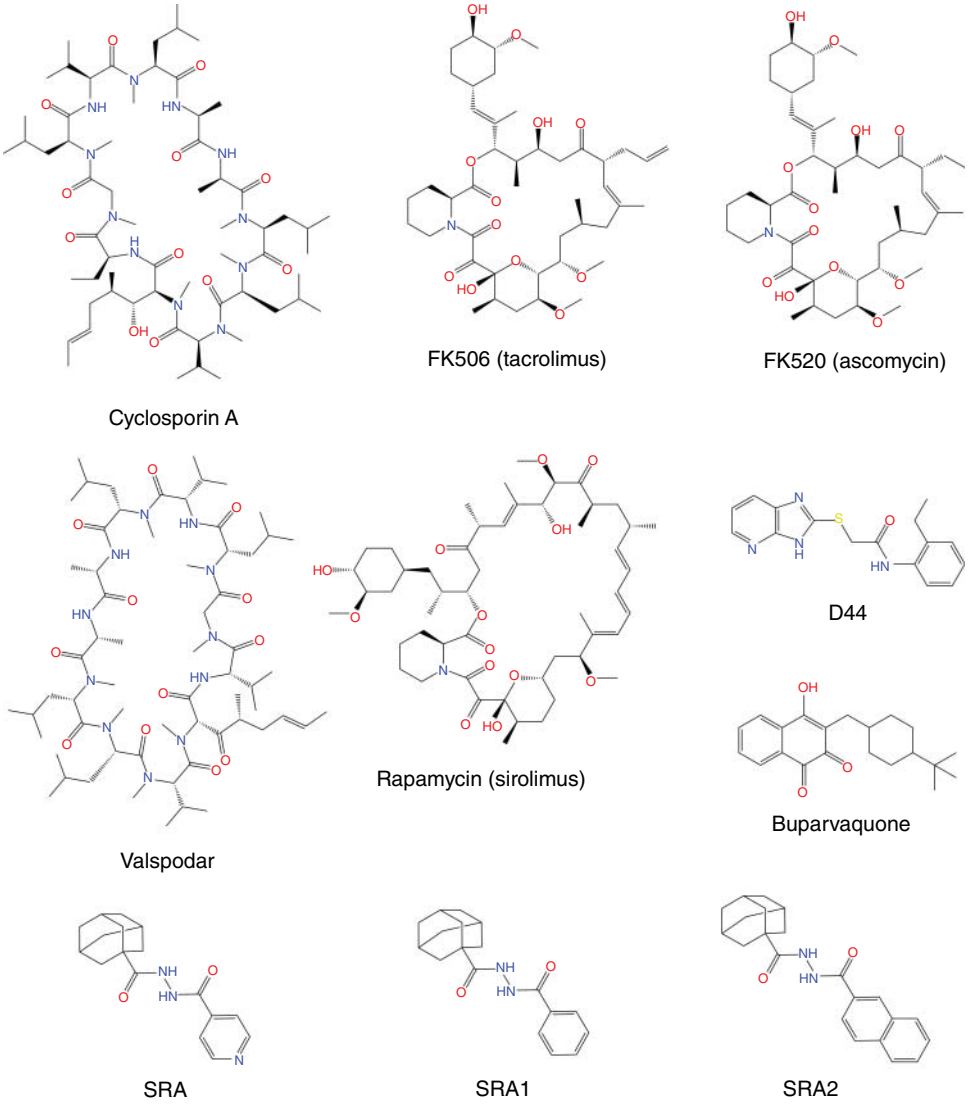


Figure 8.1 Chemical structures of ligands mentioned.

isomerases (PPIases) [3], which include not only cyclophilins (CYPs) and FK506-binding proteins (FKBPs) but also two smaller protein families, the Pin1/parvulins and trigger factors [4].

The history of immunophilins starts in the 1980s when German researchers isolated an approximately 17-kDa protein from pig kidney cortex that accelerated protein folding *in vitro* [5]. Based on the substrate specificity, the novel enzyme was classified as a PPIase [5, 6]. Around the same time, US researchers purified a specific cytosolic binding protein from bovine thymocytes responsible for the concentration by lymphoid cells of the immunosuppressant drug cyclosporin A

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(CsA; Figure 8.1), a cyclic peptide of fungal origin [7]. They named the protein cyclophilin (later cyclophilin A or cyclophilin 18), and it is still considered the archetype of the CYP family. Finally, in 1989, it was shown that the two proteins were the same [8, 9]. This was an intriguing finding because until then, the molecular details of CsA's immunosuppressive action had been largely unknown. More exciting still, a distinct PPIase protein was discovered in bovine thymus and human spleen as the receptor for the newly introduced immunosuppressive drug FK506 [10, 11]. The *Streptomyces*-derived macrolactone FK506, while chemically unrelated to the cyclosporins, was known to exert a similar effect on T lymphocyte signal transduction to that of CsA. This new enzyme was named FKBP, and the 12-kDa human FKBP12 is considered the archetype. This protein was also the receptor for another immunosuppressive macrolactone, rapamycin (sirolimus, Figure 8.1) [12]. In 2005, a novel class of dual family immunophilins was identified, FCBP (FK506- and cyclosporin-binding protein) in *Toxoplasma*, *Flavobacterium*, and *Treponema* [13] and subsequently in other organisms [14].

Immunophilin genes are known to be ubiquitous in nearly all forms of bacteria, archaea, and eukaryotes and often encode multiple CYPs and FKBPs [4]. The human genome, for example, encodes 16 CYPs and 15 FKBPs. In terms of architecture, these proteins are quite diverse, with some composed of a single PPIase domain and others having more than one PPIase domain and/or others such as tetratricopeptide repeat (TPR) domains. The presence of specific subcellular location signals in some immunophilin sequences suggests that, while the predominant CYPs and FKBPs are often cytosolic, there are commonly several distinct immunophilins in a cell, targeted to different compartments [4].

Currently, there is little evidence that immunophilins are generally required for survival in biological systems. In *Saccharomyces cerevisiae*, immunophilins are not required for growth in culture, since the deletion of all 12 genes encoding the proteins, either individually or together, had no effect on physiological parameters such as temperature sensitivity, arrest and recovery from pheromones or glycogen stress, mating, or sporulation [15]. While often used as an example of nonessentiality for immunophilins, a more accurate interpretation is that they are often only required in response to stress conditions or specific environmental cues [16, 17]. In other cases, immunophilins have clear roles that are adversely affected by genetic ablation or treatment with ligands. In mice, several knockout and mutagenesis studies demonstrated some physiological roles of immunophilins, with effects on the development of erythema of the eyelid and ventricular myocardium trabeculation [18, 19]. In the human nervous system, FKBPs participate in neuronal differentiation [20] and CYPs have been demonstrated to act extracellularly contributing to inflammatory responses [21]. One well-studied immunophilin function is in steroid receptors, which require an ordered assembly of chaperone proteins including certain immunophilins such as human FKBP52 to mature into functional conformations [22–24]. Recently, immunophilins have received a lot of attention as potential drug targets due to the discovery of their role in diverse human diseases including infectious diseases. In humans, FKBPs are associated with several neurodegenerative diseases as they are particularly highly expressed in the nervous system [25]. In Alzheimer's disease, the expression of FKBP12 in the brain is lower than in brains

from age-matched control individuals [26], and its PPIase activity is essential to prevent tau peptide from aggregating [27]. In Parkinson's disease, a degenerative disorder of the central nervous system characterized by alpha-synuclein fibrils, studies on several PPIases, demonstrated that FKBP12 was the most potent enhancer of alpha-synuclein aggregation [28]. Immunophilins have been shown to be involved also in different types of cancer, such as prostatic hyperplasia [29, 30], colorectal adenocarcinoma [31], breast tumors [32], and leukemia[33, 34].

A number of obligate parasites have been shown to adopt host immunophilins for infection and growth. In human immunodeficiency virus (HIV) infections, mutations in the promoter region of CYP18 promote disease progression from HIV-positive status to acquired immunodeficiency syndrome [35], and CYP18 knockouts and regulatory mutations have a protective effect from HIV-1 infection [36, 37]. FKBP12 is hijacked by HIV, which contains an average of 25 molecules per virion and uses it for protein degradation and sorting [38]. Other infections that involve immunophilins are hepatitis B [39] and C [40, 41], influenza virus [42], vaccinia virus [43], vesicular stomatitis virus, and severe acute respiratory syndrome coronavirus [44, 45].

Immunophilins in Apicomplexa

The phylum Apicomplexa comprises single-celled eukaryotes, known to parasitize vertebrate (including humans) and invertebrate hosts. As a group, Apicomplexa are complex organisms that undergo complex morphological and molecular transitions during their life cycles, the exact details depending on the particular genus and species. Two common features that define apicomplexan parasitism are (i) invasiveness of one or more of the life-cycle stages, followed by intracellular growth, and (ii) high rates of multiplication within hosts. The major diseases caused by Apicomplexas are malaria (caused by *Plasmodium* species), babesiosis (by *Babesia* spp.), theileriosis (by *Theileria parva* and *Theileria annulata*), cryptosporidiosis (by *Cryptosporidium* spp.), and coccidioses such as toxoplasmosis (*Toxoplasma gondii*), sarcosporidiosis (*Sarcocystis* spp.), eimeriosis (*Eimeria* spp.), cyclosporiasis (*Cyclospora cayetanensis*), and isosporiasis (*Isospora belli*). All of these genera except *Theileria* and *Eimeria* are significant human pathogens. Other diseases of economic importance are those of farm animals that incur enormous costs to agriculture [46].

Immunophilins have been identified in all of the major Apicomplexa, and in some cases, they have been characterized with respect to expression patterns, biochemical activities, and/or physiological roles. Readers are referred to the previous review by Bell *et al.* for details [47]. Protozoan parasite immunophilins are also discussed by Galat and Bua [48], and the domain architectures and phylogenetic relationships of apicomplexan CYPs are thoroughly analyzed in Krucken *et al*. [14]. Before focussing on the immunophilins of the two most significant Apicomplexa of humans, *Plasmodium* and *Toxoplasma*, which are also the best characterized (Table 8.1), more recent accounts of other apicomplexan immunophilins will be summarized.

Table 8.1 Major immunophilins of *Plasmodium* and *Toxoplasma*.

Organism	Protein name	Gene accession number[a)]	Subcellular location	Biochemical functions	Sequence motifs/ domains[b)]	Amino acids	MW	Ligands	3D structures	References
P. falciparum	*Pf*FKBP35	PF3D7_ 1247400	Cytosolic (mainly), nuclear	PPIase, chaperone	FKBD, TPR, Cam-like	304	34.8	FK506, rapamycin, ascomycin and related, D44, SRA and related	2FBN, 2OFN, 4J4N, 2VN1, 4QT2, 4QT3	[49–58]
	*Pf*CYP19A	PF3D7_ 0322000	Cytosolic	PPIase, chaperone	CYP	171	19	CsA and related	1QNG, 1QNH	[59–63]
	*Pf*CYP19B	PF3D7_ 1115600	Mainly cytosolic	PPIase, chaperone	CYP, SP	195	21.7	CsA and related	—	[59–61, 64]
T. gondii	*Tg*CYP18	TGGT1_ 221210	Secreted	PPIase	CYP, SP	179	19.6	CsA	—	[65]
	*Tg*CYP20	TGGT1_ 205700	nd	PPIase	CYP	348	38.2	CsA	—	[65]
	*Tg*FCBP57	TGGT1_ 283850	nd	PPIase	CYP, TPR, FKBD	503	57.3	FK506, CsA	—	[13]

a) Gene accession number in EuPathDB.
b) Sequence motifs/domains: FKBD, FK506-binding domain; TPR, tetratricopeptide repeat; CaM-like, putative calmodulin-binding motif; CYP, cyclophilin; and SP, signal peptide.
MW, molecular weight (thousands) and nd, not determined.

An unusual89-kDa cyclophilin from *Eimeria tenella* [66] belongs to the 40-amino-acid domain Trp/Asp containing β-propeller (WD40) repeat containing CYPs [14]. In addition to the WD40 repeats, and a C-terminal cyclophilins domain that contains all of the residues known to contact CsA, *Et*CYP89 possesses N-terminal His- and Ser-rich regions and a putative nuclear localization signal. Its closest relative appears to be an 82-kDa *T. gondii* CYP, but the latter lacks the His- and Ser-rich regions. *E. tenella* CYP mRNA was found in sporozoites, infected chicken caeca, and sporulated oocysts [66].

Previous work on *T. gondii* and *Neospora caninum* has suggested roles for secreted parasite CYPs in host immune modulation [47]. Excreted/secreted *N. caninum* antigens were chemoattractive to murine bone-marrow-derived dendritic cells, and the negation of this effect by CsA suggested that a CYP might be involved [67]. In agreement with this idea, *Nc*CYP was found to be released into the extracellular environment by *N. caninum* tachyzoites, and a recombinant form produced in *Escherichia coli* was chemotactic to murine spleen cells and bovine peripheral blood mononuclear cells, albeit only at high concentrations [68]. This protein had previously been shown to stimulate interferon-γ production [69]. In view of these immunomodulatory effects, recombinant *Nc*CYP was tested as a vaccine, and it elicited an antibody response and protection from neosporosis in mice [68].

Secreted parasite products are also of interest in the tick-born disease theileriosis, in which they may play roles in the transformation of lymphocytes of infected cattle. One candidate for such an effector is a 26-kDa CYP from *T. parva* that is expressed (at the mRNA level) in piroplasms and in schizont-infected lymphocytes [70]. The protein has a putative signal sequence and was translocated into the ER in a wheat germ cell-free system, but the signal peptide remained uncleaved. The transformative capacity of the (nonimmunophilin) PPIase PIN1 is, by contrast, now well established [71]. *Ta*PIN1 is secreted and contains a signal peptide that appears to be obligatory for proliferative capacity. It causes the degradation of the host ubiquitin ligase FBW7, leading to stabilization of c-JUN, which promotes transformation. Interestingly, *Ta*PIN1 is directly inhibited by the antiparasitic drug buparvaquone [71] (Figure 8.1), a naphthoquinone similar in structure to the antimalarial drug atovaquone that is used in combination with proguanil.

Recently, a host cyclophilin was reported to be important for the development of *Babesia* parasites in *Haemaphysalis longicornis* ticks [72]. The result of the study suggests that *Hl*CYPA might be involved in the tick immune response to *Babesia* parasites, and recombinant *Hl*CYPA showed a growth-inhibitory effect *in vitro*, suggesting the possibility of an unconventional approach to antibabesial drug design.

Plasmodium falciparum carries 13 immunophilin genes, 11 cyclophilin or cyclophilin-like genes [14, 59] and 2 FKBP or FKBP-like genes [49]; see also www.plasmodb.org. In 1994, it was shown that both FK506 and rapamycin

exhibit antimalarial activity [73], although it was not possible to demonstrate the presence of an FKBP in parasite extracts. Later, *Pf* FKBP35 was the first FKBP to be identified [50] and characterized [49, 51] in Apicomplexa and was detected as a binding protein for the FK506 derivative ascomycin [51] (Figure 8.1). *Pf* FKBP35 (Table 8.1) is encoded by a 915-bp gene located on chromosome 12. Its expression begins during the late ring stage, that is, just under half-way through the 48-h asexual, intraerythrocytic cycle, with a peak during the (metabolically active) late trophozoite stage before falling off during the (dividing) schizont stage [74]. The protein is predominantly cytosolic during the ring stage but as the parasites mature into trophozoites and schizonts, immunofluorescent microscopy shows that it migrates to the nucleus [51].

Pf FKBP35 possesses a multidomain architecture, starting with an N-terminal FK506-binding domain (FKBD), followed by a TPR domain, and a putative calmodulin binding site. The FKBD exhibits both PPIase and chaperone activities [49], while the TPR can also act as a chaperone in isolation and, based on findings on other TPR-containing proteins, is presumably responsible for protein–protein interactions. *Pf* FKBP35 has been demonstrated to interact with *Pf* Hsp90 via its TPR domain [52], indicating a possible role in high-molecular-weight chaperone complexes, as in higher organisms. Other interacting partners confirmed for *Pf* FKBP35 include several core histones, suggesting a possible role of the FKBP in regulating chromatin structure and gene expression [75]. In agreement with this idea, a posttranslational modification (trimethylation) of the lysine-36 residue of histone H3 was increased by treatment of cultured parasites with FK506.

In humans, FK506 forms a ternary complex with the FKBP12, the Ca^{2+}-dependent phosphoprotein serine/threonine phosphatase calcineurin (CaN; PPP3) and calmodulin, which blocks the dephosphorylation activity of calcineurin. When the cytosolic subunit of the nuclear factor of activated T cells (NF-ATc) is not dephosphorylated by CaN, it cannot enter the nucleus of T-lymphocytes and activate interleukin 2 (IL-2) synthesis. Thus, FK506 blocks T-cell activation by blocking the nuclear translocation of NF-ATc. In contrast, the rapamycin–FKBP12 complex inhibits the protein serine/threonine kinase mammalian target of rapamycin (mTOR). This inhibits the biochemical pathways required for cell cycle progression and blocks cytokine signal transduction: in this way, rapamycin blocks T-cell proliferation. Similar interactions are responsible for other pharmacological effects of FK506 and rapamycin that are unrelated to immunosuppression [76]. Calcineurin has also been assumed to be the target of FK506 in *Plasmodium* [77], but *Pf* FKBP35 (both recombinant full-length and FKBD alone) interacts with calcineurin (from bovine or recombinant *Plasmodium* sources) independently of the presence of FK506 [49, 51], at least *in vitro*. This (albeit disputed: [78]) finding may represent an important point of difference between *P. falciparum* parasite and host FKBPs that could be exploited. The potent antimalarial activity of a number of nonimmunosuppressive FK506 congeners [49] also argues against the involvement of calcineurin in *Plasmodium*,

which incidentally lacks an mTOR homolog. Together with the potent activity of designed *Pf*FKBP35 inhibitors [53, 54], this leads us to the hypothesis that *Pf*FKBP35 itself is likely to be the relevant target (see the following text).

There are 11 CYP and CYP-like genes in *P. falciparum*, located on various chromosomes (3, 5, 8, 9, 11, 12, and 14). All contain a CYP or CYP-like domain (CLD) ranging in size from 160 to 219 amino acids and, in some cases, joined to other domains [14, 60]. All of them are expressed at some point during the parasite life cycle [14]. Probably, the most abundant in blood stages are *Pf*CYP19A and *Pf*CYP19B (making up 1.2% and 0.5% of total cellular protein, respectively). They are both located predominantly in the cytosol of the erythrocytic stage of the parasite and were the only two CYPs pulled down on cyclosporin-coupled affinity columns [61]. Studies on recombinant forms of several of the CYP and CYP-like proteins showed that while all of those tested were capable of acting as molecular chaperones on model substrates, only *Pf*CYP19A and *Pf*CYP19B demonstrated PPIase activity and bound CsA, a PPIase substrate analog [59, 60]. Studies of the CYP interactome of *P. falciparum* by co-immunoprecipitation and yeast-2-hybrid screening identified a number of putative interacting partners of *Pf*CYP19A and *Pf*CYP19B, of which Hsp70 and rhoptry-associated protein (RAP)-1 were confirmed [75]. Since rhoptries are key invasive organelles and RAP-1 known to be important in host erythrocyte invasion by the merozoite form of *P. falciparum*, the latter observation suggested a role of one or more CYPs in this process.

CsA's mechanism of action in humans is well defined. The complex of CsA and CYP inhibits the dephosphorylation activity of calcineurin, and the result of this missed dephosphorylation of NF-ATc is blockage of T-cell activation [76]. In other words, CsA-hCYP18 and FK506-hFKBP12 have similar actions at the molecular level – even in the absence of any chemical similarity between the drugs or structural similarity between their receptors. As with FK506, it is unlikely that *Plasmodium* calcineurin is the target of CsA in view of the potent antimalarial activity of cyclosporins that lack binding to either CYP or (in complex with CsA) calcineurin [73] (see the following text).

T. gondii expresses 18.5- and 20-kDa CYPs, both of which exhibit PPIase activity [65]. *Tg*CYP18 is secreted by tachyzoites to up to 2.4% of total protein in cell culture supernatant fluid [79]. This protein is significant in host–parasite interaction, inducing production of IL-12 in dendritic cells but not in neutrophils. *T. gondii* possesses multiple molecules triggering distinct signaling cascades that lead to distinct profiles of cytokine production [80, 81]. Studying the effects of *Tg*CYP18 on the dynamics of the infection revealed that high doses of the protein enhanced the proliferation of macrophages and spleen cells in a cysteine–cysteine chemokine receptor 5 (CCR5)-independent way. The recruitment of cells and the improvement of growth of host cells at the site of infection maintain the interaction between the parasite and the host [82, 83]. *Tg*CYP18 may play a crucial role in parasite-infected host cells and in parasite migration in the host, so affecting completion of *T. gondii* infection cycle [84].

Another important aspect of*Tg*CYP18 and its involvement with CCR5 is on HIV-1. *T. gondii* is a HIV copathogen, and *Tg*CYP18 is an inhibitor of HIV-1 cell fusion and infection with cell-free virus. It efficiently blocks syncytium formation between human T-cells and effector cells expressing CCR5, an activity that neither human nor *P. falciparum* CYPs possess [85]. Structural studies identified critical sequences in the N-terminus of CCR5 involved in *Tg*CYP18 binding and anti-HIV activity [86, 87]. *Ex vivo* studies demonstrated that live *T. gondii* may influence the outcome of viral infection by preferentially suppressing CCR5 variants in coinfected tissues, an effect that can be reproduced by treatment of the tissue blocks with recombinant *Tg*CYP18 [88]. A recent study demonstrated that BCG and pVAX1 vectors expressing the target antigen *Tg*CyP (rBCGpMV361-*Tg*CyP and pVAX1-*Tg*CyP) generate specific humoral and cellular immune responses protecting against experimental *T. gondii*, representing an alternative system for the production of effective vaccines to prevent toxoplasmosis [89, 90]. *In vivo* induced antigen technology, which identifies the pathogen antigens specifically expressed during the infection, confirmed *Tg*CYP18 in pigs infected with *T. gondii* as having strong immunogenicity [91].

Perhaps the most intriguing immunophilin expressed in *T. gondii* is a novel dual-family protein that contains both CYP domain and FKBD, called *Tg*FCBP57 (FCBP) [13]. It appears to be essential for the growth of *T. gondii*, as judged by RNAi experiments [13]. *Tg*FCBP57 is composed of an N-terminal FKBD and a C-terminal CYP domain joined by a TPR region. The order of the two immunophilin domains is reversed in bacterial FCBPs from genera such as *Flavobacterium* and *Treponema*, which have replaced the TPR domain with a shorter linker. Both of the immunophilin domains showed PPIase activity and exhibited family-specific drug sensitivity [13]. CsA and FK506 additively inhibited FCBP, and the relative positioning of the CYP and FKBD domains did not affect drug–PPIase interaction in each domain, confirming the modular nature of the FCBPs. Rapamycin, instead, led to the recruitment of a 290-kDa protein, close in size to mTOR. The individual domains of FCBP inhibited calcineurin but only in the presence of FK506.

Immunophilins as Drug Targets

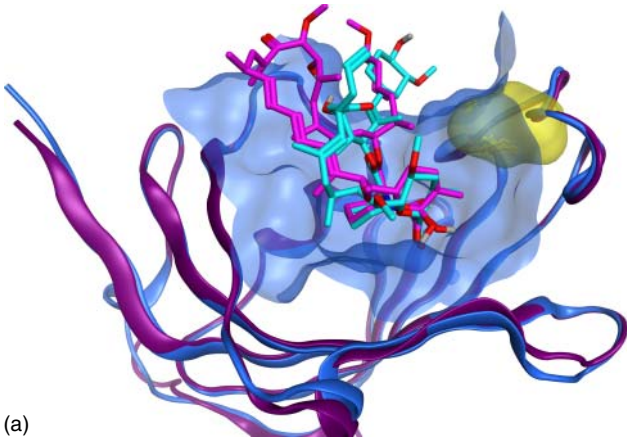
Among the four classes of proteins that mediate *cis–trans* isomerization through their PPIase activity (CYPs, FKBPs, Pin1/parvulins, and trigger factors), there is no significant sequence similarity, but there is an overlap in sequence specificity for X–P bonds in peptide substrates. Their active sites also have dissimilar architectures and bind ligands with different structures. In this section, the immunophilin active sites and the interactions and activities of ligands are described. Chemical structures of some key ligands are shown in Figure 8.1.

FKBPs

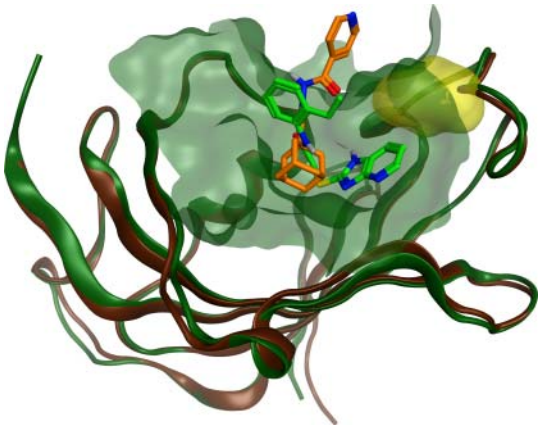
Pf FKBP35 (Table 8.1) is the unique FKBP present in *P. falciparum*. There is also a second FKBP-like protein that has been named *Pf* FKBP25 (encoded by the gene PF3D7_1313300). Its *P. vivax* ortholog *Pv*FKBP25 maintains an FKBP-like fold but lacks key residues required for ligand binding and PPIase activity [92]. As discussed earlier, the *Pf* FKBP35 structure contains an N-terminal FKBD linked to a TPR-containing domain and a putative calmodulin-binding motif. The FKBD has high-similarity (44% amino-acid sequence identity) to the human archetypal FKBP, hFKBP12, but its overall domain architecture is closer to other human FKBPs, namely FKBP38, FKBP51, and FKBP52 [49].

Human FKBP12 is composed of a single FKBD, which is composed of five β-strands wrapped around a short α-helix forming a conical shape with a hydrophobic groove to which FK506 and X–P substrates bind. hFKBP38 by contrast is composed of an N-terminal FKBD, a TPR domain, a calmodulin-binding motif, and a transmembrane region. The domain architectures of *h*FKBP38 and *Pf* FKBP35 are strikingly similar, but unlike hFKBP38, the PPIase activity of *Pf* FKBP35 is not calmodulin-dependent [49]. *Pf* FKBD and hFKBP12 differ in their active sites where Cys106 and Ser109 are replaced by His87 and Ile90, respectively. This is important because these are the specific amino acids identified as hot spots for drug design strategies, and it is expected that interaction of a ligand with these residues might be expected to give specific inhibition of the *Plasmodium* protein [55, 56].

The antiparasitic activity of FK506 and analogs on FKBPs is characterized by a significant conservation of the 14 residues that have been shown in hFKBP12 to contact the ligand FK506 [93]. Studies on FK520 (ascomycin) and a number of synthetic nonimmunosuppressive analogs suggested that inhibition of chaperone activity correlated better with antimalarial activity than PPIase inhibition [57]. The physiological relevance of PPIase activity of other FKBPs remains controversial, as their biological functions appear to be independent of PPIase activity [1, 94]. More recently, Yoon's laboratory has developed a novel FK520 analog (D44 or *N*-(2-ethylphenyl)-2-(3*H*-imidazo[4,5-b]pyridin-2-ylsulfanyl)acetamide, Figure 8.1) that inhibits the PPIase activity of both *P. falciparum* and *P. vivax* FKBP35 [56]. D44 exhibited submicromolar activity on cultured *P. falciparum* and blocked parasite development in a manner similar to that of FK506 [53]. Other interesting *Pf* FKBP35 ligands newly discovered are adamantyl derivatives (Supradamal or SRA or *N*′-(1-adamantylcarbonyl)pyridine-4-carbohydrazide (Figure 8.2), and analogs) that inhibited *P. falciparum* growth with IC_{50} in the 10–100 nM range, arresting growth at the trophozoite stage [54]. As with D44, the compounds inhibited PPIase activity but (in combination with hFKBP12) had no measurable effect on calcineurin [54]. It has not yet been proven that *Pf* FKBP35 binding is the event that leads to growth inhibition, but the antimalarial potency of three distinct chemical classes of (non-calcineurin-binding) *Pf* FKBP35 ligand supports this notion.



(a)



(b)

Figure 8.2 *Pf*FKBP35 ligands. (a) FK506 (in blue 2VN1 crystal structure [57]) and rapamycin (in purple 4QT2 crystal structure [94]) on *Pf*FKBD active site. The binding site map (blue) and residue Cys106 (yellow) are highlighted. (b) D44 (in green 4J4N crystal structure [56]) and SRA (in orange 4MGV crystal structure [93]). The binding site map (green) and residue Cys106 (yellow) are highlighted.

X-ray cocrystallographic structures confirm the binding poses for these FKBP inhibitors, and they are an important key to understand the interactions between the ligands and the active site and to improve the design of novel compounds. FK506 and rapamycin (2VN1 and 4QT2-4QT3 crystal structures [55, 56]) share the same location in the active site, interacting with Asp56, Ile75, Gly72, Glu73, and Tyr101, all generally conserved amino acids. D44 and the adamantyl derivative (4J4N and 4MGV crystal structures, [53, 54]) are smaller ligands, but even if the active site occupancy is low, they are good inhibitors. D44 interacts with Asp56, Tyr101, and Ile75 and SRA shows a network of hydrogen bonds with Tyr101, Ile75,

and two water molecules, stabilizing the protein–ligand complexes. New drug design strategies should be addressed by the scaffold of known ligands and by novel specific interactions with Cys106 and Ser109.

Cyclophilins

As mentioned earlier, *Pf*CYP19A and *Pf*CYP19B (Table 8.1) are the two most abundant cyclosporin-binding proteins in blood stage *P. falciparum* and are the only two *Plasmodium* CYPs known to possess both PPIase activity and the ability to bind CsA [59, 61]. The structure of human CYP18 contains eight antiparallel β-strands forming a right-handed β-barrel with an α-helix at each end. The core of the β-barrel is closed, and CsA and X–P substrates bind to the outer surface of *h*CYP18 via a number of critical hydrophobic residues. The major difference between the *P. falciparum* CYPs and *h*CYP18 lies in "insertions" of four to six amino acids situated around position 43–44 (*h*CYP18 numbering), which is located within the linker region between helixα-1 and strand β-3 [95]. This corresponds to a region of substantial diversity among CYPs in general [96]. *Pf*CYP19A and *Pf*CYP19B, in complex with CsA, are potent inhibitors of *P. falciparum* calcineurin *in vitro* [97, 98]. The three-dimensional structures of wild-type and a mutant *Pf*CYP19A in complex with CsA were solved to 2.1 Å resolution [62]. The wild-type *Pf*CYP19A crystal structure (1QNG) shows the active site defined by 13 residues that have van der Waals interactions with CsA. These residues, Arg62, Phe67, Met68, Gln70, Gly79, Ala108, Asn109, Ala110, Gln118, Phe120, Trp128, Leu129, and His133, are strictly conserved in hCyPA/CsA and *Pf*CyP22 [99].

While CsA is the best-known ligand of CYPs, other CYP ligands and analogs with potent and selective antimalarial activity [99, 100] (Angus Bell, unpublished data) are interesting for their different mechanism of action. For example, valspodar ([3′-keto-MeBmt]^1-[Val]^2-cyclosporin; SDZ PSC 833, Figure 8.1), a nonimmunosuppressive derivative of cyclosporin D, is a potent antimalarial agent ($IC_{50} = 32$nM) but has low affinity for CYPs [73]. Moreover, no correlation was found between PPIase inhibition (in crude parasite extracts) and inhibitory potency against cultured parasites among a group of naturally occurring and semisynthetic cyclosporins [73]. These observations were confirmed using recombinant *Pf*CYP19A [63] and *Pf*CYP19B [54]. Another nonclassic example is [MeVal]^4-CsA, another nonimmunosuppressive cyclosporin, that strongly binds *P. falciparum* CYPs, but then as complex has very low affinity for calcineurin [73]. Taken together, these data suggest that the calcineurin model of CsA action does not explain the antimalarial activities of nonimmunosuppressive cyclosporins (see discussion in [60]), nor could it account for the activities of noncyclosporin CYP ligands that have no known relationship with calcineurin. It is not known at present whether inhibition of one or more *P. falciparum* CYPs would be a lethal event; the lack of phenotype of *Plasmodium berghei* parasites disrupted in the gene encoding a protein orthologous to *Pf*CYP19B (PBANKA_093220) suggests not, at least for this CYP (www.pberghei.eu) [101].

Both *T. gondii* CYP18 (accession no. TGGT1_221210 in strain TG1) and CYP20 (TGGT1_205700) [65] are highly similar to hCYP18 in the core region, but *Tg*CYP20 differs in its 7-amino-acid "insertion" in the same region as in the *P. falciparum* CYPs. *Tg*CYP18, but neither hCYP18 nor *Pf* CYP19A appears to induce IL-12 production by interacting directly with CCR5, an effect that was blocked by the addition of CsA. Structural determinants related to CsA binding, but peculiar to *Tg*CYP18, are responsible for the effect on IL-12 synthesis. This was confirmed by modeling of the *Tg*CYP18 structure on *Pf* CYP19A and site-directed mutageneses of putatively surface-exposed residues that were absent in *Pf* CYP19A [86]. As for *P. falciparum*, in *T. gondii*, no correlation was found between growth inhibition and CYP binding among a series of cyclosporins including the nonimmunosuppressive SDZ 215-918 [102]. As discussed earlier, *Tg*CYP18 blocked HIV-1 cell fusion and infectivity in the viruses that use CCR5. This antiviral effect caused by competition of *Tg*CYP18 with the HIV-1 envelope glycoprotein gp120 for binding to CCR5 is sensitive to the mutations in *Tg*CYP18 that affected CCR5 binding, giving good hints on the design of new antiviral agents based on *Tg*CYP18 [86].

FCBP

The domain architecture of *Tg*FCBP57 (TGGT1_283850) is similar to that of *Pf* FKBP35, with the addition of a C-terminal CYP domain [13]. The complete architecture of these proteins, and in particular the presence of TPR motifs, suggests that their primary functions may be in protein trafficking as part of large hetero-oligomeric complexes. It shares 117 identical residues with *Pf* FKBP35 and 98 with *Pf* CYP19A. Its three-dimensional structure is not yet known, nor are any specific inhibitors.

Conclusions

Immunophilins of apicomplexan parasites are intriguing proteins with potential as drug targets. The *P. falciparum* FKBP *Pf* FKBP35 and its orthologs in other *Plasmodium* species appear, from the data available, to be promising targets, and *Pf* FKBP35 ligands of several chemotypes are effective antimalarial agents. The situation with *Plasmodium* CYPs is less clear: nonimmunosuppressive cyclosporins with high antimalarial potency are available, but the role of CYPs in parasite inhibition is not established. For *Toxoplasma, Neospora*, and perhaps others, immunophilin ligands have the potential to modulate host–parasite interactions and influence the severity of disease. Experimental parasitologists will benefit from the interest in immunophilin ligands in other disease areas, for example, in the supply of novel, nonimmunosuppressive ligands. It is to be hoped that one or more of these ligands might prove to be of clinical interest.

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9
Targeting the Atg8 Conjugation Pathway for Novel Anti-Apicomplexan Drug Discovery

*Alexia S. Miller and Jürgen Bosch**

Abstract

Autophagy is an important metabolic pathway, conserved within eukaryotes, for the degradation of superfluous or damaged proteins and organelles. In addition to its role in homeostasis and survival under starvation conditions, autophagy has been found to function in a variety of pathways including cell differentiation and development, cell-mediated death, and clearance of intracellular pathogens. Within the phylum Apicomplexa, however, autophagy has increasingly been found to serve novel roles specific to intracellular parasite biology. Apicomplexa are obligate intracellular parasitic protists that represent a significant global burden to human health and economic development. Autophagy within these parasites represents an attractive target for novel anti-apicomplexan therapies. Here, recent advances in the field of apicomplexan autophagy research are presented. We also discuss recent results from our laboratory, in which we use two different approaches to identify novel lead-like molecules that inhibit the *Pf* Atg3-*Pf* Atg8 protein–protein interaction. These compounds have activity against blood and liver stage *Plasmodium falciparum* malaria and lead to an upregulation and shift in lipidation state of *Pf* Atg8. The molecules and methods identified from these two complementary approaches have applications for apicomplexan small-molecule intervention strategies in general.

Autophagy: An Overview

Macroautophagy, or more simply autophagy, is a catabolic process within eukaryotic cells responsible for the recycling of intracellular material. It is mediated by double-membrane-bound vesicles called *autophagosomes*, which engulf cytoplasmic material and organelles before fusing with the lysosome to form autolysosomes [1]. This process is widely conserved in eukaryotes [2] and is involved in a variety of functions within the cell. Autophagy facilitates cellular homeostasis, occurring constitutively at a basal level to regularly turn over damaged or unused cellular debris and organelles [3, 4]. Under amino

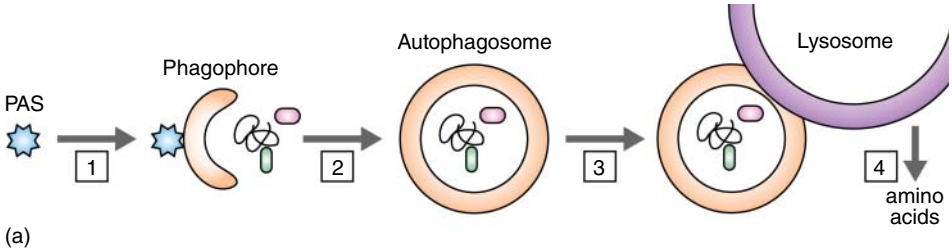
*Corresponding author.

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acid starvation conditions, autophagy is significantly upregulated in order to internally scavenge the materials necessary for continued survival [5]. In addition to functioning in homeostasis and the stress response [6, 7], autophagy is also involved in a variety of other cellular processes. For example, autophagy serves an important role in cell differentiation and development, where it facilitates rapid changes in the cellular environment through degradation of unnecessary intracellular structures and organelles [8]. As a natural extension of its role in engulfing unwanted intracellular components, autophagy is also involved in the clearance of intracellular pathogens, through a process termed *xenophagy* [9]. Finally, in a seeming contradiction to its role in cell survival under amino acid starvation conditions, autophagy also appears to be involved in nonapoptotic programmed cell death under specific cellular conditions [10].

This complex range of functions is performed by a set of more than 30 autophagy proteins that have been well characterized in yeast [11]. It is useful to have an overview of the main proteins and complexes involved in performing macroautophagy within yeast before taking a closer look at autophagy within Apicomplexa [12] (Figure 9.1a). The first step in autophagy, initiation, begins when the Atg1 initiation complex forms at the pre-autophagosomal structure (PAS), the site from which the autophagosome originates [14]. The formation of the Atg1 complex is controlled by negative regulation through target of rapamycin complex 1 (TORC1), the inhibitory function of which can be blocked by exposure to amino acid starvation conditions or treatment with rapamycin [15]. The Atg1 complex consists of the kinases Atg1 and Atg13, which bind to the ternary complex formed by Atg17 bound to Atg31 and Atg29 [16]. Once formed, the Atg1 complex activates the class III phosphatidylinositol 3-phosphate kinase (PI3K) complex (comprising Vps34, Vps15, Vps30/Atg6, Atg14, and Atg38) [17, 18]. The activated PI3K class III complex initiates phagophore assembly, converting phosphatidylinositol (PI) into phosphatidylinositol triphosphate (PI3P) at the PAS. Wortmannin and 3-methyladenine, classic autophagy inhibitors, inhibit PI3K activity [19]. The existence of PI3P at the PAS then recruits several autophagy-related proteins (Atg2, Atg9, and Atg18) to bind to the incipient phagophore [20]. Although its role remains somewhat unclear, Atg9 is thought to transport membrane to the phagophore at this stage [21]. Elongation and closure of the phagophore, to form a completed autophagosome, require conjugation of Atg8's C-terminal exposed glycine residue to phosphatidylethanolamine (PE) within the phagophore membrane [22].

Atg8 is a ubiquitin-like protein (Ubl) whose conjugation pathway involves the enzymatic function of several autophagy proteins. Atg4 is a protease that cleaves the C-terminal tail of Atg8, exposing a conserved glycine residue necessary for Atg8 conjugation with PE; Atg4 also functions to deconjugate Atg8 from the autophagosomal membrane [23]. Atg7 is an E1-activating enzyme; Atg3 is the E2-conjugating enzyme actually responsible for conjugating Atg8 to PE via Atg8's exposed C-terminal glycine; and the covalently bound Atg12-Atg5 proteins form a complex with Atg16 to significantly increase Atg8 conjugation, by acting as an E3-like enzyme [24–26]. Atg12 is also a Ubl, requiring the same E1 enzyme,



(a)

Atg1 Complex	PI3K Class III Complex	PI3P/PAS-binding proteins	Atg12 conjugation	Atg8 conjugation	Vesicle breakdown
Atg1	**Vps34**	Atg2	Atg12	**Atg8**	Atg22
Atg13	**Vps15**	**Atg18**	**Atg7***	**Atg4**	*Atg15*
Atg17	*Vps30/Atg6*	*Atg9*	*Atg10*	**Atg7***	
Atg31	Atg14		Atg5	**Atg3**	
Atg29	*Atg38*		*Atg16*		

(b)

Figure 9.1 An overview of canonical macroautophagy in yeast and *Plasmodium*. (a) Step 1: induction and phagophore assembly. The Atg1 complex forms at the PAS, leading to recruitment and activation of the PI3K class III complex. The PI3K complex converts PI to PI3P in the phagophore membrane resulting in Atg2, Atg18, and Atg9 binding. Step 2: phagophore elongation and closure to form the autophagosome. This step relies on Atg8 being conjugated to PE. Atg8-PE is the product of the Atg8 conjugation pathway, the last step of which is facilitated by Atg12-Atg5-Atg16. Atg12-Atg5 is the product of the Atg12 conjugation pathway. Step 3: autophagosome fuses with the lysosome to form an autolysosome. Step 4: digestion of autolysosomal contents and release of recycled amino acids. Atg15 and Atg22 facilitate breakdown of the membrane-bound vesicle within the autolysosome to expose the contents for digestion. The products of digestion, amino acids, are then released into the cell. (b) Conservation of canonical macroautophagy proteins within *Plasmodium* [12, 13]. Bold text on a green background indicates a conserved homolog exists; regular text on a yellow background, potential homologs identified; italics on a red background, no detectable homolog. * – Atg7 is involved in both Atg8 and Atg12 conjugation pathways.

Atg7, and its own E2 enzyme, Atg10, to covalently attach it to Atg5 [24]. Once Atg8 has been conjugated to the membrane, the phagophore elongates, engulfs the intracellular material, and then closes before fusing with the lysosome.

Autophagy is increasingly appreciated for its roles, not only within healthy cells but also within diseased or infected cells, where, in many cases, it is shown to have both pro- and antisurvival effects, depending on the specific cellular environment. For all of these reasons, research into this process is essential for developing our understanding of fundamental cell function and disease progression. However, while this pathway continues to be well explored within yeast and other higher eukaryotes, the situation is much less clear for protists, including those in the phylum Apicomplexa.

Phylum, Apicomplexa

Apicomplexa is a phylum of parasitic protists that represent a significant threat to human health and economic development. *Plasmodium*, the best-known member of the phylum, is responsible for the disease malaria, which kills about 500 000 people every year (www.who.int/topics/malaria). While *Plasmodium* is undoubtedly the deadliest organism in the phylum, many other Apicomplexa present a significant threat to immunocompromised individuals or to the livestock industry. *Toxoplasma gondii*, the causative agent of toxoplasmosis, infects nearly one-third of the world's population [27]. While *T. gondii* infection is predominantly harmless in healthy individuals, toxoplasmosis can be deadly for HIV patients and pregnant women, whose unborn children can acquire severe health complications through congenital infection (www.cdc.gov/toxoplasmosis). *Cryptosporidium parvum* is another organism that threatens the immunocompromised through the disease cryptosporidiosis, one of the major causes of water-borne illness globally [28]. For the agricultural industry, there are several organisms of considerable concern. *Neospora caninum*, *Babesia bovis*, and *Theileria annulata* all affect the cattle industry; *N. caninum* is one of the primary causes of fetal death in cows [29]. *Eimeria tenella* afflicts the poultry industry, causing caecal coccidiosis in young poultry [30]. It is difficult to estimate the cost of these various infections to the livestock industry, although *N. caninum* alone is estimated to lead to losses of over half a billion dollars annually to the US cattle industry [31]. Because of these economic losses, apicomplexan infections are not only a considerable threat to human health and well-being, but also a threat to the growth of developing nations due to the sheer burden of these diseases on a growing economy.

While various anti-apicomplexan therapies are in use, not all are wholly effective or cost-effective. Of pressing concern is the need for novel antimalarials, as increasing reports of drug resistance have been emerging against artemisinin, the first line of defense [32, 33]. To develop new classes of anti-apicomplexan therapies, it is essential to identify viable targets within these eukaryotic pathogens, targets with enough divergence from host systems to ensure specificity and limit off-target effects.

In terms of their basic biology, Apicomplexa are characterized by several shared features. They are obligate intracellular parasites, with complex life cycles spanning multiple hosts. They are classified based on a conserved "apical complex," a structure used for host cell invasion with specialized secretory organelles, including micronemes and rhoptries [34]. Most of the organisms (*C. parvum* is a notable exception) feature a unique plastid organelle called the *apicoplast*, which was acquired through secondary endosymbiosis, resulting in a structure bound by four membranes [35]. This organelle functions in the synthesis of fatty acids, isoprenoids, iron–sulfur clusters, and heme [35]. Added to the shared physical characteristics of Apicomplexa is the common feature of spending the majority of their life cycle within host cells. Because of this distinct cellular environment,

Apicomplexa differ from other unicellular eukaryotes in a myriad of ways; this extends to the process of autophagy as well.

Conservation of Autophagy in Apicomplexa

Autophagy within Apicomplexa represents an important and growing area of research, not only as a method to better understand the biology of these parasites, but also as a potential target for future drug discovery efforts. At this stage, the genomes for several parasites within this phylum are available, including those species listed earlier [36]. It is important to remember the level of diversity represented within a phylogenetic group, a point that is of particular relevance for Apicomplexa, as they have considerable intraphylum genetic variation [37]. In spite of this level of diversity, it is still possible to obtain a general sense for the degree of conservation of the autophagy proteins in the phylum. Based on a variety of genome analyses, homologs for a number of proteins have been identified [12, 38–46]. The *P. falciparum* genome, in particular, has been subjected to numerous searches for homologs. As the majority of homologs have been determined via computational rather than experimental analyses, there is a considerable amount of disagreement with the results of these investigations. From the aforementioned screens, various *P. falciparum* homologs have been proposed for the following: Atg1, Atg2, Atg3, Atg4, Atg5, Vps30/Atg6, Atg7, Atg8, Atg10, Atg12, Atg14, Atg15, Vps15, Atg16, Atg17, Atg18, Atg22, Vps34, and target of rapamycin (TOR) [12, 38–44, 46]. For any single analysis, many fewer homologs were identified (e.g., Figure 9.1b). Similarly, for *T. gondii*, Atg1, Atg3, Atg4, Atg5, Vps30/Atg6, Atg7, Atg8, Atg9, Atg12, Vps15, Atg17, Atg18, Vps34, and TOR homologs have been presented [45, 46]. Other Apicomplexa, including *N. caninum*, *E. tenella*, *C. parvum*, *Cryptosporidium hominis*, and *Theileria* spp. have also been investigated [38–40, 45, 46].

Given the range of results that have been reported, it is therefore noteworthy that the entire Atg8 conjugation pathway – Atg3, Atg4, Atg7, and Atg8 – has consistently been found to be conserved in Apicomplexa. For this reason, the identified homologs have received special focus. From a number of studies, it appears that the pathway is not only conserved but also essential to parasite survival. The crucial nature of the Atg8 conjugation pathway represents a point of divergence from other eukaryotes, as it has been shown that many autophagy protein knockout strains in yeast or mammalian organisms are still viable [8, 22]. This essentiality has been demonstrated for three autophagy proteins in *T. gondii*: interference with the Ubl *Tg*Atg8 leads to nonviable parasites [47]; elimination of the protease *Tg*Atg4 results in significant growth defects [47]; and the E2-like enzyme *Tg*Atg3 is also essential for normal organelle morphology and intracellular development [45]. Similarly, in *Plasmodium*, *Pf* Atg8 knockouts are nonviable [39, 40] and *Pf* Atg7 is essential for normal growth [44].

Functions of Apicomplexan Autophagy Proteins

From these studies, it is clear that Apicomplexa make use of, and require, a much more limited repertoire of autophagy proteins. But to what end? Are these organisms performing canonical autophagy, or is this more limited set of autophagy proteins serving different purposes altogether? A growing body of research has arisen to provide some answers to these questions. In particular, more information about the function of the autophagy proteins within *Toxoplasma* and *Plasmodium* is now available, although significant questions still remain to be answered.

Atg8 in yeast and other eukaryotes is synthesized with a C-terminal extension that must be cleaved to expose the glycine residue necessary for conjugation to PE [48, 49]. In Apicomplexa, however, Atg8 homologs contain a glycine as the C-terminal residue, implying that the protein does not need to be modified by proteolytic cleavage prior to conjugation to PE and presumably a membrane. This is true for potential homologs of Atg8 identified in *B. bovis* (EuPathDB gene id: BBOV_III005950), *E. tenella* (ETH_00016760), *N. caninum* (NCLIV_008410), *Plasmodium* spp. (e.g., *PF*3D7_1019900), *T. annulata* (TA12615), and *T. gondii* (*TG*ME49_254120) [36]. *C. parvum* Atg8 (cgd7_3990) is an exception [36]. Perhaps because it is already in this active form, Atg8 in both *Plasmodium* and *Toxoplasma* exists in a predominantly lipidated, membrane-associated form [43, 50, 51]. Although Atg8 does not need to be preprocessed by Atg4 in these parasites, Atg4 is nevertheless essential to intracellular development in *T. gondii*, and a role in Atg8 recycling from the membrane was proposed, which appears to be essential for the parasites [23, 47].

The observation that the majority of Atg8 in these organisms is membrane-bound leads to one of the most unequivocally established connections to autophagy that have been made within Apicomplexa: in numerous studies on *T. gondii* and *Plasmodium* species, Atg8 has been found to partially or completely colocalize with the apicoplast membrane [13]. Its role in this location is unclear, although it appears that Atg8 is conjugated to PE in the outermost membrane of the organelle [42, 47, 52], and it is not engulfing the apicoplast within an autophagosome [42, 50, 51]. For these reasons, and because ablation of Atg4 function leads to significant defects in apicoplast morphology [47], Atg8 is thought to play a fundamental role in the biogenesis and maintenance of the apicoplast [42]. However, this is not the only function of the protein within the parasite.

Apicomplexa undergo significant morphological changes over the course of their life cycle, changes that must be accompanied by rapid remodeling of the cellular environment with the creation and disposal of stage-specific organelles. As mentioned in the overview of autophagy in higher eukaryotes, autophagy plays a pivotal role in mammalian cell differentiation and development [8, 53]; these results also apply to certain eukaryotic pathogens, including pathogenic strains of the fungus *Candida* [54, 55]. More significantly, autophagy has also been implicated in the cellular differentiation and homeostasis of trypanosomatids [56], particularly *Trypanosoma brucei* [57], *Trypanosoma cruzi* [58, 59], and *Leishmania* spp. [60–63], organisms that undergo similarly complex changes

in morphology to adapt to diverse host cell environments. For these reasons, a role for autophagy in apicomplexan development and differentiation has been explored. There are clear indications of this in *T. gondii*, since, as mentioned earlier, *Tg*Atg3 is essential to normal cellular development: loss of *TgAtg3* led to significant morphological and growth defects [45]. For *P. falciparum*, the parasite enters the human host as an elongated sporozoite, a highly motile and invasion competent form of the parasite that penetrates a hepatocyte before transforming into a round, metabolically active trophozoite that replicates [64]. This metamorphosis is accompanied by reorganization of cytoskeletal structure and clearance of the rhoptries, micronemes, and inner membrane complex [65]. Autophagy has been proposed as a mechanism for this clearance. In support of this proposal, membranous structures enclosing micronemes have been detected via electron microscopy during this metamorphosis [65], and 3-MA delays conversion of sporozoites to trophozoites [66].

Further exploring this idea of autophagosomes being co-opted for exophagy, there is some evidence to suggest that the autophagy pathway within Apicomplexa may serve a role in trafficking to the host cell. Both *Pf* Atg8 and *Pf* PI3K have been found near the Maurer's cleft [67, 68], a structure essential to vesicular traffic to the red blood cell (RBC) membrane [69]. This idea may be further supported by the existence of *Pf* Atg8 punctate structures throughout the RBC [43], as these structures could be involved in the export necessary for extensive host cell remodeling, although these structures have also been proposed to serve other functions. However, the existence of Atg8 in these punctate structures and near the Maurer's cleft remains a disputed finding, as several studies have found no evidence of *Pf* Atg8 aside from its apicoplast association [50–52]. These contradictory results may arise from differences in protein constructs and detection methods. Either way, further investigation is clearly necessary to shed light on whether or not *Pf* Atg8 is in fact exported to the host cell, and if so, what its function may be there.

The apicomplexan response to amino acid starvation is an area of some debate, as various studies have pointed to parasites engaging in a variety of responses, including hibernation, autophagosomal scavenging, or autophagic programmed cell death. This is not unexpected: as mentioned earlier for other eukaryotes, autophagy often plays a role in both pro-survival and pro-death functions. Indications of a pro-survival role arise in response to starvation conditions within the cell. In particular, nutrient or amino acid deprivation in both *T. gondii* [45, 70] and *P. falciparum* [42, 71] parasites led to *Pf* Atg8 punctate structures throughout the cell. In *P. falciparum*, *Pf* Atg8 was found to colocalize with *Pf* RAB7 upon amino acid starvation, the plasmodial homolog of RAB7, an important protein in autolysosomal formation in higher eukaryotes [42]. Notably, these proteins were also detected in association with the food vacuole [42]. However, not every study has found the same response to starvation: in *P. falciparum*, hibernation without a detectable upregulation of Atg8 production was also observed [43, 72]. Furthermore, the identities of the various punctate structures have not been unequivocally determined, so it remains to be determined whether or not these structures correspond to classical autophagosomes.

On the other side of the life/death balance, there is evidence for autophagic programmed cell death in response to various signals, including nutrient deprivation, drug treatment, heat stress, and even normal cellular events. For example, extensive vacuolization has been observed in dying liver stage parasites in mice, although *Pb*Atg8 was not found to colocalize with these vacuoles [52]. In late erythrocytic stage parasites, heat stress led to cell death without typical signs of apoptotic cell death, but with the appearance of cytoplasmic vacuoles suggestive of an autophagic type of cell death [73]. Prolonged amino acid starvation (4 h) in growing intracellular *T. gondii* led to mitochondrial fragmentation and loss of invasion competence; these two effects were blocked by the autophagy inhibitor, 3-MA [70]. Furthermore, both *Tg*Atg8 and *Pf* Atg8 exhibited a wider punctate distribution under these conditions [67, 70]. Additionally, treatments with both chloroquine in *P. falciparum* [74] and monensin in *T. gondii* [75] have been proposed to lead to autophagy-related cell death. In particular, treatment with the anticoccidial monensin led to *Tg*Atg8 puncta and mitochondrial degradation that was similarly blocked by 3-MA [75]. Taken together, all of these details are highly indicative of an autophagic cell death mechanism in response to certain cellular environments.

Much debate still exists over the role of autophagy proteins in Apicomplexa, particularly in *P. falciparum*. Surprisingly, one of the most contested roles for autophagy proteins is in canonical macroautophagy, as numerous studies have reported both the presence and the absence of typical markers of autophagy [13]. Nevertheless, it has become increasingly clear that autophagy, even with a more limited repertoire of proteins, has been adapted and repurposed to serve novel roles within the unique life cycles of Apicomplexa, as is the case with many fundamental processes in these parasites.

Finally, it is worth commenting on the interplay between parasite and host cell autophagy. From a variety of reports, it appears that host cell autophagy may serve both pro-life and pro-death functions for apicomplexan parasites. For example, recent results from the Pierce lab have shown that inhibiting mammalian target of rapamycin (mTOR) blocks the development of experimental cerebral malaria [76]. While mTOR is involved in several different conserved pathways regulating immune responses, which could be primarily responsible for this observation, it would nonetheless be interesting to determine the extent to which upregulation of host cell autophagy is involved in this effect.

Targeting the Plasmodial (and Apicomplexan) Atg8-Conjugation Pathway

While much remains to be determined about the function of the Atg proteins within *Plasmodium* and the other Apicomplexa, one fact has become clear: whatever their functions, many of the autophagy proteins are essential in the organisms that have been examined. As mentioned earlier, efforts to inhibit or knockdown Atg8 in particular apparently result in nonviable parasites [39, 40, 47]. Inhibiting the association of Atg8 with parasite membranes, such as the apicoplast outer

membrane, is predicted to be lethal to the cell, as Atg8 is required for proper cell function, and Atg8's functional form within the parasite is its lipid-conjugated form. Therefore, interfering with the conjugation pathway that leads to Atg8 lipidation is an attractive target for drug discovery; the Atg3-Atg8 interaction is particularly relevant, as Atg3 is directly responsible for conjugating Atg8 to PE. To obtain insights into the particular protein–protein interaction between *Pf* Atg3 and *Pf* Atg8, we cocrystallized *Pf* Atg8 with a peptide of *Pf* Atg3 [77]. The peptide (NDWLLPSY) was identified by sequence similarity to be the Atg8 family interacting motif of *Pf* Atg3, and it bound, as predicted, to the conserved W/L-site on *Pf* Atg8 [77]. This crystal structure (PDB ID: 4EOY), solved to 2.2 Å resolution, revealed the presence of a loop region in *Pf* Atg8 that does not exist in the homologous human or yeast Atg8 protein structures (Figure 9.2a) [77]. We termed this loop the apicomplexan loop or A-loop, as it is conserved in apicomplexan Atg8s but not in human Atg8. It was further determined, through mutagenesis and deletion studies, that the A-loop is necessary for the interaction between *Pf* Atg3 and *Pf* Atg8. The existence and necessity of this loop makes *Pf* Atg8 a feasible target for drug discovery, as it provides the required divergence from homologous human structures to help prevent off-target effects. This point of divergence is important, since the main binding pocket on Atg8 (the W/L-site) is well conserved between *Plasmodium* and human Atg8 protein structures (Figure 9.2b). This structural knowledge allowed us to take two approaches toward novel lead discovery against the *Pf* Atg3-*Pf* Atg8 interaction.

First, the open-access Medicines for Malaria Venture (MMV) Malaria box of 200 drug-like and 200 probe-like compounds was screened [78]. Using a surface plasmon resonance (SPR) setup, we screened all 400 compounds against the *Pf* Atg3-*Pf* Atg8 interaction, identifying several compounds that met our criteria for sufficient inhibition of this protein–protein interaction [79]. Of these compounds, three shared a 4-pyridin-2-yl-1,3-thiazol-2-amine (PTA) scaffold and were found to inhibit the *Pf* Atg3-*Pf* Atg8 protein–protein interaction in a dose-dependent manner [79]. The most soluble of these hits was compound **1**2-methylsulfanyl-*N*-(4-pyridin-2-yl-1,3-thiazol-2-yl)benzamide, with an *in vitro* IC_{50} of 18.2 μM. Compound **1** bound directly to *Pf* Atg8 and via virtual docking was predicted to bind to the main binding pocket, the W/L-site (Figure 9.2c,d). The plasmodicidal activity of this compound was also tested against liver and blood stage parasites and showed an IC_{50} of 1.6 μM against blood stage *Plasmodium*. Treatment of infected liver cells for 96 h with 30 μM compound **1** led to a 50% decrease in the proportion of infected hepatocytes, while no effect was detected on liver cell viability. Of significant interest is the fact that treatment with compound **1** led to a dose-dependent upregulation of *Pf* Atg8 protein level along with a shift in lipidation state to primarily unlipidated *Pf* Atg8 as assayed via western blot. These data support the suggestion that compound **1** is acting against the predicted target and inhibits the *Pf* Atg8 and *Pf* Atg3 protein–protein interaction. Based on these promising results, we synthesized a 240-compound derivative library, using the PTA scaffold with a functional handle, in order to improve binding and specificity and to extend the molecule toward regions

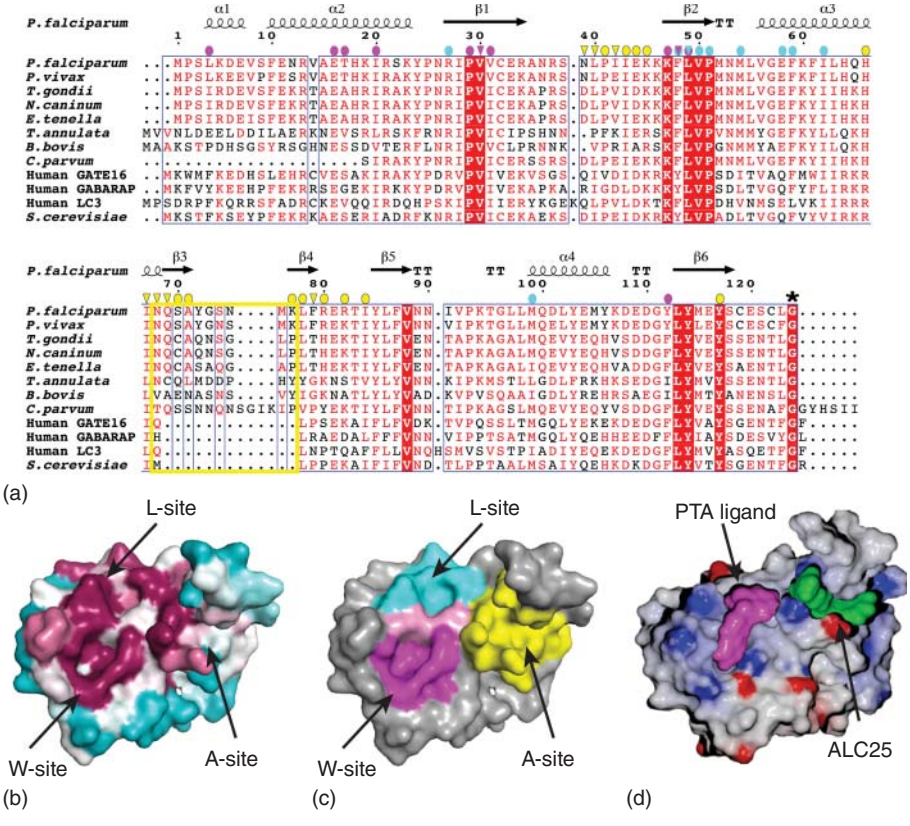


Figure 9.2 *Plasmodium* and apicomplexan Atg8 proteins. (a) Multiple sequence alignment of various apicomplexan, human, and yeast Atg8 homologs generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Visualized with ESPript 3.0, with global score set to 0.4 (espript.ibcp.fr). Ovals indicate side-chain contacts in the three pockets on *Pf*Atg8; triangles indicated backbone contacts in these pockets. Magenta corresponds to the W-site pocket; cyan, the L-site pocket; and yellow, the A-loop pocket. The A-loop itself is outlined in yellow, revealing conservation of the A-loop within apicomplexan, but not in human or yeast homologs. The C-terminal glycine is indicated by a black asterisk, illustrating that this glycine is exposed in most apicomplexan Atg8 homologs, but not in *C. parvum*, human, or yeast Atg8. (b) Conservation of pockets on *Pf*Atg8 (PDB ID 4EOY) generated using ConSurf with the default values (consurf.tau.ac.il). The W- and L-sites are highly conserved, while the A-loop pocket is not. (c) Structure of *Pf*Atg8 color-coded as in (a): magenta, W-site; cyan, L-site; yellow, A-loop pocket. The pink region between the W- and L-sites indicates residues involved in both sites. (d) Electrostatic surface representation of PfAtg8 rendered using OpenEye Vida and POV-Ray (povray.org). Predicted binding modes of one of the PTA ligands, bound in the W- and L-sites, and ALC25, bound in the A-loop pocket. From these poses, it appears there is the potential to link the two sites.

of the protein that are less conserved with the human homologs. In terms of the implications for drug development against other Apicomplexa, compound **1** showed growth inhibitory effects on *Cryptosporidium in vitro* [80], which suggests that it is also effective against the *Cp*Atg8-*Cp*Atg3 interaction. This result is not unexpected given the degree of conservation in the main binding pocket, which implies that the PTA compounds are also predicted to be active against other Atg3-Atg8 interactions in Apicomplexa.

In our second approach, we made direct use of the solved crystal structure to target the A-loop pocket of apicomplexan Atg8 using a virtual library screening (VLS) approach [82]. Using the OpenEye suite of software (www.eyesopen.com), we virtually docked the ChemBridge Express library (www.chembridge.com) into the volume defined for the A-loop pocket (Figure 9.2c), specifying two protein contacts and two hydrogen bonds to anchor the hits within the pocket. From this initial screen, we cherry-picked 14 of the top hits based on visual inspection and cost. All of the hits produced some inhibition of the plasmodial protein–protein interaction and no inhibition of the homologous human Atg3-Atg8 interaction when tested by SPR binding assays. Apicomplexan Loop Compound 25 (ALC25) had the most potent effect and was used in follow-up assays to determine the *in vitro* IC_{50} against the *Pf* Atg3-*Pf* Atg8 interaction and to test against *P. falciparum* 3D7 blood and liver stage malaria. Its *in vitro* IC_{50} was 18.5 μM, and its effect on blood stage malaria was similar, with an IC_{50} of 19 μM. As was the case with PTA compound **1**, treatment of blood stage parasites with ALC25 led to an increase and shift in the lipidation state of *Pf* Atg8 as determined by western blot. ALC25 also showed activity against liver stage parasites, leading to a 30% decrease in the number of infected liver cells at 96 h posttreatment with 30 μM ALC25, while being well tolerated by human hepatocyte cell line. We also performed a secondary SPR assay to provide evidence for the predicted binding site of ALC25 within the A-loop pocket. With the established SPR assay [82], we immobilized a biotinylated version of the *Pf* Atg3 peptide to an SPR chip via NeutrAvidin capture – we used the same peptide that was cocrystallized with *Pf* Atg8 (PDB ID: 4EOY). The expected K_D between the peptide and *Pf* Atg8 was reestablished and then it was demonstrated that ALC25 at inhibitory concentrations did not disrupt the peptide–*Pf* Atg8 interaction. As the peptide was shown to bind to the W/L-site in the crystal structure, this provides strong evidence that ALC25 binds to an alternative site. As suggested earlier, these A-loop compounds may have cross-reactivity against other Atg8 homologs in the same phylum since they target the conserved loop pocket. Either way, a similar approach would be likely to prove fruitful in generating novel leads against other apicomplexan Atg8 targets.

Both of our approaches resulted in the identification of novel compounds with activity *in vitro* and in parasite cultures against the *Pf* Atg3-*Pf* Atg8 interaction. Our next step is to optimize these leads to provide increased binding affinity and specificity. Interestingly, there is the possibility of linking these two molecules, or these two sites, as the functional handle of the PTA compound derivatives is predicted to point toward the A-loop pocket (Figure 9.2d). A drug that would bind

to both of these sites could have the exact desired qualities of increased specificity and affinity. As mentioned earlier, the greater degree of conservation within apicomplexan Atg8 homologs supports our hypothesis that the methods suggested here may have broader implications for the discovery of new intervention strategies against Apicomplexa in general.

Concluding Remarks

The past few years have witnessed a substantial increase in what is known about apicomplexan autophagy and the parasites' interactions with their host cells. As apicomplexan autophagy represents a conserved and essential pathway in these parasites, it is of significant importance on two fronts. First, the sheer fact of its necessity to parasite function indicates that autophagy proteins within these organisms have been repurposed to serve novel functions, as many autophagy proteins are not essential within other higher-level eukaryotes [8, 22]. This hypothesis is well supported by the highly documented association of Atg8 with the apicoplast, as an association with nontransient organelles is well outside of the extent of classical macroautophagy. Second, as was made clear in the third section of this review, this conserved pathway and the Atg3-Atg8 interaction in particular represent fertile ground for the discovery of novel anti-apicomplexan small-molecule interventions. Two such small molecules inhibiting the *Pf* Atg3-*Pf* Atg8 interaction were described earlier with evidence to support the theory that these molecules, and the methods by which they were discovered, could have broader applications within the Apicomplexa phylum as a whole [77, 79, 81].

As more is learned about this fundamental pathway, it is likely that new targets and intervention strategies will arise, with an emphasis on seeking low-cost solutions to the global health issues presented by Apicomplexa. Such low-cost interventions are of critical importance on the global scale, as many of the countries continually plagued by malaria and other apicomplexan diseases do not have the financial means to afford the high-cost therapies that arise from pharmaceutical brute force drug discovery. For this reason, virtual docking will continue to be a useful tool within this field, as making use of computational tools could be expected to reduce drug development costs by up to 50% [83].

On a final note, it is also worthwhile to comment on the connection between this research and potential applications to human autophagy interventions. Defects in autophagy have been linked to a number of important human diseases, including immune disorders [84], heart disease [85], neurodegenerative diseases [86], and most notably, cancer [87, 88]. As appears to be a repeating trend, autophagy has been found to serve both pro-survival and pro-death functions within various cancer types [87, 88]. Due to its pro-survival role, autophagy has recently become an important target for new cancer therapies. Because of this, our research has had potential applications to human cancer, a connection also made for other antimalarials [89, 90]. For example, in the process of screening the VLS compounds against the human LC3-Atg3 interaction, we discovered a compound that

would not be appropriate as an antimalarial due to its activity against the human interaction [81]. As regions of Atg8 are well conserved between *Plasmodium* and human homologs, it is only natural that small-molecule screening against one target will have the added benefit of potentially uncovering inhibitors against the other target.

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10
Turnover of Glycosomes in Trypanosomes – Perspectives for Drug Discovery

*Ana Brennand, Eva Rico, Melisa Gualdrón-López, and Paul A.M. Michels**

Abstract

A unique feature of trypanosomatids is the compartmentalization of the majority of the enzymes of glycolysis and gluconeogenesis, as well as several other important metabolic processes within peroxisome-related organelles designated as glycosomes. Considerable variations can be observed between the enzyme content of glycosomes from different developmental forms of trypanosomatids. These differences reflect how the parasites adapt the metabolic repertoire of the organelles to the distinct environments encountered during the successive stages of their life cycle. Several glycosomal pathways have been validated as drug targets, and drug discovery exploiting the unique properties of their enzymes is actively being pursued. But the correct sequestering of the enzymes inside glycosomes and the life-cycle-dependent tuning of the organelles' enzyme content are also crucial, rendering proteins involved in glycosome turnover – biogenesis and degradation – additional potential drug targets. In this chapter, we discuss the current knowledge about the proteins and mechanisms involved in the assembly and degradation of glycosomes, highlighting their differences with peroxisomes in the host cells. We assess the potential for development of compounds that selectively inhibit glycosome turnover in the parasites and could lead to development of new drugs against trypanosomatid-borne diseases.

Glycosomes of Trypanosomatid Parasites Are Unique, Peroxisome-Related Organelles

One of the remarkable features of trypanosomatid parasites, shared with other protists belonging to the Kinetoplastea clade, is the presence of organelles called *glycosomes*. These are peroxisome-like organelles characterized by possessing the majority of the enzymes of the glycolytic pathway. Glycolysis is, in almost all organisms, a process that is confined to the cytosol, although occasionally also a transient or permanent form of compartmentalization of some glycolytic enzymes may be found in different organelles, including peroxisomes [1–4].

*Corresponding author.

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However, the unique feature of glycosomes is the fact that these organelles play a crucial role in the glycolytic flux in kinetoplastids, whereas in the other cases where some glycolytic enzymes are sequestered in organelles, other functions are served by the activity of these enzymes, such as participation in the Calvin cycle (in plastids), buffering of perturbations in redox and phosphate potentials (in fungal peroxisomes), or providing specific metabolites to the organelles (e.g., in some mitochondria). Additionally, compartmentalized glycolytic enzymes may fulfill moonlighting functions unrelated to their glycolytic activities [5].

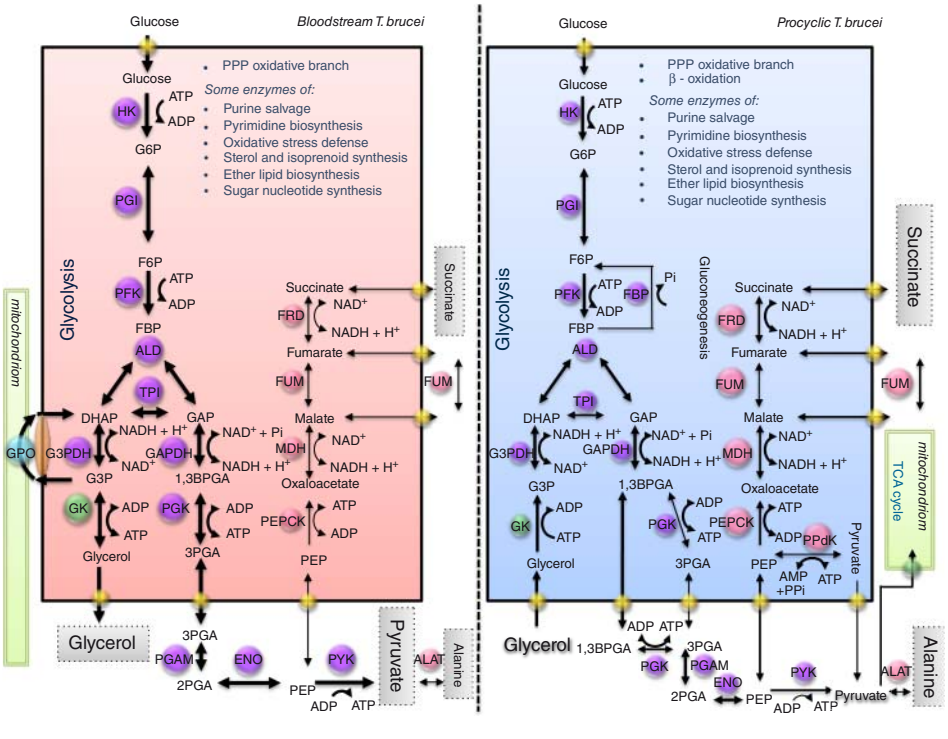
Peroxisomes form a family of organelles that display considerable heterogeneity in size, number, and the nature of their protein content in different organisms or within organisms under different environmental or developmental conditions. Despite this diversity, all members of this organelle family seem to be evolutionarily linked to a common organelle that must already have been present in the Last Eukaryotic Common Ancestor (LECA); they all follow a similar principle of biosynthesis, involving a set of homologous although often poorly conserved proteins called *peroxins* (PEXs), and possess common features such as a single-boundary lipid bilayer membrane [6]. Many members share enzyme systems,

Figure 10.1 Major glycosomal processes and enzymes in bloodstream-form (a) and procyclic-form *T. brucei* (b). Metabolic end products are boxed. Thickness of arrows indicates relative importance of fluxes. The yellow circles represent solute translocation systems (channels or transporters [10, 11]) in the glycosomal membrane. In bloodstream-form cells, glucose catabolism by aerobic glycolysis results predominantly in pyruvate production and may yield smaller, variable amounts of succinate and alanine. Electrons from NADH formed in the GAPDH reaction are transferred to O_2 via a shuttle involving a mitochondrial GPO complex (shown in blue), which is not coupled to oxidative phosphorylation. Under anaerobic conditions, approximately equimolar amounts of pyruvate and glycerol are produced, the latter with concomitant ATP production by reversal of the GK reaction (shown in green). Other processes and enzymes found in the glycosomes but quantitatively minor compared to the glycolytic enzymes and flux are listed; several of these processes and enzymes may have (variable) dual localization in glycosomes and cytosol. In procyclic trypanosomes, levels of glycolytic enzymes and the glycolytic flux are reduced compared to the situation in bloodstream-form parasites; production of succinate and alanine from glucose is increased. Under glucose-depleted conditions, gluconeogenesis may occur to produce G6P. Note that one of the enzymes in the succinate-production pathway, Fum, is found predominantly in the cytosol. Abbreviations: PPP, pentose-phosphate pathway; enzymes: ALAT, alanine amino transferase; ALD, aldolase; ENO, enolase; FRD, fumarate dehydrogenase; FUM, fumarase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK, glycerol kinase; G3PDH, glycerol-3-phosphate dehydrogenase; GPO, glycerol-3-phosphate oxidase system; HK, hexokinase; MDH, malate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PGAM, phosphoglycerate mutase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PPdK, pyruvate phosphate dikinase; PYK, pyruvate kinase; and TPI, triosephosphate isomerase; metabolites: 1,3BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; Pi, inorganic phosphate; and PPi, inorganic pyrophosphate. For a more detailed description of glycosomal processes and enzymes, see reviews in [12, 13].

such as peroxide-producing oxidases and peroxide-detoxifying peroxidases and catalase, as well as pathways of ether-lipid biosynthesis, fatty-acid oxidation, and the oxidative branch of the pentose-phosphate pathway, but no universal common peroxisomal proteome can be identified; the enzyme content is highly diverse. For example, trypanosomatid glycosomes do not contain the peroxisomal hallmark enzyme catalase. Peroxisome evolution seems to have involved massive enzyme acquisitions and losses in different taxonomic lineages. In a considerable number of eukaryotic microorganisms peroxisomes appear to have been lost altogether [6, 7].

Glycosomes are Essential Organelles

The sequestering of the majority of the enzymes of the glycolytic pathway is the most prominent feature of kinetoplastid glycosomes. Bloodstream-form *Trypanosoma brucei* are entirely dependent on glucose catabolism by glycolysis for their ATP supply. Almost all glucose taken up from the blood is converted into pyruvate that is excreted [8, 9] (Figure 10.1a). In the glycosomes of these bloodstream-form trypanosomes, over 90% of the protein content represent glycolytic enzymes [14]; also in *Phytomonas* spp., equally relying on the catabolism of plant-derived sugars, glycosomes are almost solely devoted to glycolysis [15, 16]. In contrast, in the different life-cycle stages of other parasites such as



Trypanosoma cruzi and *Leishmania* spp., or insect-stage *T. brucei*, which all may rely more on other metabolic processes for their free energy, the glycolytic enzyme content of the glycosomes is considerable lower [6, 12]. For example, in procyclic *T. brucei*, the glycolytic enzymes represent approximately 50% of the glycosomal protein content [17]. Compared to the bloodstream-form parasites, expression of other glycosomal enzymes is also upregulated including those pathways that are involved in the formation of succinate and via pyruvate phosphate dikinase generate pyruvate as well as purine salvage and pathways responsible for oxidative stress protection [17–21] (Figure 10.1b). Moreover, in glucose-starved procyclic *T. brucei* [22] and in other parasites such as *Leishmania* amastigotes, enzymes of gluconeogenesis, the reverse process of glycolysis was shown to be more prominent inside glycosomes [23].

Strikingly, almost all glycosomal enzymes participate in core metabolic processes: glycolysis, gluconeogenesis, pentose-phosphate pathway, purine salvage, biosynthesis of pyrimidines, and sugar nucleotides. Many of these enzymes have been shown to be essential in both bloodstream- and procyclic-form *T. brucei*, irrespective of their expression levels. Such glycosomal enzymes are therefore considered as potential drug targets in different trypanosomatid parasites. This has been discussed in an earlier volume of this book series [13] and elsewhere [12].

Interestingly, the correct and complete sequestering of several glycosomal enzymes has been shown to be essential. Expression of even a small amount of active phosphoglycerate kinase or triosephosphate isomerase in the cytosol of bloodstream-form *T. brucei* was shown to be detrimental to the parasites [24, 25]. The essentiality of correct compartmentalization of glycosomal enzymes has been attributed to the peculiar kinetic properties of the organellar enzymes, which are the reasons that these enzymes can only function properly within the organelle that is bounded by a membrane that may contain pores allowing the passage of small solutes such as many metabolites, but not cofactors such as NAD(H), ADP, and ATP [10]. This implies that the enzymes are regulated by different redox and phosphate potentials whether inside the glycosomes or in the cytosol and, consequently, function differently [26–28].

The realization that correct compartmentalization of glycosomal enzymes is crucial for trypanosomatid parasites renders the process of turnover of the organelles and the proteins responsible for their biosynthesis and degradation, as potential drug targets.

Glycosomal Metabolism Changes during the Life Cycle of the Parasites

All human parasites of the trypanosomatid family have elaborate life cycles. They spend successive periods in different hosts – being transmitted by insects between humans and, as is the case of some species, other mammals – and in different parts of the bodies of both their mammalian hosts, extra- or intracellularly, and the insects. During the sequential stages of their life cycle, the parasites adapt to

the different environments encountered by undergoing major morphological and metabolic changes. Among the different forms of adaptation is the reprogramming of the metabolic network to exploit the nutrients available in the different environments, to modulate the biosynthetic capacity as a function of the parasite's needs, and to develop the molecular tools for surviving the host's antipathogen defense system such as its antibodies and reactive oxygen species and for its propagation in the hostile environment. Glycosomes play a crucial role in this metabolic reprogramming of the parasites; importantly, the protein content differs between the life-cycle stages, as is most clearly demonstrated for *T. brucei*, as described earlier for the glycosomes of bloodstream and procyclic trypanosomes (Figure 10.1). The compartmentalization of core metabolism inside glycosomes and the rapid adaptation of the glycosomes during differentiation of the trypanosomes led to the hypothesis that glycosomes serve as vehicles of crucial metabolic units that could readily be replaced, allowing the parasites to adapt rapidly and efficiently to sudden, drastic environmental changes such as those that occur when the parasites are transferred between very different hosts (such as the human bloodstream and the insect intestinal tract) [29]. This hypothesis was based on the remarkable turnover of peroxisomes under changing nutrient conditions, most prominently observed in methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris*, but similarly reported for the peroxisomes in other yeasts and fungal, plant, and mammalian cells (reviewed in [30, 31]). Changes in the enzyme content of the organelles appear to be inducible. For example, glucose-grown methylotrophic yeasts contain only one or a few very small peroxisomes. When these cells are transferred to medium with methanol as sole carbohydrate and energy source, the small peroxisomes increase in size as a result of the import of newly synthesized enzymes involved in methanol metabolism, and multiple new, large organelles occupying the majority of the cell volume are formed. The adaptation to new growth conditions does involve not only the induction of a new set of peroxisomes with a different enzyme content but also the active and rapid degradation of the old, redundant organelles by a mechanism called *autophagy*. When peroxisomes are the specific target of autophagy, it is called *pexophagy*; this process is most clearly observed when yeast grown previously on methanol is transferred back to glucose as sole carbon source.

The possibility of induced biogenesis and degradation may be considered a key property of organelles of the peroxisome family. Sequestering of crucial metabolic processes, of which the functions are environmentally "sensitive" in such organelles with controlled turnover, endows a cell or organism to adapt rapidly and efficiently to new situations. It is therefore conceivable that, similarly as in the methylotrophic yeasts, particularly stage-specific, important metabolic processes have been sequestered within glycosomes of trypanosomatids. Degradation of old glycosomes with a content important for the survival of one parasite stage by autophagy or its specific form of pexophagy and induction of biogenesis of glycosomes with enzymes important for the survival of the next parasite stage possibly play crucial roles in the efficient and rapid adaptation of parasites to the

new host or a new location within a host during maturation and transmission. Indeed, experimental evidence for the existence of such processes has been obtained [32, 33] and is discussed in more detail in the next sections of this chapter. The turnover of glycosomes may be controlled by the developmental program that the parasites undergo as well as by the environmental conditions they encounter. Indeed, it has been proposed that the compartmentalization of glycolysis or gluconeogenesis within peroxisomes in the common ancestor of the Kinetoplastea and Diplonemida allowed for optimization of the adaptation of this – at this stage of evolution still free-living – protist to regularly changing nutrient situations in its environment [6]. At later stages, the ability to readily and inducibly turnover glycosomes may have facilitated the development of parasitism in these organisms.

Biogenesis of Glycosomes

In recent years, major contributions have been made to the understanding of peroxisome biogenesis in various species of yeasts, mammals, and plants (for recent reviews, see [34–36]). Most researchers currently accept the notion that all members of the peroxisome family have, eventually, their origin in the endoplasmic reticulum (ER) and are assembled by essentially the same molecular mechanisms: by the *de novo* synthesis from the ER membrane or fission of pre-existing peroxisomes (Figure 10.2a), the latter being the main mode of peroxisome multiplication in yeasts and mammalian cells [41].

So far, 34 PEXs that participate in different stages of peroxisome biogenesis have been identified; most of them are conserved in all or most peroxisome-containing eukaryotes studied to date, while others are restricted to specific taxonomic lineages. A few somewhat different models have been proposed for *de novo* peroxisome biogenesis [35]. Although species-specific variations may exist, the predominant notion is that the process starts with the insertion of PEX3 and PEX16, peroxisomal membrane proteins (PMPs), into subdomains of the ER from which small vesicles ("pre-peroxisomes") bud to form fully functional organelles. PEX16 may be involved in recruiting PEX3 (and other PMPs) to the ER [42]. Other PMPs and matrix proteins are later imported to form mature peroxisomes. Biogenesis by fission of pre-existing peroxisomes involves three consecutive steps: the initial organelle elongation mediated by proteins of the PEX11 family, growth and constriction of the elongated organelle, and the actual fission process (Figure 10.2a). The principles of constriction are unknown but may be related to the function of the ER in conjunction with actin filaments. Scission is mediated by dynamin-related proteins.

PMPs are grouped into two classes based on the requirement of the soluble receptor PEX19 for their insertion in the peroxisomal membrane: Class I PMPs are PEX19-dependent and Class II PMPs are PEX19-independent. Class II PMPs comprise PEX3 and PEX16. Once inserted into the peroxisomal membrane, PEX3 acts as a docking site for PEX19–cargo complexes.

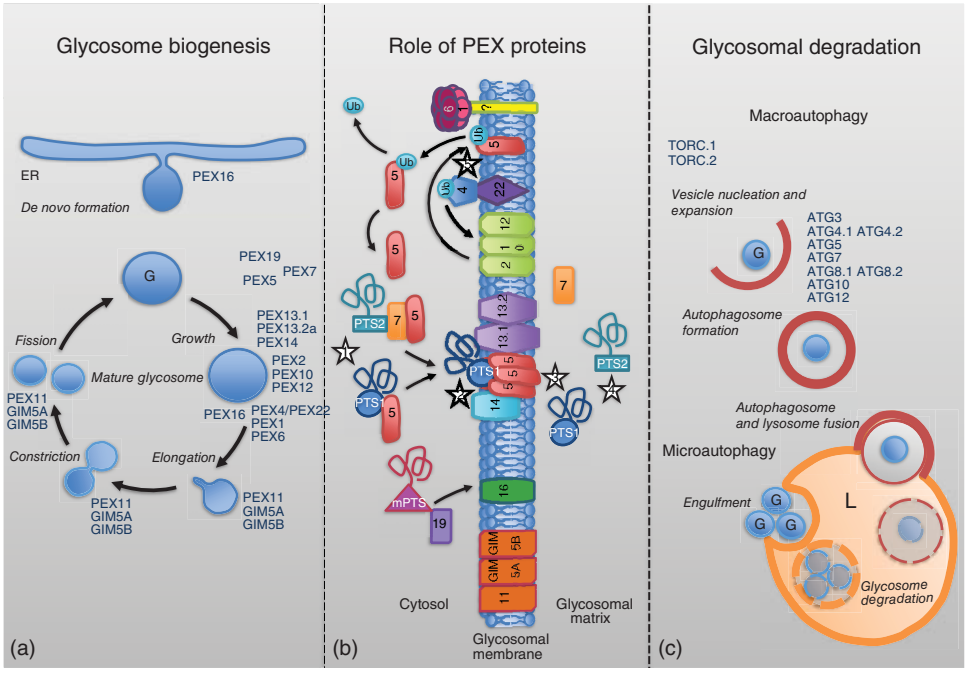


Figure 10.2 Turnover of glycosomes in trypanosomatid parasites. (a) Glycosome biogenesis can involve both *de novo* formation starting from the endoplasmic reticulum and growth and fission. (b) The different steps of glycosome biogenesis and the PEX proteins involved therein as identified in *T. brucei*. The numbers given in asterisks correspond to those of the five consecutive stages of peroxisomal matrix protein import as mentioned in the text mPTS corresponds to peroxisomal membrane-targeting signal. (c) Glycosome degradation may occur by selective autophagy ("pexophagy") or nonselective autophagy; either via the sequestering of the organelles by formation of autophagosomes, which are subsequently directed to the lysosome where their contents are released for degradation, a process called *macroautophagy/-pexophagy*, or by their direct engulfment at the lysosomal membrane, called *microautophagy/-pexophagy*. Autophagy-related proteins (ATGs) functionally identified in trypanosomatid parasites as being involved in steps of glycosome degradation are indicated. For more details, see text and reviews in [11, 37–40] for glycosome biogenesis and degradation, respectively.

Protein import into the lumen of peroxisomes differs from that of mitochondria and chloroplasts in being capable of importing fully folded and even oligomerized proteins. However, recent studies provided evidence that the peroxisomal machinery imports preferentially monomeric substrates [43]. Proteins destined for import into peroxisomes possess a peroxisomal-targeting signal (PTS). Type 1 PTS (PTS1) comprises a tripeptide located at the C-terminus of the great majority of matrix proteins. The consensus sequence of this tripeptide, -(SAC)-(KRH)-(LM), but also some other variants, are recognized in the cytosol by the peroxisomal matrix-protein receptor PEX5. This PEX comprises a C-terminal

tetratricopeptide repeat (TPR) domain responsible for the interaction with the PTS1 motif and an unstructured N-terminal domain containing several pentapeptide motifs involved in the receptor's docking to the peroxisomal membrane. Type 2 PTS (PTS2) is a nonapeptide found close to the N-terminus of a smaller subset of peroxisomal matrix proteins, with a consensus sequence -R-(LIVQ)-X-X-(LIVQH)-(LSGA)-X-(HQ)-(LA)- that is recognized by the cytosolic receptor PEX7. Different from the PEX5-mediated protein import, PEX7 requires the assistance of species-specific coreceptors to achieve the PTS2 protein import: PEX18 and PEX21 in *Saccharomyces cerevisiae*, PEX20 in *Yarrowia lipolytica*, *P. pastoris*, *H. polymorpha*, and *Neurospora crassa*, and, in the case of mammals and plants, a longer splice variant of PEX5 called PEX5L (reviewed in [34–36]).

A minority group of matrix proteins are imported in a PTS1/PTS2-independent manner. This may be accomplished by a piggyback mechanism where proteins hitchhike on PTS-containing complexes to be translocated into the peroxisomal lumen (for a review, see [44]).

Based on the dynamics of the proteins involved, the process of peroxisomal matrix-protein import can conceptually be divided into five consecutive stages: (i) PTS1/PTS2-dependent recognition in the cytosol by PEX5 and PEX7; (ii) docking of the receptor–cargo complexes at the peroxisomal membrane, involving the interaction of the loaded receptors with the docking complex formed by PEX13-PEX14 and in baker's yeast also by PEX17; (iii) translocation of the cargo through the peroxisomal membrane, a process that involves the formation of a dynamic transient pore composed of PEX5 and PEX14, which opens upon binding of the receptor–cargo complex and disassembles after its translocation; (iv) release of the cargo into the lumen; and (v) receptor ubiquitination and its retrieval into the cytosol, an event mediated by the so-called recycling complex, which consumes ATP; it involves PEX5 ubiquitination via the concerted activity of the ubiquitin-conjugating enzyme PEX4, anchored to the membrane through PEX22, and the RING-finger complex comprised of PEX2-PEX10-PEX12, three membrane proteins with ubiquitin-ligase activity. PEX5 monoubiquitination is the signal that targets PEX5 for its ATP-dependent export from the membrane by a cytosolic, membrane-associated complex of the AAA-ATPases PEX1 and PEX6. Once back in the cytosol, PEX5 is deubiquitinated by specialized ubiquitin hydrolases that free the receptor to perform new rounds of protein import. A model has recently been proposed in which the ATP-dependent extraction of the receptors is mechanistically coupled to the matrix-protein import into the lumen [45]. PTS2-protein import differs from the PTS1 process in that the receptor PEX7 is introduced into the lumen of peroxisomes, as well as the coreceptors PEX20 and PEX21. They are exported again in an ubiquitination-dependent manner, similarly to PEX5, but may be constitutively degraded by proteasomes [46]. A scheme of the consecutive stages, as unraveled for *T. brucei* glycosomes, is shown in Figure 10.2b.

Glycosomal matrix proteins possess PTS motifs similar to those of peroxisomes. Glycosomal matrix localization has been predicted for about 250 trypanosomatid proteins based on the possession of a potential PTS. These include enzymes

from all known glycosomal metabolic pathways [18, 21, 47]. So far, homologs of various mammalian and yeast PEXs have been identified in the genomes of trypanosomatids. In *T. brucei*, the following ones have been studied: PEX2, PEX4, PEX5, PEX6, PEX7, PEX10, PEX11 (and GIM5A and GIM5B, isoforms of PEX11), PEX12, two isoforms of PEX13 (PEX13.1 and PEX13.2), PEX14, PEX16, and PEX19 (Figure 10.2b); in *Leishmania* spp., PEX2, PEX5, PEX7, and PEX14 have been studied (for reviews, see [11, 12, 37, 38], where references to the individual studies can be found). These trypanosomatid PEXs display generally a low overall sequence identity with their counterparts in other organisms: between less than 15% and maximally 35%. Moreover, they often possess unique sequence features (Figure 10.3), for example, the presence of long N- or C-terminal extensions with a remarkable sequence, such as the proline-rich C-terminus of PEX7 [48] or the long YGX-repeat region in the N-terminal half of trypanosomatid PEX13.1 and the absence of the proline-rich motif with a still unknown function usually located N-terminally of this repeat [49]. Furthermore, while all organisms analyzed to date possess only a single PEX13 of which the C-terminal half is a SH3 domain, trypanosomatids also have a second isoform, PEX13.2, that lacks this domain. Nonetheless, its role in glycosomal protein import was experimentally confirmed [50]. Functional characterization of the trypanosomatid PEXs indicated that, overall, glycosome biogenesis, despite all these differences, is mechanistically similar to that of peroxisomes in yeast, plant, and mammalian cells.

Glycosomes are essential organelles, as demonstrated by the RNAi-induced depletion of all so far identified *T. brucei* PEXs. The RNAi-dependent downregulation invariably affected trypanosome growth greatly and always resulted in parasite death, regardless of whether the gene product was involved in organelle division such as PEX11, GIM5A, and GIM5B; in PMP membrane insertion such as PEX19; or in matrix-protein import such as PEX5, PEX7, PEX14, PEX13, PEX2, PEX10, PEX12, and PEX6 (reviewed in [11, 37, 38]). The toxic effect of PEX depletion has been attributed to mislocalization of newly synthesized glycolytic enzymes to the cytosol, where the enzymes cannot work properly, causing disturbance of metabolism [26–28]. A notable exception is the ubiquitin-conjugating enzyme PEX4, involved in *T. brucei* PEX5 ubiquitination; deletion of both alleles of its gene in procyclic *T. brucei* resulted in partial maintenance of glycosomal protein import, reduced cell growth, and defects in morphology and motility, but not in parasite death [51].

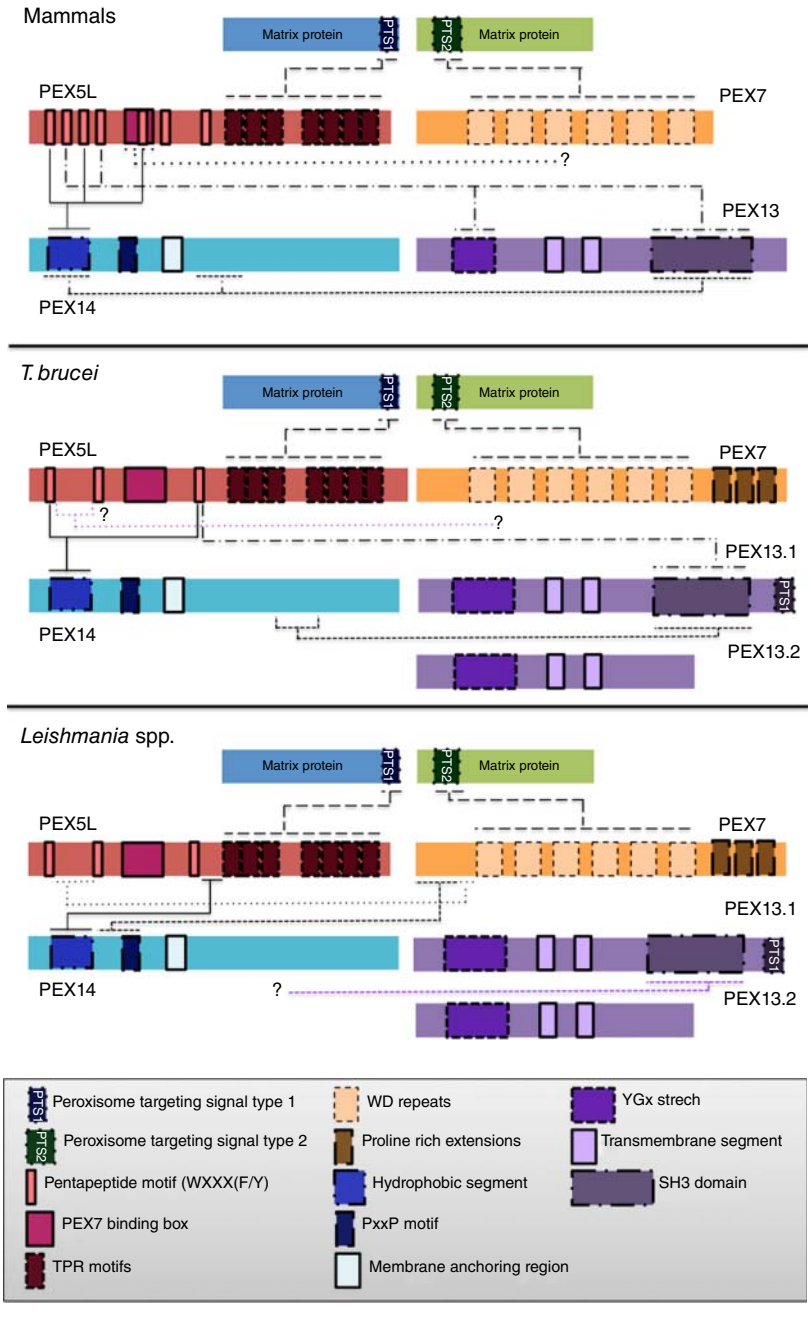
A number of remarkable differences have been found with regard to interactions between different PEXs involved in matrix-protein import, when comparing trypanosomatids with other organisms. These findings are summarized here and are highlighted in Figure 10.3.

First, the crystal structure of the PTS1-binding TPR domain of *T. brucei* PEX5 in complex with several PTS1-containing peptides has been solved [52]. The overall structure and binding mode were well conserved when compared to human PEX5, but important differences in the structure near the PTS1-binding site were noticed. In mammalian cells, a long isoform of PEX5 acts as a coreceptor of PEX7 for proper PTS2 protein import. This involves a PEX5L segment called

the PEX7-binding box that is also found in plant PEX5 as well as in the PEX7 coreceptors of yeasts. Since the PEX7-binding box sequence was also identified in the N-terminal domain of trypanosomatid PEX5s, PTS2 protein import into the glycosomes of the parasites was predicted to occur in a similar way to mammalian cells. Indeed, an interaction of *T. brucei* PEX5 and PEX7 has been experimentally demonstrated [48], but the region of PEX5 responsible for this does not include the PEX7-binding box. For *Leishmania donovani* PEX5, the interaction with PEX7 also occurs outside the predicted PEX7-binding box but involves a region between the first and second of three pentapeptide motifs (WXXXF/Y) present in the N-terminal half of the protein. These pentapeptide motifs are, in variable number, characteristic for PEX5s from all organisms analyzed to date [37, 38]. Whether *T. brucei* PEX5 binds to PEX7, in a similar manner as binds to PEX7 in a similar manner as its ortholog in *L. donovani*, is still an open question.

Jardim and coworkers studied interactions of *Leishmania* spp. PEX7 with various other proteins: PTS2 proteins, PEX5, and PEX14 [53]. PEX7s, which belong to the family of the so-called WD repeat proteins, comprise six of such repeats that cover almost the entire protein and, together with the poorly conserved N-terminal sequence, form a ring structure with a seven-bladed propeller fold [54]. Trypanosomatid PEX7s also possess a C-terminal extension containing many proline residues. PTS2 proteins bind to recombinant *Leishmania* PEX7 preferentially when the receptor is in a monomeric or dimeric form, whereas in the absence of ligand, PEX7 preferentially adopts a tetrameric state. The binding

Figure 10.3 Comparison of corresponding PEX proteins involved in the biogenesis of peroxisomes in humans and glycosomes in *T. brucei* and *Leishmania* spp. and their interaction maps. Characteristic regions and motifs – many involved in specific interactions as shown in the figure – are as follows. **PTS1** and **PTS2** (**P**eroxisomal **T**argeting **S**ignals) are two different motifs, present at the C-terminus and close to the N-terminus, respectively, of proteins to be imported into the peroxisomal matrix. **PEX5**s contain pentapeptide motifs (defined as WXXX(F/Y)) in their N-terminal half and seven TPR motifs, organized in two clusters (TPR1-3 and TPR5-7) with the fourth motif as a hinge connecting them in their C-terminal domain. The major part of the **PEX7** polypeptide is made up of six WD repeats; trypanosomatid PEX7 contains a long C-terminal extension enriched in proline residues. **PEX13** has an N-terminal region enriched in Tyr- and Gly-residues. Moreover, in most groups (but not trypanosomatids), a short Pro-rich region is found near the N-terminus. However, these Tyr/Gly- and Pro-rich regions do not form specific conserved motifs. The central part of PEX13 contains predicted transmembrane regions and is followed by a C-terminal SH3 region. Only trypanosomatids have been reported to contain two PEX13 isoforms, which are very divergent. The first isoform (13.1) possesses uniquely a PTS1 motif adjacent to the SH3 domain, while the other isoform (13.2) lacks the SH3 domain and has no PTS1. The N-terminal half of **PEX14** contains a typical hydrophobic region; more downstream is a PXXP motif and a hydrophobic membrane-interaction region. Question marks indicate that regions involved in specific interactions have not yet been identified. For more details, see the text and the reviews in [37, 38].


Mammals
Matrix protein
PTS1
PTS2
Matrix protein
PEX5L
PEX7
?
PEX13
PEX14
T. brucei
Matrix protein
PTS1
PTS2
Matrix protein
PEX5L
PEX7
?
?
PEX13.1
PTS1
PEX14
PEX13.2
Leishmania spp.
Matrix protein
PTS1
PTS2
Matrix protein
PEX5L
PEX7
PEX13.1
PTS1
PEX14
?
PEX13.2
Peroxisome targeting signal type 1
Peroxisome targeting signal type 2
Pentapeptide motif (WXXX(F/Y)
PEX7 binding box
TPR motifs
WD repeats
Proline rich extensions
Hydrophobic segment
PxxP motif
Membrane anchoring region
YGx strech
Transmembrane segment
SH3 domain

regions of PEX7 to PEX5 and PEX14 were located in the N-terminal part of the protein (for PEX14, within the N-terminal 56 residues; for PEX5, downstream of this). The yeast PEX7-PEX21-PTS2 cocrystal structure revealed the cooperative nature of PEX7 and auxiliary proteins in PTS2 binding, in which the variable N-terminal sequence plays an important role. How PTS2 binding is achieved in trypanosomatids remains to be determined.

Surprising was the report that *L. donovani* PEX7 interacts with PEX14 through a region of about 30 residues also comprising a PXXP motif of PEX14 [55], quite different from the PEX7-PEX14 interaction in other organisms. Moreover, a PxxP motif usually acts as an SH3 ligand, and indeed, this has been reported for the interaction of PEX14 of yeasts with PEX13. However, in mammalian cells, two different regions of PEX14 have been described for PEX13 binding. One of these is located in the conserved, hydrophobic region of the N-terminal half of the protein while the other one is found downstream of the membrane-binding region of PEX14 (Figure 10.3).

A most remarkable feature of all trypanosomatid PEX13s is the presence of a predicted PTS1 motif at the C-terminus, the tripeptide TKL [49]. The function of this candidate targeting signal is still unknown, and since PEX13 is a PMP, its insertion into the membrane is independent of a PTS, so it is likely to have a distinct role that requires further investigation. *T. brucei* PEX13 and PEX5 interact via the SH3 domain and the third pentapeptide motif in N-terminal domain of PEX5. The same motif was also shown to be involved in the interaction with PEX14 [56].

Trypanosomatid PEX14s have been shown to interact with PEX5s; however, in *L. donovani* the regions of PEX5 involved in this interaction seem to differ, surprisingly, from those in *T. brucei*, where the situation is more similar to that of other organisms. The N-terminal region of *T. brucei* PEX14 interacts efficiently with the first and third pentapeptide motifs in PEX5 [56], while the interaction with *L. donovani* PEX5 does not involve any of the pentapeptide motifs, but a downstream region close to the start of the C-terminal TPR domain [57]. In contrast, on the PEX14 site, a conserved region in the N-terminal half was responsible for PEX5 binding, involving some critical hydrophobic residues, as in several other organisms [58].

Intriguing observations were also made for *T. brucei* PEX4, the ubiquitin-conjugating enzyme associated to the cytosolic face of glycosomal membrane, through an interaction with the PMP PEX22 [51]. *T. brucei* PEX5 was shown to be monoubiquitinated in a PEX4-dependent manner. However, this modification appeared to be dithiothreitol-resistant, indicating that it occurs probably on a lysine residue unlike the mammalian and yeast PEX5 where a conserved cysteine is the target of monoubiquitination. Interestingly, a stable ubiquitinated PEX5 form was found in the cytosol of *T. brucei*, representing an authentic intermediate of the receptor during its recycling, a feature that differs from its counterpart in yeast.

Autophagy and Pexophagy of Glycosomes

The term *pexophagy* refers to the specific autophagy of peroxisomes or, in the case of kinetoplastids, the glycosomes. Autophagy is a largely conserved mechanism by which cells recycle their constituents. Usually, two main routes of this process can be distinguished: macroautophagy (the major mechanism, involving formation of a double-membrane-bounded autophagosome containing the material destined for degradation in the lysosomes/vacuole) and microautophagy, where the sequestering occurs directly by engulfing cellular material by the lysosomal membrane (Figure 10.2c). The process serves as a housekeeping tool by which misfolded proteins or no longer required proteins or organelles, individually or in bulk, are discarded as part of natural processes such as sporulation or differentiation (for a review, see [59]). However, autophagy can be upregulated under different types of cellular stresses such as starvation, hypoxia, or heat shock. Proteins involved in autophagy are denoted as ATGs (from AuTophaGy), and genes for a nonredundant set of more than 30 of these proteins have been identified so far in different organisms, mainly *S. cerevisiae*. ATGs work cooperatively with other proteins through the consecutive steps of the various forms of the autophagy process [59] that culminates with the final degradation of autophagosome cargo in lysosomes in mammalian cells or the vacuole in yeast.

Searches for ATG candidates in the genome databases of trypanosomatid parasites in 2006 [60, 61] and further annotations [62–64] indicated that these parasites have homologs for approximately half of the ATGs known in yeasts and mammals but also that, in many cases, the gene copy numbers in the parasites are expanded.

The molecular mechanism of autophagy is generally conserved, but some specialized types of autophagy are shared only by a small group of closely related organisms. Pexophagy occurs in many organisms, but it has been mainly studied in methylotrophic yeasts, where changes in nutrient source and availability lead to increased pexophagy. Several pexophagy-specific ATGs were identified, but appear to be poorly conserved among the different phylogenetic groups.

In trypanosomatids, several studies report that autophagy and pexophagy are important stress-response mechanisms. Autophagy is increased when parasites are amino-acid-starved and responses were found to be life-cycle stage-specific and were also distinct in different trypanosomatids [32, 33, 61, 63, 65, 66]. Interestingly, using the autophagy markers TbATG8.1 and TbATG8.2 (which contribute to the autophagosome expansion), Schmidt and Bütikofer [67] observed that autophagy is induced when cultured procyclic *T. brucei* parasites reach the stationary growth phase and that the cell density affects their capacity to undergo starvation-induced autophagy, which can be rescued by adding the amino acid histidine to the culture medium otherwise devoid of amino acids. Moreover, compounds such as dihydroxyacetone [68] or neuropeptides [69] also increase autophagy in *T. brucei*. Similar observations were made in

T. cruzi parasites exposed to trifluralin and chloralin [70] or in *L. donovani* and *Leishmania major* treated with antimicrobial peptides [71] or cysteine cathepsin inhibitors [72], respectively. Autophagy is associated to acidocalcisome acidification [73], and importantly, it has also been shown to be upregulated during differentiation in trypanosomatids [32, 61, 63, 66].

In *T. brucei*, strong indications for pexophagy were obtained by the observation of colocalization of glycosomal aldolase with lysosomal p67 and the surrounding of the lysosome by glycosomes during the transformation of slender to stumpy bloodstream forms, in agreement with the notion that the latter require preadaptation of their enzymatic contents for transmission into the tsetse fly. Pexophagy increased even more dramatically during the differentiation from stumpy to procyclic forms, which are completely adapted to living in the insect midgut, for which they require a different set of metabolic enzymes to thrive in the new environment [32]. Surprisingly, Proto *et al*. [74] observed that virulent monomorphic bloodstream *T. brucei* where ATG5 (instrumental for autophagosome formation) was knocked down by RNAi still differentiates *in vitro* into procyclic forms. This suggested that autophagy may not be essential for differentiation under these *in vitro* conditions or that the knockdown is insufficient to exert a clear effect on parasite differentiation. Therefore, a deletion of ATG5 and/or studies with a pleomorphic cell line may be required to fully understand the role of ATG5 and the process of autophagy for the conversion of bloodstream to procyclic *T. brucei*. In this context, it is important to mention that microautophagy still occurs in the absence of ATG5 in *S. cerevisiae* [75] and in mammalian cells [76] and that Herman *et al.* [32] found that glycosome degradation in *T. brucei* possibly involves microautophagy, especially micropexophagy, rather than macroauto-/pexophagy. In *T. cruzi*, autophagy is required during the differentiation from epimastigotes to metacyclic trypomastigotes, and ATG8.1 was shown to be involved in this process [63]. Similarly, *L. major* metacyclics that differentiate into amastigote mammalian forms also display increased autophagy [33]; in addition, it was demonstrated that in these trypanosomatids during this particular differentiation process, glycosome degradation was elevated due to an overall increase in autophagy, not in pexophagy. If genuine pexophagy occurs in trypanosomatids, it is probably induced in a way distinct from the process in yeast, especially as a PEX3 homolog (which is instrumental for the process in yeast and other organisms) is lacking from the genome of trypanosomatids.

Another interesting aspect of autophagy in trypanosomatids is related to TOR kinase. In most cells, TOR complex 1 (TORC1) controls aspects of cell growth and autophagy and is the target for the autophagy-inducer rapamycin, while TORC2 controls the cytoskeleton organization, but is not involved in autophagy and does not respond to rapamycin. In contrast, in *T. brucei*, rapamycin only prevents TORC2 formation, but TORC1, which in these parasites is also responsible for control of autophagy, is not susceptible to this classic autophagy inducer [77].

Proteins Involved in Glycosome Biogenesis and Degradation are Potential Drug Targets

As explained earlier, biogenesis of glycosomes resulting in correct sequestering of enzymes into the matrix of the organelles is essential for the viability of trypanosomatids. Moreover, autophagy was demonstrated to be crucial for the differentiation of trypanosomatid parasites and their virulence. This appears to extend to the degradation of glycosomes by pexophagy or by the more ordinary, nonspecific autophagic process, as suggested by the observations made in *L. major* [33]. This essentiality, together with the finding that proteins involved in the turnover of peroxisomes are poorly conserved, offers promise for discovery of antiparasitic drugs that interfere with the processes involved. Among the set of 34 PEXs identified in different organisms, mainly in yeast, only 13 have been identified and (partially) characterized in trypanosomatids; 2 of these occur in multiple isoforms (2 for PEX13 [50], 3 for PEX11 [78]). Most of the identified trypanosomatid PEXs are involved in import of matrix proteins, two (PEX16 and PEX19) in the insertion of proteins into the membrane of the organelles, and one (PEX11) in elongation and proliferation of the organelles, including the generation of membrane curvature during fission of the organelles.

Almost all of these PEXs have been genetically validated as drug targets in bloodstream-form *T. brucei*; their depletion by RNAi resulted in mislocalization of glycosomal matrix proteins to the cytosol that is highly detrimental for the parasites (reviewed in [11, 12]). Moreover, the conservation is generally very low [11]. No trypanosomatid candidates have been identified as yet for some seemingly essential functions in biogenesis, which are performed by PEXs such as PEX3, which is conserved in all other organisms studied so far. Additionally, some functions may be executed by trypanosomatid proteins not homologous to their counterparts in other organisms.

Several potential routes have been proposed for the development of selective inhibitors of glycosome biogenesis that should not interfere with the formation of peroxisomes in the host cell [12, 37, 38]. These proposals involve compounds disrupting (i) the binding of glycosomal matrix proteins to be imported with the cytosolic PTS receptor, (ii) the piggybacking transport involving the binding of matrix proteins without conserved PTS through a sequence-internal motif to a PTS1- or PTS2-containing protein, and (iii) one of the transient interactions of PEXs executing consecutive steps in the processes of matrix-protein import or membrane-protein insertion. Some of these strategies are being pursued, guided by available crystal structures of matrix proteins to be imported (reviewed in [12]) as well as the structure of the PTS1-binding domain of *T. brucei* PEX5 [52]. However, no compounds interfering with glycosome biogenesis and possessing trypanocidal activity have been reported as yet.

Nonetheless, compounds have been discovered that affect the integrity of *T. brucei* glycosomes and kill the parasites. Various endogenous neuropeptides,

produced by the host as part of their natural innate defense, are taken up by bloodstream-form (but not procyclic) *T. brucei* and kill the parasites with EC_{50} values in the low micromolar range [69, 79, 80]. In trypanosomes – in contrast to *Leishmania* species and bacteria [80] – these peptides do not act as typical antimicrobial peptides by forming pores in the surface but are endocytosed and accumulate intracellularly where they disrupt both glycosomes and the lysosome. Glycosomal glycolytic enzymes are then released in the cytosol and the cellular ATP levels drop dramatically. Eventually, the trypanosomes undergo autophagy-like intracellular degradation followed by their lysis.

Almiramides have also been reported as compounds that likely perturb glycosomal function through disruption of the organelle's assembly machinery [81]. These compounds, which are N-methylated linear lipopeptides isolated from marine cyanobacterial extracts, were shown to have antiparasitic activity in a screen with *L. donovani*, while exhibiting little toxicity to mammalian cells [82]. Different almiramides and synthetic derivatives displayed growth inhibition with EC_{50} values in the low micromolar range; compounds that were most potent in inhibiting bloodstream-form *T. brucei* showed values of 0.4–0.6 μM, while affecting growth of Vero monkey cells with EC_{50}'s of 40–220 μM. The likelihood that these almiramides interfere with glycosome assembly was evidenced in affinity capture experiments that yielded the glycosomal integral membrane proteins PEX11 and GIM5A, as well as some glycosomal matrix proteins.

As for PEXs, ATGs, and other proteins involved in the degradation of the peroxisomes in yeast and mammalian cells, only a limited set of homologous genes was detected by genome database searches in conjunction with more elaborate analyses in the trypanosomatids [60–63]. At most only half of the repertoire of proteins characterized in yeasts is present in trypanosomatids, but components for each of the successive stages of the autophagy pathway were recognized and are functional. This suggests that a streamlined version of the process occurs in these protists. The cytoplasm-to-vacuole targeting (Cvt) system found in yeast for delivery of cargo to the vacuole appears to be entirely absent from trypanosomatids. It should be noted that for a number of yeast ATGs, no homologs have been found so far in organisms other than fungi, suggesting these are proteins that have evolved only in fungi [83]. Similarly, novel lineage-specific autophagy-related proteins have also been found in animals [84]. It is therefore likely that lineage-specific autophagy-related proteins, still to be identified, have also been developed in other supergroups than the Opisthokonta comprising animals and fungi, such as the Excavata to which the trypanosomatids belong [39, 40, 62]. If indeed unique proteins are involved in autophagy, including pexophagy, in trypanosomatids, they would be interesting candidates for development of selective inhibitors of these vital processes and exhibit trypanocidal activity [40].

When compared to *S. cerevisiae*, the percentage of amino-acid identity of most components identified in the parasites was below 35%, and the autophagosomal marker ATG8 is about 50% identical to the yeast counterpart [60]. This low percentage of identity, together with the fact that possibly other components,

not homologous to yeast ATG proteins, are involved in the process in trypanosomatids, offers possibly perspectives to targeting autophagy in the parasites with selective drugs.

Little information is available yet about conservation of pexophagy factors. Most studies on pexophagy were performed on yeasts and showed the involvement of peroxisome-selective, species-specific autophagy receptors (ATG30 for *P. pastoris* and ATG36 for *S. cerevisiae*) at the organellar surface. These are phosphorylated and then interact with ATG8 and ATG11 involving other factors such as ATG17 and ATG37, as well as PEX3 and possibly also PEX14. These interactions ultimately direct the peroxisomes to the vacuole [85]. Only homologs of some non-pexophagy-specific components have been identified and characterized in trypanosomatids (ATG8, PEX14), but their involvement in glycosome degradation has not yet been demonstrated, while no trypanosomatid homologs have been identified to date for most of the other factors that are crucial in yeast pexophagy. Data from glycosomal membrane proteome studies [18, 19, 21] would be a useful source to identify potential pexophagy-specific proteins in the trypanosomatids. Testing cell lines in which proteins with unknown function are depleted by reverse genetics techniques for defects in pexophagy could help to identify such proteins in the parasites and test their suitability as drug targets.

Glycosome biogenesis has to occur continuously in proliferating trypanosomatid parasites and is, therefore, probably an essential process throughout most of the life cycle of each of the species. It is thus likely that the proteins involved in the assembly of the organelles are good drug targets for the human pathogenic forms of each of the parasites, although the efficacy of such drugs may quantitatively vary dependent on the nature of the metabolism in the different life-cycle stages and the impact of mistargeting of glycosomal enzymes. In contrast, autophagy, including pexophagy, may usually occur at a basal level in the parasites, to remove incorrectly synthesized or damaged cell constituents, but seems only highly upregulated during transitions in the life cycles, when the parasites have to adapt to new conditions [40]. This suggests that the pexophagy machinery for glycosome degradation may only be a suitable drug target during differentiation events occurring in the human hosts. Another possibility is, instead of inhibiting autophagy, to use autophagy inducers and cause the uncontrolled degradation of important cell components. However, little is known about the so-called autophagic cell death in these parasites.

Discussion and Conclusions

Glycosomes are highly relevant for drug discovery against trypanosomatid-borne diseases. The organelles contain important metabolic pathways that are validated drug targets and are being successfully used for drug discovery projects, as reported in a previous volume of this series [13]. As discussed in this chapter, glycosome biogenesis is another essential process for the viability of the trypanosomes, and the low conservation of the PEX proteins and variations

in their activity mechanisms offer promise for drug discovery. Also relevant is the high likelihood that the biogenesis of the organelles is a very active process in proliferating and differentiating parasites, thus, during essential stages of their life cycle. Moreover, basal levels of PEX protein expression and activity, for maintenance of the organelles by regular turnover, may be expected in quiescent pathogenic parasites such as the *T. brucei* short-stumpy bloodstream forms and *T. cruzi* trypomastigotes.

Degradation of glycosomes also involves a number of poorly conserved ATG and other proteins that are likely amenable to the development of compounds that selectively interfere with the process without affecting peroxisome degradation in host cells. Whether this degradation of the organelles in trypanosomatids involves the specific autophagy process of pexophagy or (only) nonspecific autophagy remains to be confirmed. Pexophagy relies on factors responsible for the specific recognition of peroxisomes to target them for degradation. Such factors are generally very poorly conserved; even in different yeasts, different pexophagy-specific ATG proteins have been identified. Future research is required to identify such factors in trypanosomatids. Similarly to their biogenesis, degradation as part of regular glycosome turnover is expected to play a role throughout the life of parasites, whereas the process can be highly upregulated during life-cycle transitions. Crucial differentiation steps with major changes of glycosome metabolism in the human pathogenic stages of parasites, such as probably in the *T. cruzi* amastigote–trypomastigote–amastigote or *Leishmania* spp. metacyclic promastigote–amastigote transitions, may thus be targeted with autophagy-/pexophagy-interfering drugs [40]. Alternatively, compounds that activate the glycosome degradation may be harmful for each of the parasites irrespective of their life-cycle stage.

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11
Glideosome of Apicomplexans as a Drug Target

*Lauren E. Boucher and Jürgen Bosch**

Abstract

As intracellular parasites, members of the phylum Apicomplexa invade host cells where they replicate, forming daughter parasites in order to continue their life cycle. Many of these parasites are responsible for human diseases such as malaria, toxoplasmosis, and cryptosporidiosis, in addition to affecting livestock including cattle and chickens. In order to invade host cells, apicomplexans use a conserved actin–myosin motor to generate force to enter the cells. Protein components that assemble to form the motor and bridge the motor to the parasite cytoskeleton are conserved across apicomplexans. These proteins are often highly divergent from human homologs or completely unique to the parasite. The unique and divergent proteins and the protein–protein interactions needed to assemble the motor components are attractive targets for developing novel antiparasitic small-molecule drugs. Here, we discuss how parasites invade host cells and the function and essentiality of the proteins that compose the motor assembly, also known as the glideosome. Furthermore, we discuss the current attempts to develop small-molecule inhibitors of invasion by targeting protein–protein interactions and insights into currently unexplored areas such as targeting of actin polymerization or myosin inhibition.

Economic and Public Health Burden of Apicomplexans

The clade of apicomplexan species comprises a multitude of pathogens that have a direct health burden on the human population, including the malaria-causing *Plasmodium* spp., and *Cryptosporidium* spp. and *Toxoplasma gondii*, which cause infections in immunocompromised individuals [1, 2]. Other apicomplexans, such as *Babesia* spp., *Theileria* spp., and *Eimeria* spp., impair livestock production, and the annual economic burden globally is approximated to be several billion USD [3]. The largest human toll, however, is caused by *Plasmodium falciparum*, through the transmission of malaria.

*Corresponding author.

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Phylogenetic Relation of Human and Livestock Apicomplexan Pathogens

When comparing various apicomplexan human and livestock pathogens, it becomes apparent that these organisms have many conserved pathways. As obligate, intracellular parasites, invasion is a necessary action in their life cycle, and it is unsurprising that the machinery and mechanism of invasion are conserved features [4]. The underlying mechanism and the proteins involved in this process, therefore, represent an attractive target for small-molecule intervention strategies. Figure 11.1 shows the average phylogenetic distance of different apicomplexans from humans, based on three key proteins required for the invasion process that have human homologs: actin, myosin, and aldolase. With the high degree of conservation of the invasion components among different apicomplexan parasites, *Plasmodium* spp. and *T. gondii* often serve as model organisms for the less well studied livestock pathogens from this clade (e.g., *Eimeria* spp. (chicken) and *Babesia* spp. (cattle)). Since the highest mortality rate is attributed to the malaria-causing parasites, *Plasmodium* spp., we will focus the discussion on the findings in this organism and how they relate to the other apicomplexans. Additionally, we will refer to studies in *T. gondii*, which, due to a larger number of genetic tools available and easier manipulation, often serves as a model organism in parasitology.

Burden of Malaria

According to the WHO [5], it is estimated that approximately 1.2 billion people are at a high risk of contracting malaria, and there were 198 million cases of malaria, resulting in approximately 584,000 deaths in 2013; most of these deaths occurred in children under the age of 5. Over 90% of the total global malaria burden is in sub-Saharan Africa, and the disease primarily affects lower income countries that are the most vulnerable due to lack of access to preventative measures, diagnosis, and treatment.

Malaria control and elimination programs have managed to lower the number of cases of malaria by 30% and deaths by 47% between 2000 and 2013 [5]. However, a new threat to these programs comes in the form of emerging drug resistance [6]. The first-line treatment of malaria is with artemisinin combination therapies (ACTs); however, treatment failure has been reported over the last decade in Southeast Asia, an area where both chloroquine and sulfadoxine–pyrimethamine resistance developed and later spread to Africa [7]. With the timeline for new drug development stretching on average 12 years from discovery to FDA approval [8], it is necessary to start developing novel antimalarials now, to target unique mechanisms and pathways. A previously unexplored area of drug development in malaria surrounds targeting protein–protein interactions. An attractive multiprotein-complex target composed of several essential protein–protein

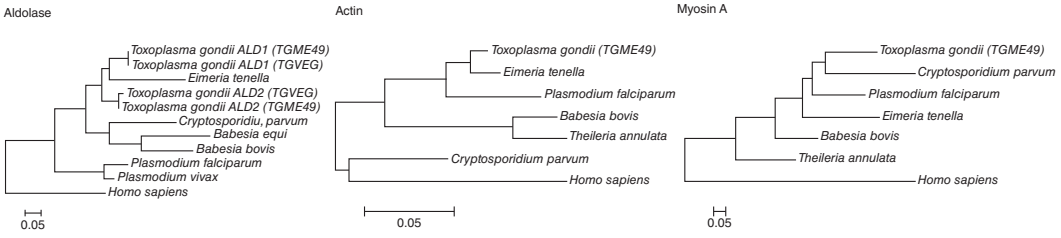


Figure 11.1 Phylogenetic tree of apicomplexan pathogens based on the amino acid sequences of aldolase, actin, and myosin A. Distances between phylogram leaves are indicated with a scale bar.

interactions and conserved among apicomplexan species is the invasion machinery, also known as the *glideosome*. The glideosome contains several proteins that are unique to the apicomplexan parasites (e.g., glideosome-associated proteins (GAPs)) or with high degrees of structural and sequence divergence (e.g., actin, myosin, myosin light chain (MLC)) compared to their human homologs.

Life Cycle of the Parasite

Malaria is transmitted through the bite of an infected female *Anopheline* mosquito. When a mosquito takes a blood meal, the *Plasmodium* spp. sporozoites, a motile form of the parasite, are deposited into the dermis (Figure 11.2).

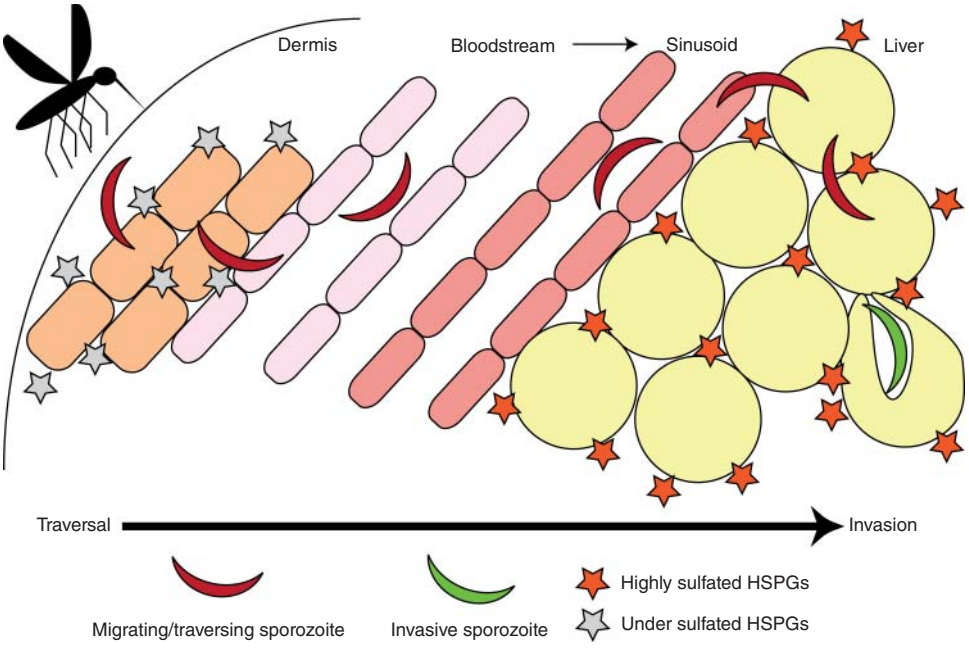


Figure 11.2 Migration, traversal, and invasion in the skin and liver stages. When the mosquito takes a blood meal, sporozoites (red) are injected into the dermis. These "migratory" sporozoites are capable of traversing and migrating the dermis, comprised of cells with under-sulfated HSPGs on their surface (gray stars), in a random motion. When they encounter the endothelium of a blood vessel, they can enter the bloodstream. The sporozoite then travels to the liver where it exits the sinusoid and can traverse several hepatocytes. Here, the sporozoite encounters highly sulfated HSPGs (orange stars) on the surface of the hepatocytes, which triggers signaling inside the parasite and cleavage of the circumsporozoite protein (CSP), activating the sporozoite for invasion (green).

In the dermis, the sporozoites glide through the skin in a random motion [9], eventually encountering the endothelial cells of a blood vessel or the lymph vessels, and cross this barrier to enter the bloodstream or lymphatic system, respectively [10]. Two extracellular membrane-bound surface receptors necessary for skin migration and dermis exit [11] are the thrombospondin-related anonymous protein (TRAP) [12] and TRAP-like protein (TLP) [13]. TRAP mutants display gliding defects *in vitro*, and TLP-deficient parasite lines exhibit reduced infectivity both *in vitro* and *in vivo* [14].

Crossing into the lymphatic system represents a dead end for the parasites; however, those that cross the endothelium and enter the bloodstream travel through the circulatory system until they reach the liver. Upon encountering hepatocytes, the parasites can traverse multiple liver cells. Hepatocyte surfaces are marked with highly sulfated heparan-sulfate proteoglycans (HSPGs), and this marker seems to trigger the parasite to invade a hepatocyte, where it establishes residence and develops into the blood stage form, the merozoite [15]. Once mature, the merozoites are released into the bloodstream to begin the asexual stage of the life cycle by invading red blood cells, replicating, and causing the clinical symptoms of malaria, including fever and anemia [16]. The process of gliding through the dermis, traversing hepatocytes, and invading host cells, such as red blood cells and hepatocytes, requires an orchestrated multiprotein motor complex, the glideosome.

Invasion Machinery (Glideosome)

For motility and invasion, apicomplexan parasites employ an actin–myosin (actomyosin)-based motor that is part of the glideosome (Figure 11.3), see Boucher and Bosch for more details [17]. Parasites attach to their target host cell via a variety of receptors that are essential at different stages of the parasite life cycle. Depending on the life-cycle stage, different proteins are used to attach to the host cell, (e.g., TRAP for liver stage, MTRAP for blood stage) [4]. These extracellular receptors contain cytoplasmic tails that are connected to the motor via a bridging protein. This enables locomotive force to be transmitted from the cytoplasmic motor to the parasite's attachment point at the host-cell surface. Most of the cytoplasmic proteins that are part of the machinery are conserved across apicomplexa and represent potential targets for drugs aiming to disrupt this machinery and the parasite. In the following sections, we discuss the proteins that compose the glideosome including the motor proteins, proteins that link extracellular receptors to the motor, and others that connect the motor to the internal structure of the parasite. We also explore how some of these proteins and interactions have been targeted using small molecules in an attempt to disrupt parasite invasion and motility.

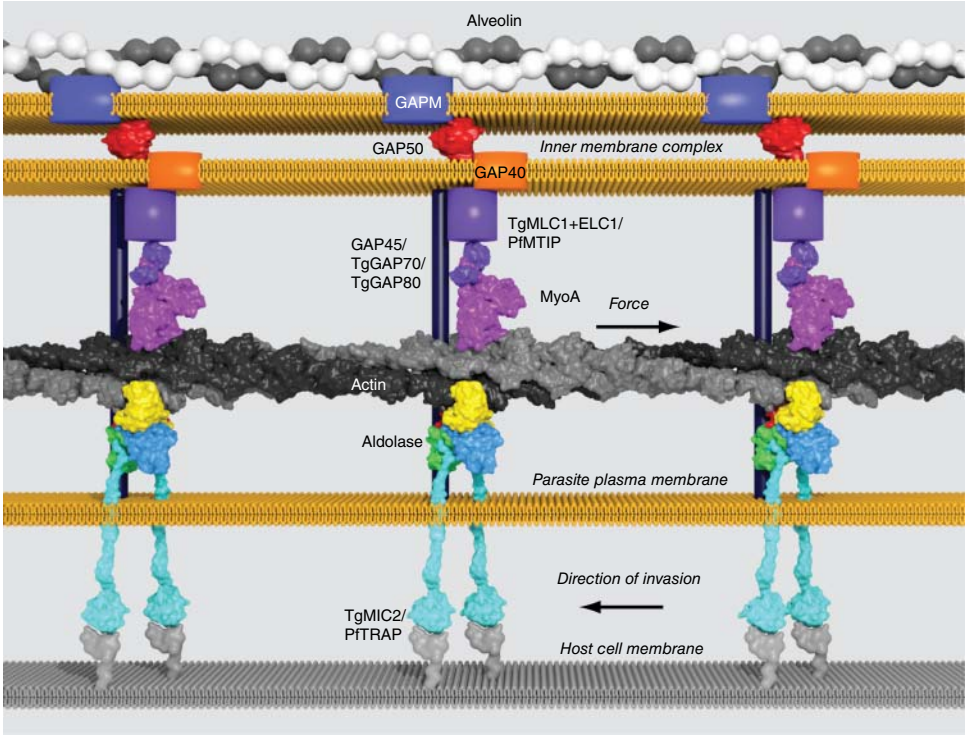


Figure 11.3 Schematic of apicomplexan glideosome. Protein structures solved from both *T. gondii* and *Plasmodium* spp. and homologous protein structures were combined with the results of biochemical and genetic data to depict the glideosome model. The actin–myosin motor (gray/black and magenta/light pink) is bridged by tetrameric aldolase (multicolor) to extracellular adhesins such as TRAP (*Plasmodium* spp.) or MIC2 (*T. gondii*) (cyan), which connect to unknown cellular receptors on the host cell membrane (gray). The motor is anchored to the inner membrane complex (IMC) via its interaction with MTIP (*Plasmodium* spp.) or MLC1/ELC1 (*T. gondii*) (purple). The conserved GAP45, acylated at the N- and C-terminus, spans the supra-alveolar space between the IMC and parasite plasma membranes and interacts with the MyoA-MTIP complex. The motor complex also interacts with the polytopic integral membrane protein, GAP40 (orange), and GAP50 (red) located in the lumen of the IMC and attached via a C-terminal membrane helix. The integral membrane protein GAPMs (blue) are located on the cytosolic side of the IMC membrane and interact with the alveolins (white/gray). (Reproduced from Boucher and Bosch [17], with permission from Elsevier.)

Conservation of the Glideosome in Apicomplexans

Although many key proteins of the glideosome exist in other organisms such as aldolase, myosin, and actin, the conservation of the respective surfaces varies. In general, the substrate-binding sites for fructose-1,6-bisphosphate (FBP) on aldolase, and ATP on actin and myosin, exhibit a higher degree of conservation

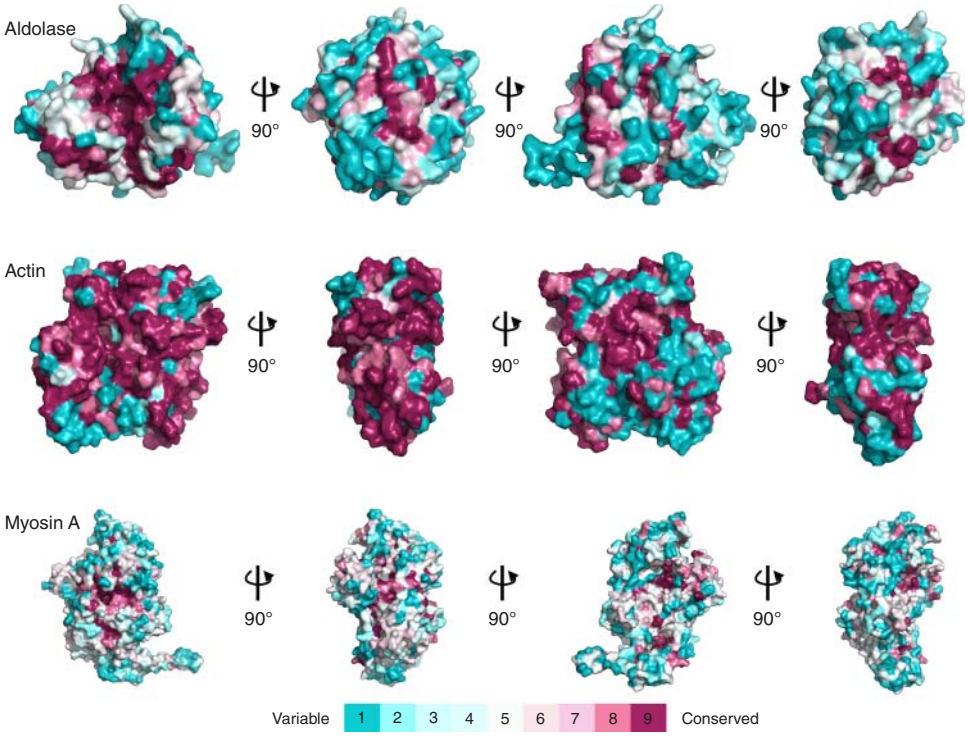


Figure 11.4 Conservation of key glideosome proteins. Conservation is depicted on a gradient colorimetric scale from cyan to magenta indicating low to high conservation, respectively. Actin is the most conserved protein within the glideosome assembly while the catalytic site of aldolase is completely conserved.

compared to the remaining protein surface. These areas would be difficult to specifically target through a small-molecule approach due to conservation with essential human proteins; however, the interaction surfaces between two protein partners exhibit more variability, which allow a selective targeting mechanism to be exploited (Figure 11.4)

Components of the Glideosome: Function and Potential as Drug Targets

Aldolase

As previously mentioned, the parasite employs extracellular receptors, which engage the host cell and are specific to the stage of the life cycle and cell type. The intracellular, cytoplasmic tail of these membrane-bound proteins is connected to the actomyosin motor via a bridging protein. This key bridging protein is FBP

aldolase [4, 18, 19]. In addition to catalyzing a reaction in the glycolytic pathway, aldolase has a second nonenzymatic function in the parasite; the aldolase tetramer interacts with actin on one face [20] and the C-terminal tails of multiple receptor proteins via its active site [4, 18]. In *Plasmodium* spp., aldolase interacts with receptors responsible for invasion and motility, such as the TRAPs, AMA1 (apical membrane antigen), and RH1/2b/4 (reticulocyte binding-like homolog) [21], and in *T. gondii*, it interacts with AMA1 and MIC2/6/12 (microneme protein) [4]. This interaction creates a connection between the motor and the extracellular adhesins so that force can be transmitted.

The cocrystal structure of the C-terminus of TRAP in complex with aldolase has been solved [18]. This structure showed that the tail binds the catalytic active site and that a key tryptophan is sandwiched between two arginines. In pursuit of a molecule to disrupt the machinery by targeting protein–protein interactions, a ternary cocrystal structure with TRAP and a small-molecule stabilizer bound in the aldolase active site has been deposited [22, 23]. This compound was first identified using an *in silico* screen against the aldolase–TRAP cocrystal structure that had been deposited earlier. As the active site of aldolase is highly conserved between humans and parasite (Figure 11.4), development of a compound that interfered with the dissociation kinetics of the receptor tail–aldolase complex was pursued instead of trying to inhibit the complex formation by targeting a small molecule to the active site. The initial hypothesis was that by preventing dissociation of the bridging protein and extracellular adhesin as invasion progressed, the parasite would have motility and invasion defects. The approach was successful in identifying a small molecule that reduced liver cell invasion by 95%; however, this compound had no activity against blood stage parasites. In blood stages, the parasite employs a different extracellular adhesin, MTRAP, which has a different cytoplasmic tail, extended by two amino acids at the C-terminus. This extension likely interferes with binding of the compound to the aldolase–MTRAP complex. The ternary cocrystal structure of the aldolase–TRAP compound could allow for structure-guided optimization to improve the inhibitory effect on parasite motility and invasion [23].

Actin

Actin forms the linear track of the glideosome motor. Typically, actin polymerizes in a head-to-tail fashion forming helical, parallel strands that are highly stable and range from several hundred nanometers to microns in length. However, both *Plasmodium* spp. and *T. gondii* form unstable actin polymers around 100 nm in length [24]. *Plasmodium* spp. express two variants of actin [25], whereas most apicomplexans, including *T. gondii*, express only one [26]. Actin 2 forms filaments similar to those of canonical F-actin, whereas actin 1 is responsible for the short, fragile filaments observed in apicomplexans [27]. The dynamics of apicomplexan actins are also unique. *Plasmodium* spp. actins 1 and 2 can hydrolyze ATP more

efficiently and are able to oligomerize in response to ADP [27], while TgActin can form filaments at critical concentration three to four times lower than F-actin [24] and polymerize without a lag phase [28].

The divergence of the actins from canonical actin found in humans indicates that this may be a druggable target. In spite of high sequence identity (Figure 11.4) and structural work that has indicated there are not large structural difference compared to canonical actin [27], smaller structural divergences in areas responsible for contacts between actin monomers and binding interfaces for regulatory proteins indicate that it may be possible to target actin protein–protein interactions. Small molecules could be used to target the interface of monomeric actin, preventing formation of the short actin filaments that make up the motor for assembly or by targeting an accessory-protein-binding interface. Additionally, inhibitors of actin accessory proteins necessary for capping, polymerization, and depolymerization may affect the pools of filamentous versus globular actin or the dynamics of assembly, preventing parasite gliding or invasion [29].

Myosin A

Myosin A (MyoA) is the motor protein of the complex, pulling on the actin filament to move the parasite forward by generating mechanochemical force through the hydrolysis of ATP. TgMyoA and *Plasmodium* spp. MyoA are part of the class XIV myosin family. This class possesses a head domain but lacks a neck composed of IQ motifs and a tail. Apicomplexan myosins diverge from this architecture by possessing a tail domain [30–33]. PfMyoA possesses a noncanonical, degenerate IQ-motif (VQxxxRKxxx(A/V)) in the tail domain [34–36] responsible for anchoring the motor head to the parasite's internal structure. It has been shown that despite lacking features of typical myosins such as a conserved glycine in the motor domain, a conserved residue in an actin binding loop, and possession of the degenerate IQ-motif that TgMyoA moves at speeds of approximately 5 μm/s *in vitro* [37].

Unfortunately, structures of the MyoA motor domain do not exist due to difficulty in expression, but recently, the successful expression of TgMyoA in *Sf9* insect cells was accomplished via coexpression with TgMLC1 (myosin light chain 1), TgELC1 (essential light chain 1), and a chaperone, TgUNC, a myosin-specific cochaperone from the UCS family, Unc45b [38]. This strategy will hopefully enable structural characterization of the protein in the future and an idea as to whether it would be possible to specifically target the protein with small molecules. It is likely that due to the unusual features of the apicomplexan myosins already mentioned and divergence from human myosins (Figure 11.4) that compounds could be developed against this target. Myosin inhibitors are being developed by some groups to treat hypertension and heart disease, so it may be possible to target the parasite myosin to prevent invasion [39].

MTIP/MLC1

To anchor the actomyosin motor to the internal structural network of the parasite, *T. gondii* encodes for the MLC protein [37] and *Plasmodium* spp. express the ortholog, myosin A tail interacting protein (MTIP) [35]. These proteins are distantly related to the MLC proteins, but lack the calcium-binding motif common to the family. Unique to the apicomplexan interaction of MyoA with MTIP/TgMLC1 is that it is mediated via the MyoA tail containing the single degenerate IQ motif, instead of by a neck domain containing multiple, canonical IQ motifs [35].

A structure of PfMTIPΔ60 bound to a helical MyoA tail peptide was crystallized at physiological pH [40]. The closed MTIP structure possesses a kinked central helix that connects the N- and C-terminal domains, forming one helix in each domain. The N-terminal domain of the truncated construct consists of four helices, forming two degenerate EF hands (no Ca^{2+} binding was observed), while the C-terminal domain is composed of four helices, forming two degenerate EF hands, as well as a small antiparallel β-sheet. The MyoA peptide binds to the C-terminal domain via residues extending into hydrophobic pockets and via complementary electrostatic interactions. The N- and C-terminal domains latch via an electrostatic interaction forming the closed complex around the MyoA tail. This state enables a tight interaction of the MyoA peptide with both the C- and N-terminal domains and hinge region. This kinked, or compacted/closed structure, is in contrast to an open structure that had been previously described with MTIP bound to the MyoA tail at low pH [36], where protonation of MyoA residue H810 favored the open state. The closed conformation is believed to be the functionally competent state [40]. MTIP is also phosphorylated via CDPK1 [41], a key kinase regulating egress and invasion. Phosphorylation of a key serine alters the charge state of the residue, disrupting the latch interaction, which prevents formation of the closed state and destabilizes the MyoA-MTIP complex [42].

As the MTIP-MyoA interaction is necessary to anchor the motor protein to the inner membrane complex (IMC) connected to the parasite cytoskeleton, experiments have been performed to identify small molecules that disrupt the protein–protein interaction. Using the crystal structure of the MyoA-MTIPΔ60 complex, a hybrid virtual screening approach was implemented [43]. First, a template pharmacophore was built based on key interactions of MyoA with MTIP, and then a large library was screened for compounds matching the pharmacophore. Identified molecules were then docked against the crystal structure of MTIP, and several compounds tested using growth inhibition assays for *P. falciparum* exhibited $IC_{50} < 25\ \mu M$. This work led to the identification of a pyrazole–urea compound that resulted in decreased parasite proliferation and altered gliding phenotypes. However, proof of a direct interaction of the compound with MTIP was not established.

A more recent study used a fragment-based approach to screen molecules against the MTIP-MyoA protein–protein interaction using differential scanning fluorimetry to screen an initial library, looking for fragment compounds that

bound and stabilized apo-MTIP [44]. This was followed by cross-validation with ITC and NMR. Unfortunately, crystal structures of the compounds in complex with MTIP alone were unable to be obtained, likely due to the low binding affinities ($K_D \sim$ mM) typical of fragments. To overcome the low binding affinity, an MTIP-stabilizing compound fragment, which was demonstrated to interact with residues implicated in MyoA binding, was linked to the N-terminus of a truncated MyoA peptide to form a chimera. A crystal structure of MTIP in complex with the compound peptide chimera was solved, providing further insight into how the weakly binding compound interacts with MTIP. This structure may allow for rational expansion of the fragment to target other regions and residues of MTIP and development of a tighter-binding compound.

Inner Membrane Complex (IMC)

The actomyosin motor is attached to the internal structural support of the parasite via the IMC comprised of alveoli. These alveoli underlay the entirety of the parasite's plasma membrane, except at the base and apex, and are connected to the parasite's cytoskeleton [45]. Several proteins connect the actomyosin motor to the IMC, and others are involved in attachment to the cytoskeleton. For the most part, these proteins are unique to apicomplexans or diverge significantly from their closest human homologs, indicating that they may make good therapeutic targets.

Glideosome-Associated Proteins (GAPs)

Three GAPs are conserved between *T. gondii* and *Plasmodium* spp. [46] and are notated by their apparent molecular weight, as determined by SDS-PAGE: GAP40, GAP45, and GAP50. It has been demonstrated that GAP40, GAP45, and GAP50 colocalize with the MyoA-MTIP/MLC1 complex [47]. GAP45, MyoA, and MTIP/MLC1 form a precomplex mediated by the N-terminal domain of MTIP/MLC1 interacting with the C-terminus of the peripheral membrane protein GAP45. An induced knockout of *gap45* results in the mislocalization of the MyoA/MLC1 complex [48]. The structure of GAP45 is unknown; however, it is predicted that GAP45 consists primarily of a coiled-coil domain and acylation at the N-terminus [49] targets GAP45 preferentially to the plasma membrane, preventing GAP45 and the motor from prematurely associating with the nascent IMCs [46]. GAP45 has additional predicted palmitoylation sites at its C-terminus, and this modification is expected to facilitate GAP45 localization to the IMC, allowing the MyoA-MTIP/MLC1 complex to also associate with the IMC [50]. Phosphorylation is the final step that controls association of the complex with the IMC [51]. Phosphorylation of residues in the C-terminal domain of GAP45 controls association with the IMC. The induced knockout of *gap45* and resulting defect in motor localization indicate that there is a potential to target assembly of the motor complex by preventing GAP45 and MTIP/MyoA association. Small

molecules specifically targeting this interaction would have a small likelihood of off-target effects due to the divergent sequence of MTIP and MyoA and because GAP45 is unique to apicomplexans.

GAP45 also interacts with GAP50, a protein secreted to the IMC, which possesses a C-terminal transmembrane helix for insertion into the IMC membrane [47]. The likely orientation of GAP50 is with the N-terminal domain located within the lumen of the IMC [52]. The structure of GAP50 [53] is most closely related to the human purple acid phosphatase; however, the recombinant protein shows no protein phosphatase activity *in vitro*, but does have activity against small molecules such as ATP and IPP (isopentenyl pyrophosphate), with a preference for di- and triphosphates [54]. Also, copurifying with GAP45 and GAP50 is the last member of the family to be discussed, GAP40, a polytopic membrane protein [46]. GAP40 copurifies with the MyoA-MTIP/MLC1-GAP45/50 complex and is comprised of nine transmembrane spanning domains. The direct interactions of the GAP proteins with each other are still under investigation as most evidence comes via co-immunoprecipitation experiments and may involve other binding partners. The glycosylation and phosphorylation states of these proteins are also known to play a role in their interactions with the other IMC components [55], so it may be possible to disrupt the interactions by interfering with kinases/phosphatases, glycosylases, or proteins responsible for acylation and targeting.

GAPMs and Alveolins

The actomyosin motor is attached to the outermost side of the IMC membranes. In cryo-EM freeze fracture studies, 9 nm particles on the innermost cytoplasmic side of the membrane of the IMC were observed [56]. Investigation of the makeup of these particles led to the discovery of the glideosome-associated proteins with multiple membrane spans (GAPMs) [57]. There are three GAPM variants (GAPM 1–3), which possess six transmembrane domains and are present at the IMC in large, detergent-resistant oligomeric complexes. GAPM copurifies with components of the subpellicular network, a 2D lattice made up of 8–10 nm fibers to which the IMC is anchored, and may serve to anchor the IMC and motor complex to the parasite's cytoskeletal network [57]. The main components of this subpellicular network are the alveolins [45]. The alveolins, which vary in size, contain multiple repeat domains with a common motif of (E/D)(K/R)ΦΦ(E/D)ΦPx (Φ = hydrophobic and x = any amino acid), flanked by nonconserved regions on either side [58]. Knockout of these proteins results in defects in sporozoite and ookinete motility and parasite invasion [59–61]. While little is known structurally about these proteins, it may be possible to target the protein–protein interactions necessary for anchoring the IMC to the subpellicular network, leading to disruption of invasion and motility.

Exploring the Essentiality of the Adhesins and Glideosome Components

To proceed with a targeted approach in designing novel inhibitory compounds, it is important to determine whether the proteins and targets of interest are essential to the disease process. While the process of invasion is essential for parasite invasion of host cells and continued survival, questions have arisen regarding essentiality of key receptors and motor proteins. To begin to answer this question, several groups have published data regarding conditional/induced (iKO) and clonal knockouts of proteins key to the presented glideosome model. Conditional knockouts, using a DiCre-recombinase system, of *ama1*, *aldolase*, *act1*, *gap45*, and *myoA*, have been generated in *T. gondii*, and invasion progresses, albeit at severely reduced levels [62]. These experiments and what the results mean in our understanding of the actomyosin-based invasion by *T. gondii* and how this work translates to other apicomplexans, specifically *Plasmodium* spp. are discussed as follows.

A clonal knockout of *ama1* in *T. gondii* tachyzoites was produced along with a nonclonal knockout in *Plasmodium berghei* (rodent malaria) merozoites to study the essentiality of this protein thought to be necessary for tight-junction formation, a key step in the invasion pathway [63]. This knockout negatively affected the parasite host-cell attachment but did not prevent invasion or tight-junction formation. It was concluded that AMA1 was dispensable for *T. gondii* and *P. berghei* invasion. However, a conditional knockout of *ama1* in *P. falciparum* did not support invasion, and it was determined that AMA1 is not dispensable for *P. falciparum* merozoite invasion [38]. An explanation offered to reconcile the *P. falciparum* results with the *P. berghei* and *T. gondii* data [63] is that both *T. gondii* and *P. berghei* possess orthologs/paralogs of AMA1, which could act as redundant adhesins; however, no redundancies have been identified in *P. falciparum*, which would make it essential for the model in which AMA1 plays a key role in formation of the tight junction [64].

In addition to disruption of a key adhesin, induced knockouts were generated in *T. gondii* for *myoA*, *gap40*, *gap45*, *gap50*, *mlc1*, and *act1* [62], thought to be essential components of the glideosome. A clonal knockout of *myoA* was isolated but could not be obtained for *gap40*, *gap45*, *gap50*, *mlc1*, or *act1*. The *myoA*iKO reduced invasion by 84% compared to wild-type (WT) and delayed host-cell invasion by up to 10 min. Gliding motility was reduced by approximately 65% and altered the smooth gliding pattern typically observed [62]. The *mlc1* iKO caused mislocalization of MyoA, reduced egress by 95%, and led to a approximately 70% reduction in invasion [48]. The experiments suggest that MyoA is necessary for efficient invasion and gliding but is not absolutely essential for *in vitro* experiments. An explanation is that other myosins could act as a redundancy mechanism and experiments have shown that MLC1 is able to interact with MyoC [65], indicating that other myosins could facilitate invasion. A triple conditional knockout of *myoA/B/C* was produced, resulting in a more severe phenotype than the single

*myoA*iKO; however, the parasites could still invade [48]. There were several other myosins that were not knocked out, including *myoD* and *myoE,* and it is reasonable to assume until proven otherwise that these could compensate for the loss of the other myosins. Similarly to the myosins, other MLCs may be able to compensate for the loss of MLC1. The data suggests that MLC1 is important, yet not essential, for host-cell egress and invasion; however, the severe phenotype indicates that other MLCs are unable to fully compensate for the loss of MLC1.

Regarding the IMC-associated component knockouts, it was not possible to induce a knockout of either *gap40* or *gap50* and retain viable parasites. It was possible to induce a knockout of *gap45*, which resulted in cytosolic localization of the MyoA/MLC1 complex and affected the parasite morphology, likely due to separation of the IMC from the plasma membrane [48]. Along with a change in localization and morphology, depletion of GAP45 resulted in approximately 95% reduction in egress as well as invasion. However, gliding remained at 100% that of WT parasites, suggesting that altered morphology is the cause of invasion defects and not motility.

A conditional knockout strain of *act1* [62] was generated, resulting in a defect in apicoplast segregation. Unlike in *Plasmodium* spp., it is not possible to supplement *T. gondii* cultures with IPP to determine if the *act1* clonal KO is unable to be obtained solely due to an apicoplast defect. The conditional *act1* knockout displayed gliding and invasion defects but was still motile and could invade at approximately 10% that of WT. A follow-up study, however, showed that after knockout induction, the residual actin in the parasite was sufficient to support propagation of induced actin-depleted lines for several replication cycles [66]. The severity of invasion defect was correlated with time after the knockout of *act1* was induced; wild-type strains invaded within 1 min while approximately one-third of the population of actin-depleted strains were unable to complete invasion within 4 min [66]. This study emphasizes the need for careful controls and quantification of protein levels in induced knockout strains.

A conditional knockout *aldolase* strain in *T. gondii* was able to invade fibroblasts under low glucose conditions [67]. The low glucose conditions prevented accumulation of toxic glycolysis intermediates, enabling the *aldolase* iKO tachyzoites to invade in the absence of aldolase. This led to the conclusion that aldolase was dispensable for invasion; however, GAPDH, another molecule that has been shown to interact with cytoplasmic tails of adhesins and can bind actin, could compensate for the loss of aldolase.

When using these inducible knockout strains to explore essentiality of proteins, it is important to take into account the efficiency of the gene deletion and the timing of the deletion with stage-specific promoters. In the *P. falciparum* AMA1 study, the recombinase was expressed after replication of parasite DNA but before parasite segmentation and there was imperfect deletion efficiency, resulting in some expression of AMA1 [38]. However, AMA1 was expressed after DNA replication but prior to parasite segmentation, so the result was a mixture of genetically null and WT parasites, with the "knockout" parasites containing a range of AMA1 levels. Also, keeping in mind the results from the actin conditional knockout in

T. gondii, protein stability in the parasite should be assessed for the potential knockout. Proteins with long half-lives may not be well suited for these conditional knockout studies as the time period may never be sufficient to obtain undetectable or nonfunctional levels of protein, allowing for parasite growth and propagation even after the gene is knocked out. In the absence of clonal knockout lines, control experiments should be devised using sensitive and well-validated antibodies to detect low, residual protein levels after inducing knockouts and protein levels measured over time and related to the degree of altered phenotype. But beyond questioning the essentiality of the components and information gained from some knockouts is the realization that there are redundant proteins that can assume roles in this machinery to continue invasion if a key protein is lost and that must be considered when choosing a small-molecule target. However, whether these parasites that lose a key protein due to a small-molecule intervention could sustain the disease process *in vivo* is still under investigation.

Concluding Remarks

Discovery and optimization of small-molecule inhibitors are often a primary research goal in order to develop new antiparasitic drugs to treat disease. Discussed earlier are some potential targets of small-molecule inhibitors that have been explored or are waiting to be explored. Specific to the invasion machinery, targets include key motor components such as myosin, protein–protein interactions that bridge adhesins to the motor, and interactions that link the motor to the IMC and parasite's internal cytoskeletal network. Other potential and indirect targets are enzymes responsible for posttranslational modifications, phosphorylating, glycosylating, and acylating these proteins and controlling motor assembly by affecting protein–protein interactions and localization. Early research success in establishing the model for the actomyosin-based glideosome essential for invasion along with work to validate the essentiality of different components means that continued work to develop therapeutics to target this complex and inhibit parasite motility and invasion could be rewarding. There have been preliminary successes in targeting the adhesin–aldolase complex and MyoA/MTIP complex, and this work may encourage further exploration of protein–protein interactions as drug targets or other glideosome components as targets, instead of relying on the traditional approach of targeting metabolic pathways and enzymes.

In addition to having a beneficial impact on disease, small-molecule inhibitor development can also help to elucidate critical functions of the parasite. Knockout lines of truly essential genes cannot be established for obvious reasons. Our genetic tools are therefore limited to a temporal action rather than permanent. To help elucidate critical biological functions of pathogens, small-molecule probes are desirable, as they provide an on/off switch that can be tunable, and they are typically fast-acting. However, the limitation of small-molecule inhibitors can be their pleiotropic nature, with potential off-target effects. Careful validation is needed

with orthogonal methods to limit potential misinterpretation of *in situ* data. The glideosome provides multiple established *in vitro* and *in situ* assays that can be used to validate that a small-molecule inhibitor is acting specifically. Experiments include and are not limited to measuring the gliding speed, curvature, and radii of isolated sporozoites in live imaging studies [68], levels of total gliding in sporozoite assays, and HepG2 and RBC invasion efficiency [69], to help confirm glideosome targeting. *In situ* cross-linking of compounds could confirm interactions with a particular target but would require compound modification with a handle that may interfere with its binding and function as an inhibitor. Therefore, providing direct evidence of an inhibitor interacting *in situ* with a particular target of interest will always be extremely difficult to achieve. There are pros and cons to both the knockout and small-molecule approaches to study the parasite function.

Overall, small-molecule targeting of the glideosome components, including the multiple protein–protein interactions and enzymes responsible for controlling the dynamics and localization of key components, is possible and should be pursued. It has been established that this invasion and motility machinery is critical for apicomplexans and can be targeted with inhibitors. Identification, validation, and optimization of compounds may be increasingly guided by the wealth and availability of structural data and exploited for more rapid drug development.

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12
N-Myristoyltransferase as a Target for Drug Discovery in Malaria

*James A. Brannigan and Anthony J. Wilkinson**

Abstract

Resistance has undermined the use of chloroquine and antifolates in malaria treatment and is threatening the future use of current front-line therapies based on artemisinin combination therapy. The need for new antimalarial drugs is being addressed with cell-based and targeted inhibitor screens. Here, we discuss the discovery of potent inhibitors of two new drug targets phosphatidylinositol-4 kinase and elongation factor 2 identified following phenotypic screens. We then review studies of *N*-myristoyltransferase (NMT) in *Plasmodium*, which have led to the validation of NMT as a drug target in malaria, the discovery and development of new lead compounds, and insights into the role of protein myristoylation in these parasites.

Introduction: Malaria and the Need for New Drugs

Malaria is a serious threat to human health and a leading cause of morbidity and mortality worldwide with nearly half of the world's population at risk. According to WHO statistics, there were an estimated 214 million cases of malaria in 2015, leading to 438 000 deaths with children under 5 years of age being the most severely affected [1]. Malaria is caused by parasites belonging to the genus *Plasmodium*, with four main species *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax* responsible for disease in humans, with *P. falciparum* accounting for the highest numbers of deaths, predominantly in Africa. *P. falciparum* infection can cause severe malaria, which is associated with organ failures, and cerebral malaria in which the parasite accumulates in the brain capillaries leading to coma and eventually death. *P. vivax* is geographically the most widely distributed cause of malaria, and while it rarely kills, it is associated with high morbidity and significant socioeconomic impact [2].

*Corresponding author.

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P. falciparum has a complex life cycle. It is transmitted by the bite of the female *Anopheles* mosquito as it takes a blood meal. Sporozoite forms of the parasite, introduced from the insect's saliva, make their way rapidly to the liver where they proliferate and differentiate to form merozoites. In this 5–10-day liver phase, there is expansion of the parasite population without symptoms of disease. The merozoites next invade the red blood cells in which they undergo a 48 h cycle of infection characterized by ring, trophozoite, and schizont stages. This results in 20 or so daughter merozoites, which, upon rupture of the host cell, are released into the bloodstream to reinvade new red blood cells. These asexual blood stages are associated with the symptomatic high fevers and pathology. A subpopulation of the parasites differentiates to form male and female gametocytes, which are the agents of disease transmission. These are taken up by the feeding mosquito and undergo fertilization in the insect gut before passing through a series of developmental stages completing the life cycle with the formation of sporozoites in the salivary glands.

As well as being effective and safe, drugs used in the treatment of malaria must be low cost since malaria is a disease strongly associated with poverty [3]. Chloroquine (Figure 12.1), the front-line treatment for malaria for several decades [4], is an inexpensive synthetic derivative of the natural product quinine isolated from the bark of the *Cinchona* tree. Chloroquine and related

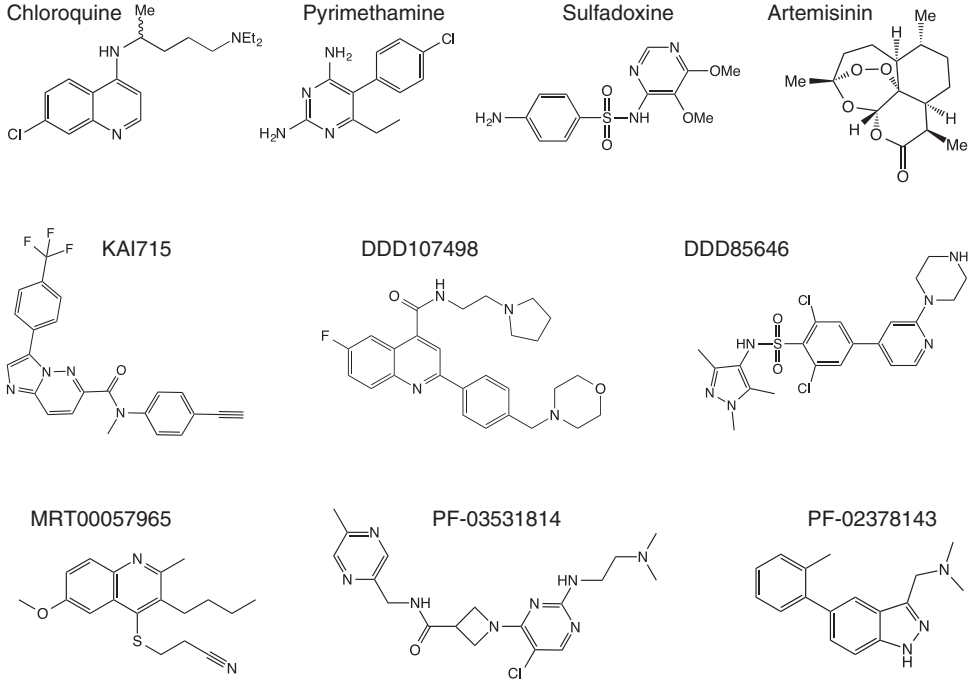


Figure 12.1 Structures of clinically important antimalarial drugs and molecules identified through screening programs discussed in the text.

4-aminoquinolines target a biochemical process that is unique to the parasite. In the blood stages, the parasite imports nutrients from the host-cell cytoplasm by pinocytosis into the vesicles, which are trafficked to a subcellular organelle referred to as *the digestive vacuole*. Here, the vesicle contents, which are rich in hemoglobin, are broken down by proteases yielding peptides and amino acids for biosynthesis. Hemoglobin digestion also releases potentially toxic "free" heme as a by-product [5]. To counter this threat, the heme is deposited into an inert crystalline form known as *hemozoin* or *malaria pigment* [6]. Chloroquine accumulates to high concentrations in the food vacuole through a combination of pH and heme-binding effects [7]. It is a diprotic base with pK_a values of 8.1 and 10.2 with a mixture of states populated at physiological pH, the neutral CQ, which can diffuse across membranes and the charged CQH^+ and CQH_2^{2+}, which cannot [8]. Upon entry into the acidic environment of the digestive vacuole, the equilibrium shifts toward the doubly protonated form, CQH_2^{2+}, so that the drug is trapped in this compartment where it exerts its antimalarial action by blocking hemozoin formation. This leads to accumulation of free heme and CQ–heme complexes that are toxic to the parasite resulting in cell killing. Despite much study, the detailed molecular basis of chloroquine-induced toxicity is not fully understood and more than one mechanism may be involved [9]. Chloroquine is selective in its action since it targets a parasite process, iron-porphyrin crystallogenesis, which does not take place in the host. Moreover, resistance cannot evolve through mutation of the target, which is not genetically encoded. Nevertheless, resistance to chloroquine has emerged and is now widespread. In chloroquine-resistant parasites, there is less accumulation of the drug in the food vacuole and resistance-conferring mutations map to a transporter gene, *pfcrt*. *pfcrt* encodes the chloroquine resistance transporter (*PfCRT*), which localizes to the food vacuole membrane. The key resistance-conferring mutation in field isolates replaces lysine76 on the first of 10 predicted transmembrane helices with threonine, in a region of the protein predicted to be involved in substrate binding [10]. Chloroquine transport by *PfCRT* has been demonstrated in a heterologous *Xenopus laevis* oocyte system, where membrane translocation of CQH_2^{2+} was facilitated by *PfCRT* from resistant parasites but not by *PfCRT* from wild-type parasites [8].

Inhibitors that target the folate biosynthesis pathway of the parasite, including pyrimethamine, cycloguanil, and sulfadoxine, have also been widely used [11]. Pyrimethamine (Figure 12.1) is a potent inhibitor of dihydrofolate reductase (DHFR), which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. It binds competitively to the folate substrate-binding pocket. DHFR inhibition blocks the pathway of nucleotide biosynthesis, arresting DNA replication and killing the parasite. Pyrimethamine is also an inhibitor of human DHFR, albeit with a lower potency [12]. Selectivity is achieved principally as a result of differential translational control of the parasite and host enzymes, with levels of the latter upregulated when the active site is occupied by the inhibitor [12]. Sulfadoxine (Figure 12.1) is an inhibitor of another enzyme of the folate biosynthesis pathway, dihydropteroate synthetase (DHPS), which catalyzes

the condensation of 6-hydroxymethyl-7,8-dihydropteridine pyrophosphate and *para*-aminobenzoic acid (PABA) to form 7,8-dihydropteroate. Here, the sulfonamide drug is a competitive inhibitor of PABA binding. Selectivity is achieved because DHPS is not expressed in human cells. In combination, as *components of* the therapeutic formulation Fansidar™, pyrimethamine and sulfadoxine act synergistically to produce a powerful antimalarial treatment that was a widely used first-line therapy in Asia, Africa, and South America after chloroquine had lost its efficacy due to the emergence of resistance. This widespread use and likely misuse/overuse of Fansidar™ led inevitably, in turn, to the emergence of resistance to the antifolate combination. Resistance is associated with multiple point mutations in *dhfr* and *dhps*, the genes encoding DHFR and DHPS, respectively. In the case of DHFR, the combinations of point mutations leading to pyrimethamine resistance have been mapped onto the crystal structure of the parasite enzyme in complex with the drug. This revealed a clustering of mutation sites around the active site, which substantially lower the affinity for pyrimethamine without blocking substrate (dihydrofolate) binding [13]. These insights are underpinning the development of a new generation of drugs with activity against the resistance-compromised target [14].

The current first-choice treatment for malaria is artemisinin combination therapy. Artemisinins (Figure 12.1), derived from a natural product of the plant *Artemisia annua*, are potent antimalarials, which bring about rapid clearance of parasitemia and alleviation of the disease symptoms. In addition to their potent activity against blood-stage parasites, they have activity against early-sexual-stage parasites, which is important for blocking transmission [15]. However, artemisinins are rapidly cleared from the body, and their co-dosing with a prolonged-acting partner drug, usually a quinoline-related inhibitor such as lumefantrine, mefloquine, or amodiaquine targeting the digestive vacuole, is designed to slow the emergence of resistance [16, 17]. Artemisinins contain an unusual endoperoxide pharmacophore. Their mechanism of action is not well understood and there has been no consensus on their biochemical target, leading to suggestions that they may have multiple targets [18]. Analysis of artemisinin-resistant parasites has implicated mutations in the gene encoding *Pf* Kelch13 in resistance development [19]. Orthologs of *Pf* Kelch13 are involved in protein ubiquitinylation, suggesting a role in intracellular signaling and/or proteasomal degradation. A recent study has shown that dihydroartemisinin is a potent inhibitor of the parasite enzyme phosphatidylinositol-3 kinase (*Pf* PI3K) [20]. *Pf* PI3K phosphorylates phosphatidylinositol to produce phosphatidylinositol-3-phosphate. Interestingly, *Pf* PI3K levels are increased in artemisinin-resistant parasites correlating with mutations in *Pf* Kelch13. It is proposed that these mutations prevent *Pf* Kelch13 from binding to *Pf* PI3K and mediating its tagging for degradation. The blockade of the degradation pathway leads to accumulation of *Pf* PI3K to levels sufficient to overcome the killing effect of the drug.

The absence of a licensed malaria vaccine and the undermining of current drugs by resistance emphasize the urgent need for a pipeline to new drugs to be maintained [21]. Ideally, these drugs should prevent as well as cure malaria

and block parasite transmission. Moreover, they should have activity against all human malarial parasites and be capable of clearing the liver of *P. vivax* hypnozoites (dormant liver stages), which are responsible for frequent relapses in infected individuals. One approach is to develop new derivatives of proven antimalarials based on mechanistic knowledge of the mode of action and the basis of resistance. Another approach is to use cell-based phenotypic screening of large libraries of compounds to identify new molecules that kill parasites in infected red blood cells. For such unbiased approaches, the discovery of inhibitors is relatively straightforward, but the optimization of hits can be more laborious as the target of the inhibitor is initially unknown. Finally, there is the targeted approach exemplified here for the enzyme *N*-myristoyltransferase (NMT).

Phenotypic Screening and Inhibitors Directed toward PI4K and eEF2

There have been a number of phenotypic screens for antimalarials, and these studies have been reviewed [22, 23]. In a wide-ranging, cell-based inhibitor screen, 1.7 million compounds were tested in a high-throughput format for their capacity to block *P. falciparum* growth on erythrocytes using a fluorescent dye-based screen for nucleic acids [24]. Red blood cells lack nuclei, so increases in DNA and RNA can be attributed to parasite proliferation. In total, 17,000 "primary hits" were identified as achieving 50% inhibition of parasite growth at a concentration of 1.25 μM. In the follow-up assays, these reduced to approximately 6000 molecules based on approximately 530 scaffolds with potent antimalarial activity including known antimalarials, thus validating the approach. This set of compounds represents a rich source of starting points for developing novel antiparasitic activities [24].

A new class of antimalarials emerging from this screen were imidazopyrazines, exemplified by KAI715 (Figure 12.1), which are active against many drug-resistant strains of *P. falciparum* (IC_{50} values of ~50 nM; IC_{50} is the half-maximal inhibitory concentration) as well as field isolates of *P. vivax* (IC_{50} ~ 100 nM) [25]. In addition to the activity against asexual blood-stage parasites, the imidazopyrazines showed promising activity against liver-stage development of several *Plasmodium* species when assayed in cell and animal model systems. This translated to prophylactic protection of mice from colonization by *Plasmodium berghei* sporozoites, as well as the elimination of existing *P. berghei* infections [25]. This liver-stage activity extended to the killing of cultured hypnozoites of the simian parasite *Plasmodium cynomolgi*. Together, these observations suggest that the imidazopyrazines have the potential to clear the liver stages of human *P. vivax* malaria, thereby preventing relapse. These inhibitors were also active in transmission blocking, thus they showed gametocytocidal activity and lowering of the number of infected mosquitoes measured in membrane feeding assays.

The potency of the imidazopyrazines against multiple stages of many parasite species suggests that these inhibitors block a conserved essential cellular activity. Detailed cytological examination showed the accumulation of multinucleated

schizonts in synchronized blood-stage parasites treated with the imidazopyrazines. Inhibitor administration/washout experiments indicated that the drug target is critical at the very late stage of the 48 h intraerythrocytic life cycle [25]. Visualization of the plasma membrane of schizonts at this stage using a fluorescent protein marker revealed a disorganized structure and absence of clearly defined daughter cells, indicating a block in membrane biogenesis around the developing merozoites. The target of the imidazopyrazines was identified through *in vitro* evolution of KAI407-resistant blood-stage *P. falciparum* parasites followed by chromosomal analysis using a genome tiling microarray to identify genomic differences relative to the parent. KAI407-resistant clones shared either copy number differences or single nucleotide polymorphisms in *pfpi4k*, which encodes phosphatidylinositol-4 kinase (PI4K). Resensitization of resistant parasites encoding a variant PI4K (His1484Tyr) to the drug was associated with reversion of the mutation to wild-type sequence. In addition to *pfpi4k*, KAI715 resistance was also mapped to *pfrab11a*, which encodes *Pf* Rab11A, a small GTPase and likely target of *Pf* PI4K regulation. In *Drosophila*, PI4K and Rab11A interact during cytokinesis, regulating membrane delivery in the final steps of cell division [26]. It is therefore proposed that *Pf* PI4K acts at the Golgi to generate phosphatidylinositol-4-phosphate, which recruits effectors, the obvious candidate being Rab11A, which generate transport vesicles destined for the ingressing membrane where the membrane material is needed to complete the asexual stage [25]. Thus, as is often the case, inhibitors of cell growth can turn out to be useful chemical tools for illuminating hitherto unexplored aspects of cellular biochemistry.

In a more recent phenotypic screen of the Dundee protein kinase scaffold library (~5000 compounds) against blood-stage *P. falciparum* 3D7 parasites, a 2,6-disubstituted quinoline-4-carboxamide series was identified and optimized to give DDD107498 (Figure 12.1) [27]. This compound is highly potent with EC_{50} values of around 1 nM against a range of widely geographically distributed *P. falciparum* and *P. vivax* parasite isolates in *ex vivo* assays. Significantly, the inhibitor is active against the liver schizont stage when added during or after parasite infection in assays using *P. berghei* and *Plasmodium yoelii* model systems (EC_{50} ~1 nM; EC_{50} is the 50% effective inhibitory concentration). These experiments with *in vitro* models translated well into experiments with *in vivo* models in *P. berghei*-infected mice. Compounds active at this earliest stage of infection have potential for use in chemoprotection and for the clearance of *P. vivax* hypnozoites from the liver to prevent the frequent relapses that characterize infections with this parasite. DDD107498 is also active against the sexual stages where it blocks formation of male and female gametes with similar high potency ($EC_{50} = 2$ nM), suggesting additional potential as a transmission-blocking agent. This activity was confirmed in a *P. berghei* mouse-to-mouse transmission assay. The dual capacity to block parasite transmission and to confer protection against infection is a sought-after characteristic in drugs as part of the quest to eradicate malaria. Thus, DDD107498 has great therapeutic

potential – moreover, it has very low toxicity toward human cells, excellent drug-like properties and is believed to have the potential for use as a single-dose antimalarial treatment [27].

To identify the target of the inhibitor, blood-stage *P. falciparum* parasite strains were cultured in the presence of DDD107498 until resistant parasites emerged. The genomes of 10 resistant parasite cell lines were sequenced and compared to the genome sequences of sensitive parasites, revealing a consistent pattern of mutations in the gene encoding the translation elongation factor 2 (*Pf* eEF2) [27]. eEF2 is an essential GTP-dependent factor involved in translocating the ribosome along the messenger RNA during eukaryotic protein synthesis. Inhibition of protein synthesis by DDD107498 was confirmed in assays measuring ^{35}S-methionine incorporation into *P. falciparum* proteins with its direct targeting of *Pf* eEF2 demonstrated in experiments with transgenic parasites.

Protein *N*-Myristoylation in *Plasmodium*

N-Myristoylation of proteins is typically a cotranslational event, although it also occurs post-translationally in apoptotic cells [28]. Nascent peptides emerging from the ribosome exit tunnel are acted on sequentially by methionine aminopeptidase, which removes the initiator methionine residue and myristoyl-CoA:protein N-myrisotyltransferase (NMT) which transfers myristate from myristoyl-CoA (Myr-CoA) onto the newly exposed α-amino group of glycine residues. *N*-Myristoylation can variously stabilize protein structures, regulate protein – protein interactions, or influence the partitioning of proteins into membranes as part of protein trafficking. A single *N*-myristoyl group is considered to be insufficiently hydrophobic to direct membrane incorporation; instead, it increases the propensity for membrane association, which is normally modulated by other switches [28].

The repertoire of proteins in the cell that are myristoylated was predicted to be broad, but prior to the advent of proteomic approaches, few NMT substrates had been defined unambiguously. Part of the reason for this is the absence of a clear consensus myristoylation motif in substrate proteins, the only invariant feature being an N-terminal glycine. In *P. falciparum*, only two proteins, calcium-dependent protein kinase-1 (CDPK1) and glideosome-associated protein 45 kDa (GAP45), were known to be myristoylated with indirect evidence implicating three other proteins, GRASP1, armadillo repeats-only protein ARO, and the ADP-ribosylation factor ARF1, as NMT substrates [29].

Recently, the "myristome" of *P. falciparum* has been defined in experiments exploiting *in vivo* tagging of NMT substrates using the myristate mimetic, tetradec-13-ynoic acid (YnMyr), and multifunctional capture reagents [30]. In YnMyr, there is a triple bond between the carbons (C13 and C14) most distal to the carboxylate group. The alkyne was fed to malaria parasites *in vitro* and shown to be incorporated into YnMyr-CoA and subsequently transferred

cotranslationally by NMT onto target proteins. After 5 h of incubation, parasite proteins were extracted and the Yn-myristoylated proteins were selectively ligated by copper (I) catalyzed azide–alkyne cycloaddition to the capture reagent, azido-TAMRA-PEG-biotin (AzTB), which contains (i) an azide group for selective chemical labeling of alkyne-containing molecules; (ii) a biotin moiety for selective pull-down of tagged proteins onto streptavidin-coated beads; and (iii) a fluorophore (TAMRA) that allows direct in-gel imaging [30].

For the enrichment of amide-linked *N*-myristoylated proteins over ester-linked glycosylphosphatidylinositol (GPI)-modified proteins, which also contain myristate, the samples were treated with alkali to hydrolyze the ester bonds prior to pull-down. The streptavidin pull-downs were digested with trypsin and the products resolved and analyzed by liquid chromatography and tandem mass spectrometry, resulting in the identification of 30 NMT substrates in blood-stage *P. falciparum*. These included the previously identified and inferred NMT substrates, as well as new proteins that are known to be *N*-myristoylated in other species. The majority of these proteins are known or predicted to be localized to membranes or membrane-bound compartments. The proteins have a range of activities and roles in diverse processes including motility, protein transport, development, and signaling [30]. The integral role of these processes to cell function suggests that NMT is a promising target for antimalarial drug development.

Structure, Specificity, and Mechanism of NMT

NMT, a ubiquitous enzyme in eukaryotic cells, catalyzes the attachment of the 14-carbon saturated tetradecanoic acid (myristic acid), via an amide bond, to the amino-terminal glycine residue of a subset of proteins. The mechanism of NMT action has been studied in most detail for the enzymes from *Saccharomyces cerevisiae* and *Candida albicans*, and much of what has been learned from these studies is known or assumed to apply to orthologous enzymes, including those of parasitic protozoa and their human and mammalian hosts. *N*-Myristoylation by NMT proceeds via a compulsory order reaction mechanism (Figure 12.2a), with the binding of MyrCoA preceding the docking of the substrate protein. Following acyl transfer in a nucleophilic addition–elimination reaction, the CoA product dissociates prior to release of the acylated peptide [31].

NMTs are monomeric proteins typically of $M_r = 50$ kDa. In yeast and protozoan parasites, there is a single NMT, while in human cells, there are two enzymes NMT1 and NMT2. The crystal structures are known for the NMTs from *Aspergillus fumigatus* [32], *S. cerevisiae* [33], *C. albicans* [34], *Leishmania donovani* [35], *Leishmania major* [36], *P. vivax* [37], and *Homo sapiens* NMT1 and NMT2 [38], the majority reported as ternary complexes of the enzyme with either the cosubstrate MyrCoA or a nonhydrolyzable analog *S*-(2-oxo)pentadecyl-CoA (NHM), and an inhibitor molecule.

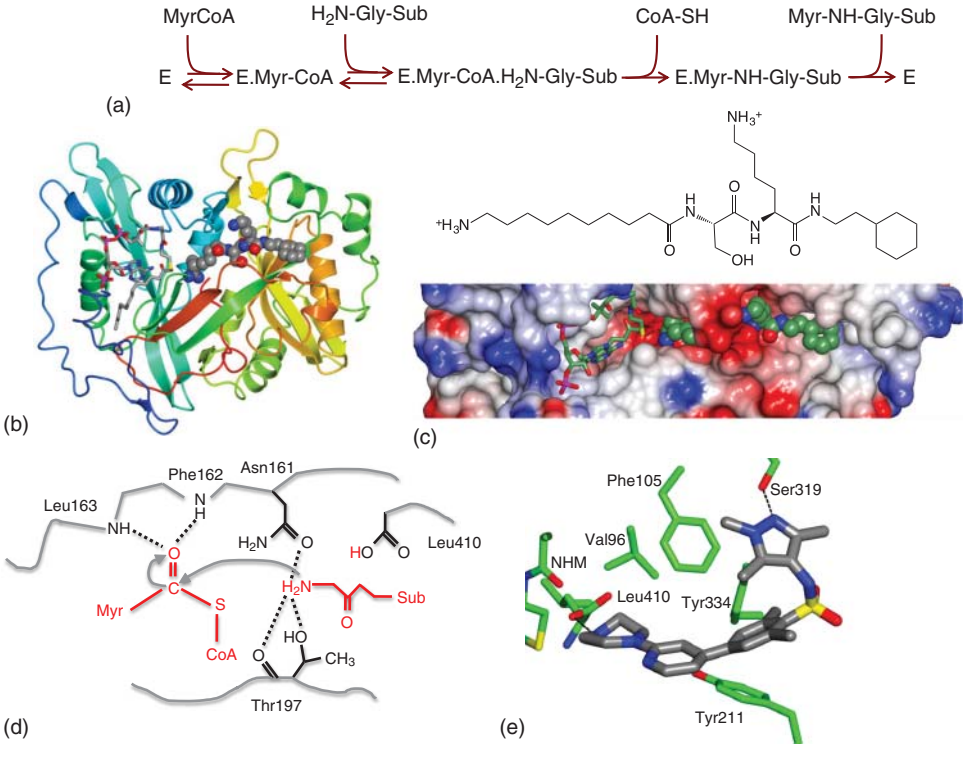


Figure 12.2 *N*-Myristoyltransferase catalytic mechanism and structure. (a) Scheme showing the ordered binding of myristoyl-CoA (MyrCoA) and the substrate protein followed by the ordered release of coenzyme A (CoA-SH) and the myristoylated substrate protein. (b) The crystal structure of the ternary complex of NMT from *P. vivax* (*Pv*NMT, PDB code 4C68) with NHM (a myristoyl-CoA analog; *S*-(2-oxo)pentadecyl-CoA); and a peptidomimetic (*N*-(10-aminodecanoyl)-L-seryl-*N*-(2-cyclohexylethyl)-L-lysinamide) inhibitor bound in the active site. Ribbon representation of protein with the chain color-ramped from the N-terminus (blue) to the C-terminus (red). NHM is represented in cylinder format and the peptidomimetic is shown as spheres. The atoms are colored by type: carbon, gray; nitrogen, blue; oxygen, red; sulfur, yellow; phosphorus, magenta. (c) Top: Chemical structure of the peptidomimetic inhibitor. Bottom: Electrostatic surface rendering of the protein molecule revealing the substrate binding groove. The ligands are represented as before except that carbon atoms are colored in green. (d) Schematic of a step in the reaction mechanism showing the active site following binding of substrates and deprotonation of the peptide's α-amino group by the carboxylate of Leu410. (e) The binding of the inhibitor DDD85646 (thick cylinders with gray carbons) to *Pv*NMT (PDB code 2YND) with selected enzyme residues shown including Leu410 and Ser319, which form important polar contacts (dashed lines). (Brannigan http://journals.iucr.org/m/issues/2014/04/00/jt5003/index.html. Used under CC By 2.0 License http://creativecommons.org/licenses/by/2.0/uk/legalcode.)

The central feature of the structure is a highly twisted 12-stranded β-sheet spanning the width of the molecule as shown for *P. vivax N*-myristoyltransferase (*Pv*NMT) in Figure 12.2b. The β-sheet together with a pair of α-helices forms a barrel that encloses the C-terminal segment of the protein chain, so that the α-carboxylate of the C-terminal residue, Leu410, is at the heart of the molecule. A prominent and extended groove runs across the front face of the enzyme, and this forms the substrate-binding site (Figure 12.2b,c). MyrCoA and analogs such as NHM bind to the left-hand region of the groove such that the long aliphatic chain of the myristate extends into a deep hydrophobic cavity in the protein interior with the adenine and pantetheine components of the CoA moiety more exposed on the surface. The pantetheine and fatty acyl moieties of the MyrCoA are folded around the adenine base, giving a shape that has been likened to a question mark [33], such that the scissile thioester bond is in the center of the complex.

The right-hand side of the groove forms the binding site for the N-terminal segment of substrate proteins (Figure 12.2b,c). The crystal structure of *Sc*NMT bound to NHM and the octapeptide H_2N-GLYASKLA-NH_2 reveals that the peptide is bound in an extended conformation with the N-terminal glycine located at the center of the complex. The amino group of this glycine is in close proximity to both the C-terminal carboxylate of the enzyme chain and the carbonyl group of NHM, which corresponds to the thioester carbonyl of MyrCoA. To date, this is the only structure of an NMT in complex with a *bona fide* peptide [39]. Structures have also been determined with peptidomimetic inhibitors such as that illustrated in Figure 12.2b,c.

The mode of substrate binding in the crystal structures is consistent with that deduced from kinetic and mechanistic analysis [39]. Upon binding of MyrCoA, a hitherto disordered loop closes over part of the active site groove. This loop forms interactions with the peptide, suggesting that compulsory-order substrate binding is the result of MyrCoA-induced ordering of this loop [33]. For *Pv*NMT, following binding, abstraction by the α-carboxylate of the C-terminal residue Leu410 of a proton from the α-amino group of Gly1 of the substrate protein takes place (Figure 12.2d). Thr197 and Asn161 form polar interactions with the amino group, which attacks the carbonyl carbon of the MyrCoA. The resulting tetrahedral reaction intermediate is stabilized by interactions with an oxyanion hole formed by the amides of Phe162 and Leu163. As this intermediate collapses, CoA would be released first from the enzyme surface followed by the modified peptide product.

The determinants of specificity in peptide substrate binding have been examined for yeast NMT. A systematic structure–activity analysis of the *S. cerevisiae* ADP-ribosylation factor-2, Arf2p, derived substrate GLYASKLS showed that a hexapeptide comprising the six amino terminal residues is the minimum sequence needed for interactions with the enzyme [40]. Alanine scanning mutagenesis of this substrate emphasized (i) that Gly1 is essential for activity (no activity was observed with Ala1) and (ii) the importance of Ser5 and Lys6, whose substitutions led to 10^4-fold and 10^2-fold increases in K_M, respectively [40]. Analysis of enzyme–peptide interactions in the structure of the *Sc*NMT-NHM-GLYASKLA complex suggested that Gly is needed at the N-terminus, as its flexibility allows

rotation of its amine following deprotonation to bring it into close proximity to the thioester carbonyl of the cosubstrate [39]. Meanwhile, analysis of the side-chain-binding pockets helps to explain broad substrate specificity, the preference for serine at position 5 being explained by a hydrogen bond to a conserved histidine residue.

NMT as a Drug Target

NMT is a promising target for drug discovery in infectious diseases. It catalyzes the cotranslational modification of proteins involved in diverse, essential processes, and genetic analyses have shown that NMT is an essential enzyme in the fungal pathogens *A. fumigatus*, *C. albicans*, *Cryptococcus neoformans* and in the kinetoplastid parasites *Trypanosoma brucei*, *L. major*, and *L. donovani* [35, 41–44]. Validating a drug target by genetic methods in *P. falciparum* is more challenging since the conditional knockout of an essential gene in this organism has not reliably been achieved to date. However, partial knockdown of NMT expression in the rodent malaria parasite *P. berghei* using a tetracycline-inducible repression system led to a dramatic reduction in parasite viability, suggesting that NMT is essential in the murine model [45]. As discussed later, *P. falciparum* NMT has been shown by chemical genetic methods to be essential [30]. The NMT-catalyzed reaction lends itself to straightforward assays that have been adapted to high-throughput screening [46]. Moreover, recombinant NMTs have been overexpressed and purified from many species, the enzymes are monomeric and stable, and the structural and mechanistic basis of NMT activity is well understood. Structure–activity studies using analogs of the MyrCoA and peptide substrates led to the conclusion that pathogen versus host enzyme selectivity can be achieved by targeting inhibitors to the peptide-binding site on the enzyme [40, 47]. Sequence comparisons guided by crystal structure data supported this notion, revealing a highly conserved MyrCoA-binding pocket and a peptide-binding site that is divergent [33].

Early studies of *C. albicans* NMT led to the development of peptide-based and peptidomimetic inhibitors that were selective in their action against the fungal target compared to the human NMT [40]. This encouraged the establishment of antifungal drug discovery programs targeting NMT in the pharmaceutical industry and the generation of selective and specific inhibitors based on benzofuran [48] (Roche) and benzothiazole [49] (Pfizer) scaffolds. These nonpeptidic inhibitors also bind in the peptide-binding pocket [50]. However, these inhibitor series were not progressed as they proved to be specific for *C. albicans* NMT and unlikely to give rise to broad-spectrum activity against the NMTs of *Aspergillus* and *Cryptococcus* species. New, higher-efficacy antifungal agents need to have cross-species activity to reduce the necessity for clinical diagnosis prior to treatment. Interest in NMT inhibitors as fungicides has been renewed recently as pulmonary aspergillosis is becoming a major problem in immunocompromised patients and NMT has been shown to be a drug target in *A. fumigatus* [44].

Compounds developed in these antifungal programs formed the basis of preliminary studies of NMTs as drug targets in protozoan parasites. Compounds containing a core benzothiazole scaffold were elaborated, which inhibited NMT from *P. falciparum* with IC_{50} values of around 50 μM, two of which reduced parasitemia of cultured parasites significantly at concentrations of 10 μM [51]. Meanwhile, a pair of compounds was identified with submicromolar IC_{50} values against NMT from *T. brucei* and EC_{50} values against mammalian parasite stages of 16 and 66 μM [52].

The NMT of *T. brucei* was validated as a drug target for treating African sleeping sickness through studies that followed high-throughput screening of a 62 000 compound library to identify new inhibitor series [36]. This led to the identification of a pyrazole sulfonamide inhibitor of *Tb*NMT, which was chemically evolved to give DDD85646 (Figure 12.1) that has high potency against the enzyme ($IC_{50} = 2$ nM) and parasites *in vitro* and *in vivo* ($EC_{50} = 2$ nM). This inhibitor cures *T. brucei*-infected animals in the hemolymphatic form (stage 1) of mouse models of human African trypanosomiasis (HAT) at doses of 12.5 mg/kg administered twice a day over 4 days; moreover, the inhibitor acts on-target in bloodstream trypanosomes [36]. Modifications to this molecule have subsequently improved the capacity to cross the blood–brain barrier, which is necessary for curing stage 2 HAT when parasites have entered the central nervous system giving rise to disease symptoms leading to coma and death [32].

Discovery of Inhibitors of *Plasmodium* NMT – "Piggyback" Approaches

Drug repurposing, using a developed compound against a new indication or disease, is a popular way to generate new leads. Where the drug is directed against a common molecular target in a different species, this approach is referred to as *piggybacking* [53]. Searle followed a peptidomimetic strategy in their antifungal campaign, maintaining the core recognition features of native peptides but incorporating modifications that conferred stability against degradation by proteolysis [40]. These have been modified and assessed for antiparasitic activity. An example is *N*-(10-aminodecanoyl)-L-seryl-*N*-(2-cyclohexylethyl)-L-lysinamide, which is shown bound to *Pv*NMT in Figure 12.2 [54]. This peptidomimetic forms many of the key interactions expected of a native substrate. The amino group of the aminodecanoyl species occupies the site of the α-amino group of the substrate peptide and is linked to the Ser-Lys dipeptide (preferred residues at substrate positions 5 and 6) by a 10-carbon aliphatic spacer, which replaces residues 2–4 of the substrate (Figure 12.2c) [54]. The lysine interacts with an acidic loop on the enzyme, which closes over the inhibitor on binding. Although potent and specific to the enzyme *in vitro*, peptide-based inhibitors tend to be large and expensive; moreover, they have limited bioavailability leading to weak inhibition *in vivo*.

The antifungal screen performed by Roche identified the benzofuran **1** (Figure 12.3) as a potent selective inhibitor of *C. albicans* NMT [48], and

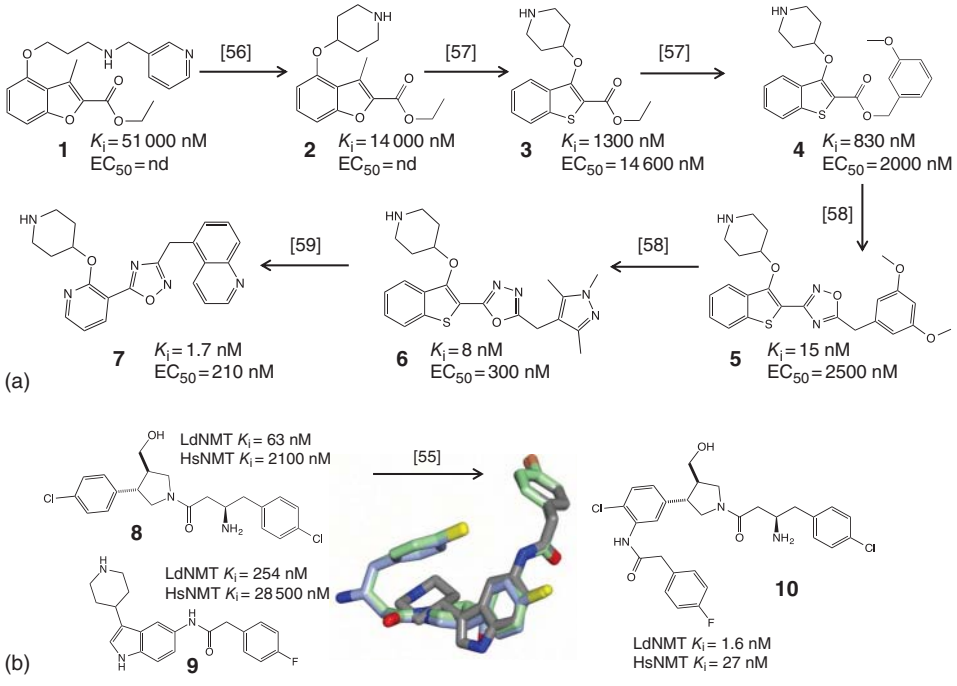


Figure 12.3 NMT inhibitor optimization. (a) The evolution of the benzofuran **1** to the potent inhibitor **7** by structure-guided medicinal chemistry. Arrows are labeled with associated references. The K_i and the EC_{50} values refer to inhibition of *Pf*NMT and the *in vitro* potency against *P. falciparum* respectively. (b) Hybridization of the binding modes of **8** and **9** to give **10** with a 40-fold increase in potency toward *Leishmania donovani* NMT (*Ld*NMT). In the center, **8**, **9**, and **10** from their complexes with *Ld*NMT and myristoyl-CoA are shown overlaid following superposition of the protein chains. The carbon atoms are colored according to the inhibitor: ice blue, **8**; gray, **9**; and light green, **10**. The PDB codes are 4CGL, 4CGN, and 4CYO, respectively [55].

this compound was later shown to inhibit *Pf*NMT with modest potency ($IC_{50} = 50\,\mu M$) and promising selectivity (HsNMT1 $IC_{50} > 1000\,\mu M$) [56]. This molecule was the basis of chemistry-driven elaborations around the benzofuran core leading to compound **2,** which has threefold higher potency and is more drug-like, having fewer rotatable bonds and a higher ligand efficiency (potency per heavy atom) while retaining selectivity over human NMT [56]. Structures of *Pv*NMT, which is closely similar to *Pf*NMT, in complex with representative benzofuran inhibitors revealed the basis of their inhibition. Each binds with the benzofuran nucleus and the attached piperidine ring occupying a volume, adjacent to the MyrCoA cofactor that would correspond to the binding site for residues 1–3 of a peptide substrate. The piperidine nitrogen forms an ion-pairing interaction with the C-terminal α-carboxylate (Leu410) of the enzyme mimicking the interaction that would be made by the substrate α-amino group [56].

"Lead hopping" in which the position of attachment of the piperidine ring to the bicyclic center is swapped from the 4- to the 3-position and a subsequent "scaffold hop" from a benzofuran to a benzothiophene core [57] led to compound **3** (Figure 12.3a) and a 10-fold increase in potency over the benzofuran parent compound **2**. Enzyme inhibition was improved by structure-guided design following the observation that, despite the similarities of their nuclei, the furan and thiophene molecules adopt strikingly different binding poses on the enzyme so as to maintain the interaction of the basic amine functionality with the enzyme C-terminal carboxylate group. Addition of a 3-methoxyphenyl group gave compound **4** (Figure 12.3a), which inhibits *Pf*NMT in the submicromolar range. Curiously, although compound **3** is equipotent against *Pf*NMT and *Pv*NMT, compound **4** has a 10-fold higher affinity for *Pv*NMT ($K_i = 80$ nM) [57].

Further compound development, including replacement of the labile ester in compound **4** with an isosteric oxadiazole ring, led to compound **5,** which has a 50-fold higher potency against *Pf*NMT; however, this does not translate to a lower effective dose against parasites *in vivo* (Figure 12.3a) [58]. To improve the characteristics of this compound, synthetic chemistry guided by crystallographic data on selected enzyme-inhibitor complexes was directed at driving down the ligand-efficiency-dependent lipophilicity (LELP = cLogP/LE where $LE = [-\log K_i] \times 1.374/$(number of heavy atoms) and P is the octanol/water partition ratio) to below a target value of 10. This resulted in compound **6** and a further twofold increase in potency ($IC_{50} = 8$ nM). Compound **6** shares with DDD85646, another tight-binding *Plasmodium* NMT inhibitor, the capacity to span the peptide-binding pocket so as to form both the ion-pairing interaction with Leu410 and a hydrogen bond to Ser319 (Figure 12.2e) [30]. Relative to **5**, compound **6** also has eightfold lower EC_{50} at 300 nM [58]. It has been shown to act on-target in *P. falciparum* parasitized red blood cells and most encouragingly to reduce parasitemia in mice infected with *P. berghei* [30].

The repositioning of the piperidine ring in the evolved *Pf*NMT inhibitors allowed their overall molecular weight to be reduced by scaffold simplification [60]. Altering the bicyclic nucleus to a monocyclic phenyl ring led to the development of a single-ring pyridyl derivative, which was less lipophilic, had a greater ligand efficiency, and displayed enzyme affinity in the low nanomolar range [59]. Further structure-guided development led to quinoline ring addition as part of a hybridization strategy, adopting the core entity from MRT00057965 derived from a high-throughput screen [37] (Figure 12.1). Compound **7** is a potent enzyme inhibitor that retains submicromolar activity in *P. falciparum* killing (Figure 12.3a).

Discovery of Inhibitors of *Plasmodium* NMT – High-Throughput Approaches

Partnerships that bring the drug discovery expertise of pharmaceutical companies and academia together, with funding from governmental or charitable sources, are an attractive model for the discovery of new lead molecules for infectious tropical diseases. The industrial partner can provide large libraries of compounds, facilities

for high-throughput screening, and expert judgment of the best potential leads for follow-up. Two such ventures have provided the resources to generate starting points for antimalarial drug development centered on *Plasmodium* NMTs.

High-throughput screening of a library containing nearly 60 000 commercially available compounds collated by Medical Research Council Technology (MRCT) led to the discovery of a small-molecule quinoline inhibitor with an IC_{50} of 1 μM against *Pv*NMT [37]. A crystal structure of the hit compound MRT00057965 (Figure 12.1) in complex with *Pv*NMT suggested a binding mode different from those of the inhibitors described earlier. The lead molecule is largely buried in a hydrophobic pocket, with the quinoline ring forming π–π stacking interactions with surrounding aromatic residues and with its nitrogen forming a polar interaction with the hydroxyl of Ser319 [37]. A nitrile group appended to the ring system interacts with the imidazole of His213, which would normally interact with the preferred substrate residue Ser5. The inhibitor thus occupies a "distal pocket" volume corresponding to the binding site of residues 4 and 5 of a substrate peptide. This is in contrast to the core elements of the benzothiophene-derived inhibitors described earlier, which interact with C-terminal carboxylate group of NMT and occlude the "proximal pocket" volume that would be occupied by substrate residues 1–3.

A complementary high-throughput screen of over 150 000 compounds from the Pfizer Global Diverse Representative Set, which included legacy compounds from antifungal NMT programs, was performed against protozoan parasite NMTs [61]. This identified a number of submicromolar inhibitors of *P. falciparum* as well as *L. donovani* NMTs, which showed selectivity over the host (human) enzyme. The *Leishmania* inhibitors have been further developed and are described in more detail here. As expected for competitive inhibitors, structural studies of the four *L. donovani*-selective compound classes in complex with the closely related enzyme from *L. major N*-myristoyltransferase (*Lm*NMT) show that each occupies the peptide-binding site. Despite the variety of molecular scaffolds, all have overlapping binding sites in the peptide-binding groove making extensive apolar contacts and a small number of polar interactions with the enzyme. Each inhibitor nevertheless exploits different features of the peptide-binding pocket [62]. In one case, two inhibitor molecules were seen in the binding groove, with their thienopyrimidine rings stacking against each other and partially filling both proximal and distal pockets.

The more potent NMT inhibitors such as DDD85646 also span both pockets, forming extensive hydrophobic interactions with important flanking polar interactions (Figure 12.2e). Structural superposition of two of the hits, compounds **8** and **9** (Figure 12.3b), suggested how features of the two compounds could be combined to exploit both major binding sites. This led to the design of the chimera **10** and a 40-fold enhancement of K_i against *L. donovani* NMT' (Figure 12.3b) [55]. The novel *P. falciparum*-selective hits from this screen, including PF-03531814 and PF-02378143 (Figure 12.1), provide excellent starting points for development of analogs and the generation of new compound series with therapeutic value against malaria [61].

Using NMT Inhibitors to Validate NMT as a Drug Target in Malaria

The lead inhibitor series discovered by the high-throughput and piggyback approaches were developed using medicinal chemistry strategies guided by structural data with the aim of improving potency. This involved driving down the IC_{50} or K_i values for *Plasmodium* NMTs. However, when these inhibitor series were tested for their efficacy in reducing parasitemia *in vitro*, the correlation between the K_i measured against the purified enzyme and the EC_{50} measured against parasites was poor [30]. One explanation for this lack of correlation is that the inhibitors are not acting on-target and are killing parasites by mechanisms other than NMT inhibition.

To address the question of whether the inhibitors are acting on-target, the high-affinity inhibitors and the chemical tagging methodology (see Section Protein *N*-Myristoylation in *Plasmodium*) were combined. Thus, compounds related to DDD85646 and **6** were added to blood-stage parasites prior to the addition of YnMyr (Section Protein *N*-Myristoylation in *Plasmodium*). After completion of the 48 h life cycle, YnMyr-tagged proteins in the parasite extracts were labeled using the azide-linked fluorophore. A dose-dependent reduction in "tagging" measured by in-gel fluorescence was observed for all the NMT substrates. Plots of protein fluorescence in gels as a function of inhibitor concentration allowed TC_{50} values (inhibitor concentration required for 50% reduction in YnMyr tagging) to be calculated. When these TC_{50} values were compared with the EC_{50} values, a strong correlation was observed [30] establishing a direct link between intracellular inhibition of NMT and efficacy in reducing parasitemia, thus suggesting strongly that the inhibitors are acting on-target. Collectively, these experiments validate *Pf* NMT as a target for antimalarial drug development. The diverse enzyme-to-cell translation of activity, indicated by the poor correlation between K_i and EC_{50} values, can be explained by differential access of the inhibitors to their site of action caused, for example, by differences in their membrane permeability or their susceptibility to efflux pump activity.

Essential Function of NMT in Plasmodium Parasites

As NMT cotranslationally modifies more than 30 cellular proteins in *P. falciparum*, its inhibition is likely to have pleiotropic effects since any or all of these proteins may become mislocalized or misregulated. Thus, the specific consequences of NMT inhibition and the resulting reduction in protein myristoylation that lead to parasite death are hard to predict. For this reason, red blood cells infected with synchronized ring-stage *P. falciparum* parasites were treated with DDD85646 and their progression through the intraerythrocytic life cycle was monitored by microscopy [30]. At 45 h postinvasion, control parasites appear as schizonts containing an average of 20 nuclei. In contrast, parasites treated with DDD85646 at concentrations that reduce without abolishing NMT activity, form

what are termed *pseudoschizonts*, with an average of six nuclei. Furthermore, the treated parasites have a greatly reduced capacity to egress from the infected red blood cell, and reinvade to form new ring stages, indicating a severe loss of viability.

An analysis of the parasite proteome at 45 h postinvasion showed significantly reduced abundance of proteins such as MyoA, MTIP, GAP45, and ISP3 that are characteristic of a distinctive subcellular structure found in *Plasmodium* and its relatives, the inner membrane complex (IMC). The IMC consists of flattened membrane compartments that are situated directly below the plasma membrane. The IMC is the site of assembly of the actin–myosin motor complex that drives the process of red blood cell invasion. Immunofluorescence imaging of parasites using an anti-GAP45 antibody allows the assembly of the IMC to be visualized [63]. The IMC normally forms as small ring-like structures at the periphery of the parasite, and these are later seen to surround each merozoite nucleus after the schizont has undergone segmentation and differentiation to form the merozoites. In parasites treated with inhibitor, the IMC structure was not visible, suggesting that its assembly is blocked with catastrophic consequences for parasite viability [30].

Conclusion

The studies described here emphasize the power of high-throughput screens to generate promising antimalarial drug leads using either cell-based or target-based approaches. The selective inhibitors emerging from these drug discovery programs have been used as chemical tools to probe parasite cell biology. Thus, mechanistic studies of artemisinin and KAI407 have revealed the vulnerability of parasites to changes in phosphatidylinositol phosphate (PIP3 or PIP4) levels while studies of the NMT inhibitors have illuminated the essential role of protein myristoylation in merozoite cell division and underlined the importance of membrane biogenesis. For inhibitors directed against any new target, resistance will emerge in the field – indeed, it is often straightforward to evolve in the laboratory through single point mutations. NMT, with its plethora of essential substrates, may prove to be a sturdier opponent, and NMT inhibitors may prove to be useful in new combination therapies for the treatment of malaria.

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Part Two
Metabolomics in Drug and Target Discovery

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13
Methods to Investigate Metabolic Systems in *Trypanosoma*

Maria Fatarova[1], *Florian Bellvert*[1], *Edern Cahoreau*[1], *Frédéric Bringaud, and Jean-Charles Portais*[1,*]

Abstract

Trypanosoma brucei possesses several unconventional metabolic traits due to its parasitic way of life involving complex life cycle and consequent need for redundant metabolic pathways. In the past decade, a number of metabolomic studies focused on the functional analysis of *T. brucei* metabolism aiming to find potential new treatments against this parasite. Metabolomics aims at the comprehensive analysis of low-molecular-weight species – metabolites – resulting from the operation of metabolism. This review is a general introduction to the field of metabolomics and its application to trypanosomatids, with a focus on *T. brucei* metabolism. It describes the various approaches applied to investigate cellular metabolomes, as well as the main steps of the experimental workflow and the main analytical platforms. It also includes the use of stable isotope-labeling experiments in the investigation of metabolic networks. Over the past years, the application of this approach to mutants impaired for specific metabolic enzymes has allowed significant progress of the topology and operation of metabolism in the parasite.

Trypanosomes, Unconventional Organisms

Trypanosoma brucei is a unicellular parasitic organism responsible for fatal Human African Trypanosomiasis (*HAT*) and Nagana in cattle. Available HAT treatment currently consists of four licensed compounds, which all have the following drawbacks: low efficacy, toxicity, susceptible to resistance, and difficult administration [1]. In order to eradicate the lethal disease, new drug targets are intensively being searched for, focusing on features that distinguish parasites and eukaryotic host cells. *T. brucei* survives in two distinct and very

*Corresponding author.

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different environments: in the midgut and salivary glands of the male or female blood-feeding tsetse fly (genus *Glossina*) and in the blood of a mammalian host. Insect forms, called *procyclic form*s (PCFs), proliferate in the insect midgut and undergo several developmental stages before migrating to the salivary glands, where they differentiate into mammalian infective (metacyclic) forms [2]. From the point of injection, parasites traverse into the bloodstream and differentiate into long slender forms. In the late stage of the infection, they enter the cerebrospinal fluid (CSF) but remain strictly extracellular. The bloodstream population is pleomorphic as it comprises of both proliferative long slender forms and nonproliferative stumpy forms. The latter are preadapted for transition to the insect host [2]. There are several prominent physiological and biochemical features distinguishing trypanosomatids from other eukaryotic cells that have shaped the search for new drug treatments, such as the unusual network of interconnected mini and maxi circles of mitochondrial DNA (kDNA) called *kinetoplast*, advanced host-defense mechanism through the variable surface glycoprotein (VSG) [3] effective in the mammalian host and the protective protein layer (procyclin) in the insect host [4]. The metabolism of trypanosomatids has been subject of intense research since it was discovered that these organisms possess a peroxisome-like organelle, called *glycosome*, in which glycolysis takes place [5]. Such an atypical feature has motivated detailed investigations of glucose metabolism, and it was further shown that this organelle contains a broad range of metabolic processes in addition to glycolysis, such as part of pyrimidine biosynthesis, nucleotide synthesis, and purine salvage; part of mevalonate pathway; and oxidative part of pentose phosphate pathway (PPP), which has dual localization between glycosomes and cytosol [6–8]. *Trypanosomes* also contain unusual pathways for lipid metabolism. They synthesize fatty acid *de novo* with three endoplasmic reticulum elongases (ELO1, ELO2, and ELO3) instead of canonical type I fatty acid synthase (*FAS*). To do so, they require malonyl-CoA as two-carbon donor and NADPH as reducing agent. Synthesis starts from butyryl-CoA instead of acetyl-CoA common in other systems [9]. In bloodstream form (BSF), the main product of the ELO pathway is myristate (C14), a key molecule exclusively used for the glycophosphatidylinositol (GPI) anchors of VSG, while PCFs were found to produce more hydrophobic stearate (C18) [10]. *Trypanosomes* are also equipped with an unusual thiol redox metabolism based on trypanothione [11]. In respect to these unique physiological and metabolic features, understanding the organism's metabolism matches the comprehensive understanding of its physiology and adaptation capabilities. In recent years, novel and powerful tools for system-level and comprehensive investigations of metabolism have been developed [12, 13]. The emergence of metabolomics, the large-scale analysis of metabolites, has allowed unpreceded investigation of metabolism at the cellular level [14]: particularly, when combined with mutants impaired for specific enzymes or exposed to conditions mimicking various environments encountered during the life cycle [15, 16]. As a result, notable progress in the understanding of the metabolism of these organisms and their adaptation capabilities has been achieved. The main purpose of this chapter is to

provide an overview of approaches and methodologies in metabolomics and to illustrate its application to the investigation of trypanosomatid metabolism, with a focus on *T. brucei* PCFs.

Metabolomics

Metabolomics focuses on the comprehensive – qualitative and quantitative – analysis of the metabolome, that is, all low-molecular-weight species (molecular weight usually <1000 or <1500 Da, nonpolymeric) present in an organism or a biological sample [17]. The qualitative and quantitative composition of the metabolome depends on the cellular activity, as it results from the expression of the organism's genome in the considered environment, and hence represents a detailed and functional phenotypic characterization of the interaction between genome and the environment.

There is no absolute cutoff in definitions of metabolomics approaches, but several complementary strategies can be distinguished according to their general purposes and considered analytical strategies. *Targeted analysis* aims at the absolute quantification of a limited number of known metabolites. Because the chemical identity of metabolites is known, highly selective and sensitive analytical methods can be applied for this purpose. *Metabolite profilin*g focuses on the quantitative analysis of a larger group of metabolites that share common chemical properties (mass spectrometry (MS)-based metabolite profiling) or represent the major compounds in a sample (nuclear magnetic resonance (NMR)-based metabolite profiling). *Metabolic fingerprinting* (intracellular metabolites) and *footprinting* (extracellular metabolites) refer to the untargeted analysis of metabolites independently of their chemical nature and are often applied for pattern screening and chemometrics [18]. The complete resolution of the metabolome is coined *global metabolomics* and aims at the identification and quantification of all metabolites. This is currently a challenge. Simultaneous determination of all metabolites is hampered by numerous factors. The first issue is the large number of metabolites (up to thousands in a single sample) and their extreme chemical diversity (e.g., sugars and fatty acids have totally opposite chemical characteristics), which can be neither extracted nor analyzed all at once with a single method. A second issue is the high turnover rates of many metabolites (subseconds to seconds range). Nonphysiological evolution of the metabolite content can occur rapidly if metabolism is not blocked immediately. A third issue is the extremely large amplitude of metabolite concentrations, which can range from trace amounts (picomolar) to highly abundant metabolites (hundreds of millimolar) in the same sample. This concentration range spans well beyond the capabilities of current analytical methods, thus cannot be resolved with a single one from this viewpoint as well. For all aforementioned reasons, the complete coverage of the metabolome cannot be achieved by a single sampling and analytical method, but through the combination of many sampling and analytical methods. This requires tremendous experimental efforts and is often

carried out by consortia of laboratories with complementary expertise. A more practical approach of global metabolomics is to apply an analytical method allowing "the broadest possible" coverage of the metabolome. With modern analytical instruments, such methods allow the detection of thousands of analytical features, which represent a few hundred compounds.

The extreme heterogeneity of the metabolome explains why analytical techniques with broad chemical coverage are preferred in metabolomics. The two most prominent techniques are the sensitive MS coupled with selective chromatographic techniques, such as gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS), and less sensitive but versatile and robust NMR. These techniques, as well as their application to metabolomics, are detailed later in this chapter. Due to the strong resolutive power of modern instruments, a huge amount of analytical data can be collected on a single metabolome sample, providing rich information about the metabolism of investigated organisms. Finally, a fourth major issue in metabolomics is related to the interpretation of such mass of analytical data, since converting the analytical information into identified metabolites is currently a huge challenge whatever the analytical platform considered. More details about these issues and how they are currently addressed are given in the following sections.

In addition to the methods aiming at the identification and quantification of metabolites, complementary approaches aim at the resolution of biochemical reactions and networks. The most widespread method in this respect is represented by isotope-labeling studies, which are currently performed mainly with stable isotopes. In metabolomics, the most common tracer is ^{13}C because the wide majority of metabolome components are organic molecules, hence contain one or more carbon atom(s). Other considered isotopes include mainly ^{15}N to investigate nitrogen metabolism, along with ^{2}H and ^{18}O. The basic principle of isotope-labeling studies of metabolism dates from the pioneering times of biochemistry. Basically, cells, tissues, or organisms are fed with an isotopically labeled substrate. The label input can be optimized according to the metabolic pathways of interest, but also to the analytical technique used to measure label incorporation [19]. The label propagates in the metabolites and leaves a specific pattern that depends on the set of reactions by which the labeled substrate is converted into the compounds. Hence, meaningful information about biochemical reactions and metabolic pathways can be obtained from the measurement (by NMR or MS) of the labeling patterns of metabolites, a strategy termed *isotopic profiling*. In some favorable cases, especially with cultivated cells, the labeling data can be combined with mathematical models of metabolic pathways to calculate metabolic fluxes ("fluxomics"), which are the actual rates of biochemical reactions *in vivo*. Because they provide functional information about the operation of metabolism, stable-isotope-labeling experiments are increasingly used in the field of metabolism. In particular, they have been significantly applied to investigate *Trypanosoma* metabolism, enabling the identification of key metabolic features in these organisms. This is why they are detailed later in this chapter.

The metabolomics workflow can be divided into four parts: experimental design, sample collection and preparation, sample analysis, and data processing and interpretation (Figure 13.1). All these steps are defined and optimized according to the biological model, purpose of the experiment, biological model, metabolites of interest, and considered analytical platform.

Sample Preparation in Metabolomics

General Considerations

The choice of the metabolite sampling method is crucial in metabolomics because it can determine both the observed metabolite content and subsequent interpretation of data. Ideally, sample preparation should be fast and reliable, avoid under-/overestimation of metabolite levels during sampling, and also prevent their degradation or loss during extraction. Overall, the sampling of metabolites consists of three main steps. The first one is the collection of the desired biological material, that is, cells for endometabolomics and cultivation medium for exometabolomics. The separation of cells from the medium is achieved by centrifugation or fast filtration, although in some approach, the whole cultivation broth – including both cells and extracellular medium – is directly sampled. During collection of biological material, washing step is required to avoid cross-contamination. It also aids in removal of salts present in the media that can impede MS detection. Because the rates of metabolic reactions can be very high, the washing solution is supplemented with all nutrients normally present in the cultivation medium to avoid substrate limitation and metabolic perturbations, which can be observed after a few seconds in some systems [20]. Some intracellular metabolites can be detected in the medium, as a result of active metabolite release (especially organic acids) or of cell lysis [21]. They can also result from the nonphysiological release of metabolites (metabolite leakage) if cell integrity is not maintained during sampling, thereby leading to underestimation of the intracellular content [22]. A number of strategies to circumvent the leakage problem have been suggested, such as quenching of metabolism before separating the cells from medium, although the quenching method can also induce some leakage (e.g., some quenching solvents can permeabilize cell membranes). Another alternative strategy consists in the so-called *differential method*, in which the concentrations of intracellular metabolites are measured from the difference between the metabolites in total cultivation broth and the metabolites in the extracellular medium [23]. The second step of sampling is the blockage of metabolic activity (quenching), to avoid nonphysiological alteration of metabolome composition during the sampling process. Quenching should be extremely fast since the turnover of metabolites is in the range of subseconds to seconds. The turnover rate is high especially for the intermediates of central metabolic pathways. In the bacterium *Escherichia coli*, the turnover time of phosphorylated sugars and most organic acids involved in TCA cycle could be measured below 4 s [23]. Quenching can be achieved by rapidly pouring the

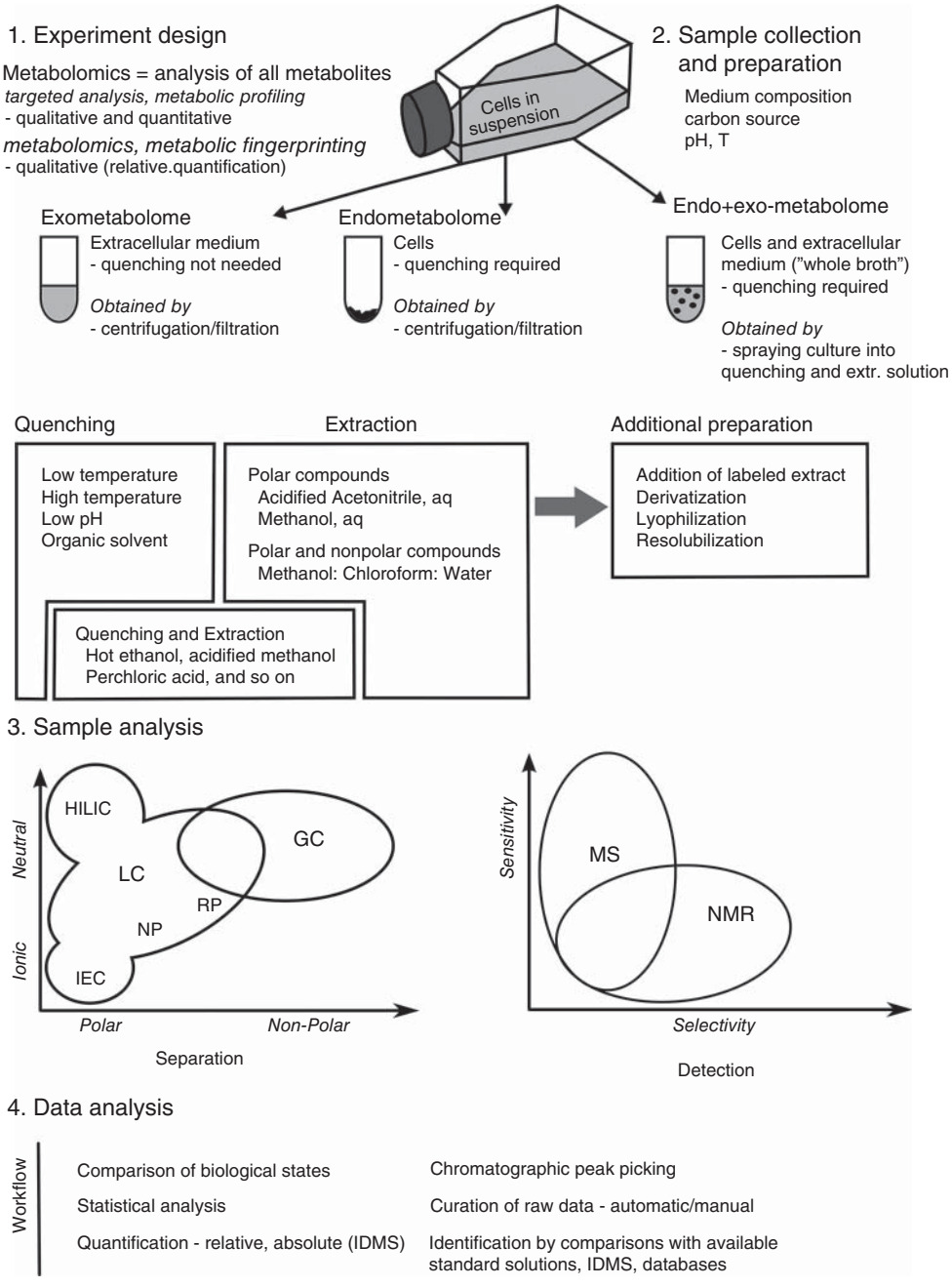


Figure 13.1 Scheme of workflow in metabolomics. Abbreviations: LC, liquid chromatography; GC, gas chromatography; IEC, ion-exchange chromatography; HILIC, hydrophilic interaction liquid chromatography; NP, normal phase; RP, reversed phase; MS, mass spectrometry; and NMR, nuclear magnetic resonance.

samples into liquid nitrogen or cold solvents (e.g., methanol at −60 °C) to thermodynamically block chemical reactions or, on the contrary, in hot solvents (e.g., boiling ethanol, boiling water) to denature enzymes or by directly performing metabolite extraction. Finally, the last step of sampling is metabolite extraction, which depends on both the targeted metabolites and analytical methods. Because of the very different purposes, organisms, samples, metabolites, and analytical platforms considered in metabolomics, a broad range of extraction methods have been developed. Unfortunately, there is no method equally applicable to all metabolites because extraction efficiency depends on the chemical properties of each compound and on cellular characteristics (e.g., membrane composition). Classification of metabolites into polar, nonpolar, acidic, or alkali-stable translates mainly to the extraction method applied. The various extraction methods can yield different and even contradictory metabolic profiles, emphasizing the importance of testing and optimizing the sampling conditions according to the metabolites of interest [24]. Moreover, metabolite sampling is organism- and strain-dependent [20], meaning that methods should be validated for each particular investigation and adapted if needed.

Sampling of *T. brucei* Metabolome

Comprehensive reviews of sampling methods applied to trypanosomatids were published a couple of years ago [25] and only the main aspects are discussed as follows. For cells cultivated *in vitro*, the exometabolome simply refers to the metabolites detected in the cultivation medium and resulting from cellular activity. The culture medium can be sampled by centrifugation or fast filtration. It is usually analyzed by NMR, which allows measurement of both consumption and production of metabolic end products. The media used for cultivation of *T. brucei* often contain buffers that can be incompatible with analytical methods such as HEPES or MOPS that can impair the chromatographic separation of metabolites in LC-MS. The buffers in general should not be an issue for the acquisition of NMR spectra; however, their signals can potentially overlap with the signals of analytes of interest. Significant information about the metabolism of *T. brucei* has been collected by isotopic profiling of the exometabolome, as is illustrated later in this chapter.

Sampling of intracellular components requires separation of media and cells, which can be achieved by centrifugation or fast filtration. Fast filtration has been successfully applied to *T. brucei* PCFs [8, 26, 27]. In this method, the cultivation broth is rapidly filtered and cells on the filter are rapidly washed with cold and diluted medium to maintain the salt and nutrient balances during the sampling process. The presence of the carbon source in the washing solution was also shown to prevent substrate limitation during sampling [28]. The metabolites can be extracted immediately after filtration, although for practical reasons, in most cases they are quenched in liquid nitrogen or other cold solvents before metabolite extraction is performed. Fast filtration takes 4–10 s, which is well adapted for maintaining the pools of fast-turnover compounds such as central metabolites (e.g., intermediates of glycolysis, TCA cycle, amino acid metabolism,

and anabolic pathways). However, metabolite leakage was reported to occur in *T. brucei* BSF during fast filtration or aqueous methanol quenching [25]. An alternative method has been applied to *T. brucei* and *Leishmania* [25, 29, 30] in which cells are separated from medium by centrifugation. In this approach, cells are rapidly chilled in a bath of dry ice and ethanol at 4 °C, centrifuged (5–10 min) at 4 °C, and subsequently, the cell pellet is extracted in methanol:chloroform:water (1 : 3 : 1). This protocol is well adapted for the analysis of metabolites with slow turnover; however, cells might undergo undefined conditions (substrate or oxygen limitation) during centrifugation [31]. So, as for all other organisms, there is currently no absolute method for sampling trypanosomatid metabolome, but different protocols that are adapted to specific objectives. Fast filtration is well adapted to the investigation of central and energy metabolism, meanwhile, centrifugation-based protocols appear better suited for analyzing biosynthetic processes. Finally, various methods have been applied to extract trypanosomatid metabolome. Extraction of polar metabolites, organic acids, and phosphorylated compounds in *T. brucei* PCF has been performed with boiling water [26, 27]. Methanol:chloroform has been used for the simultaneous extraction of both polar and nonpolar metabolites [32]. Extraction of whole *T. brucei* PCF culture medium with boiling ethanol has been used for untargeted metabolome [33]. Nonpolar lipids were reported to be extracted by chloroform:methanol (2 : 1) [34–37]. The broad range of methods applied to trypanosomatids is a direct illustration of the current necessity to adapt metabolite extraction according to the investigated metabolism/metabolites.

Analytical Platforms in Metabolomics

This section provides a brief description of the two main analytical platforms used in metabolomics, NMR and MS. For more details, the reader should refer to comprehensive reviews that have been published in recent years on analytical methods in metabolomics [38], MS [39, 40], and NMR [41]. The choice of the analytical technique(s) depends on instrument availability and on the particular objectives of the investigations.

NMR allows the detection of any atom nucleus with nonzero magnetic moments (^{1}H, ^{13}C, ^{31}P, ^{14}N, etc.), which are called *spins*, by applying a radiofrequency pulse to samples placed in a high magnetic field. The nuclei absorb the energy, excite to high-energy state, and subsequently emit detectable energy in the form of an electromagnetic radiation emitted at a specific frequency. This resonance frequency is altered by electrons surrounding the nuclei in the molecule, hence depends on the chemical environment of the nucleus. For instance, the resonance of a CH_3 group will be different from that of a CH_2 group. This chemical dependence, called *chemical shift*, is scaled according to known chemical references (e.g., trimethylsilyl, TMS, for ^{1}H NMR and ^{13}C NMR) and can be used for identification. Additional structural information about the molecules is obtained from the so-called *scalar coupling*. This phenomenon is

due to the interaction between two or more neighboring spins. It results in the splitting of the NMR resonances into two or more lines, according to the nature and number of spins in interaction. For instance, the resonance of a proton will be a singlet (one line) if it has no proton neighbor, a doublet (two lines) with one proton neighbor, a triplet (three lines) with two proton neighbors, and so on. NMR provides highly detailed chemical information from which the structure of the molecules can be resolved. Furthermore, multidimensional NMR methods can be applied to resolve chemically complex samples – such as those considered in metabolomics – by spreading the signals over 2 (2D-NMR) or 3 (3D-NMR) spectral dimensions. This "magnetic separation" of chemical groups makes possible the resolution of metabolome samples without the need for physically (chromatographically) separating the compounds. Therefore, very often, little sample preparation is needed in NMR, especially for the analysis of biofluids. In principle, all compounds occurring in the sample can be detected by NMR independently of their chemical nature and in a quantitative manner. All these features make NMR very attractive for untargeted (or without a priori) and quantitative approaches in metabolomics, such as metabolic fingerprinting or metabolic profiling, respectively. In practice, NMR has a rather low sensitivity (nanomole range) and allows the detection of predominant metabolites (around 100 species at a maximum). With current developments in NMR technology, however, the sensitivity of instruments is increasing, which provides better metabolome coverage. Since it requires minimal sample preparation, and is very robust, NMR is well suited for high-throughput metabolomics. Finally, NMR is also a valuable method for the detection of stable isotopes and is extensively used for isotopic studies of metabolism as developed further in this chapter.

MS detects ionized molecules and separates them according to the mass/charge (m/z) ratio using a variety of analyzers (quadrupole, time of flight, ion trap, Fourier transform ion cyclotron, etc.). Separation of metabolites in m/z dimension depends on the analyzer resolution, which is defined by IUPAC as the smallest difference ($\Delta\ m/z$) between two ions that can be separated at a given peak height (10%, 50%, etc.). Within routine instruments, one can differentiate between low-resolution (e.g., quadrupoles, with resolution between 1000 and 3000) and high-resolution (e.g., time-of-flight, OrbiTrap, FT-ICR) detectors, with resolution up to 1000 000 (FT-ICR). The various analyzers are applied for different purposes. For instance, quadrupoles are highly linear analyzers and very sensitive in the tandem mode (triple quadrupoles), so that they are preferred to other instruments for quantitative investigations. High-resolution analyzers allow better separation of analytes in the m/z dimension and have become pivotal for the identification of metabolites in complex samples. Mass analyzers can be combined in tandem mass spectrometry (MS/MS) to enable fragmentation of the molecule. The detailed analysis of the generated fragments provides detailed chemical information about the chemical substructures composing the molecule. MS is very sensitive, and modern instruments have detection limits around or below the femtomole range (i.e., six orders of magnitude better than NMR). This allows

the potential detection of hundreds to thousands of compounds; for that reason, MS is a method of choice for gaining the most complete view of the metabolome of a particular organism. Metabolites are mainly identified on the basis of their accurate mass, from which their elemental formula can be determined. Other MS parameters, such as the isotopic cluster, can be used in addition to get a more precise formula. Then chemical databases are searched for potential matches. One accurate mass can correspond to various elemental formulae, and one single formula can correspond to hundreds or thousands of molecules. Moreover, many biological compounds are currently unknown and thus not present in database. Therefore, the unambiguous identification of metabolites from high-resolution MS is extremely challenging and needs complementary information such as MS/MS fragmentation profiles or complementary analytical methods [42]. The metabolomics community is currently making significant efforts to develop identification strategies based on the information collected by high-resolution MS, in parallel with the development of appropriate annotation tools and (bio)chemical databases.

Although there has been significant progress in analyzer technologies, chromatographic separation of metabolites before MS analysis is necessary to resolve the strong chemical complexity of metabolome samples. Separation of metabolites prior to MS detection increases sensitivity and adds information for analyte annotation (e.g., retention time). The prevalent separation techniques for coupling with MS are GC and LC. In GC, the analytes are separated based on their boiling point in a flow of carrier gas (nitrogen, helium, or argon). GC is highly relevant to separate volatile compounds but also low-volatile compounds that can be converted into volatile species after appropriate chemical derivatization. An advantage of GC-MS over LC-MS is the availability of reproducible fragmentation patterns enabling identification from spectral databases. In LC, compounds are separated based on their interaction with a stationary phase. Because a variety of stationary phases is available, LC (and LC-MS) is very flexible and is a method of choice for separating most polar compounds. For highly polar metabolites, such as phosphorylated sugars or ionic compounds, ion-exchange chromatography (IEC) can also be applied [43]. Depending on the purpose of the experiment and instrument availability, the major GC-MS or LC-MS approaches include "targeted metabolomics," that is, the (quantitative) analysis of specific compounds using a highly selective method or "global metabolomics" using a method with broad selectivity. The chromatographic separation of molecules is chemically selective, and so far, there is no universal platform that would allow complete separation of all metabolites, that is, global metabolomics is a challenge. Currently, the best way to maximize the coverage of the metabolome is to combine multiple analytical platforms and methods (a series of "targeted approaches").

It is worthy to note from the brief descriptions of MS and NMR given earlier that the two techniques are very complementary and can be used in combination to increase metabolome coverage in both qualitative and quantitative terms.

Isotopes and Isotopic Profiling

Some basic information about isotopes and nomenclature of isotopic species are given in the following before analytical methods relevant for isotopic profiling are discussed. *Isotopes* are defined as chemical elements that differ only in the number of neutrons, such as ^{11}C, ^{12}C, ^{13}C, and ^{14}C for carbon. Some are stable (e.g., ^{12}C and ^{13}C) and others are radioactive (^{11}C and ^{14}C). Only stable isotopes are further discussed in this chapter. Depending on its chemical formula, a particular molecule can have many different isotopic forms, resulting from all possible combinations of number and positions of isotopes in the molecule. These isotopic forms can be divided into *isotopomers* and *isotopologues.* Isotopomers ("isotopic isomers") are compounds with identical isotopic formula only distinguished by the position(s) of the isotope(s) in the molecule (e.g., an alanine labeled with one ^{13}C atom in position 1 and an alanine labeled with one ^{13}C atom in position 3 are isotopomers). *Isotopologues* are chemically identical compounds that differ in the number of isotopes, for example, incorporation of 0, 1, 2, … , isotopes (e.g., an alanine labeled with one ^{13}C atom and an alanine labeled with two ^{13}C atoms are isotopologues, whatever the positions that receive the label atoms).

By MS, species with different numbers of isotopes – *i.e.* with different masses – can de distinguished, meaning that isotopologues are measured. For a three-carbon compound such as pyruvate or lactate, four ^{13}C-isotopologues can be detected by MS: nonlabeled [M0], partially labeled [M+1], [M+2], and fully labeled [M+3] species (where x in [M+x] stands for the mass excess compared to the unlabeled form [M0]). The position(s) of labeled atom(s) in [M+1] and [M+2] forms cannot be determined directly, although some information about this can be obtained after fragmentation of the molecule. The different isotopologues of the molecule give distinct signals separated by one mass unit, and together with the contribution of other, naturally occurring isotopes (e.g., ^{18}O, ^{15}N), they form a cluster of peaks called *isotopic cluster*. In practice, in ^{13}C-labeling experiments, the ^{13}C-isotopologue distribution (percentage of each ^{13}C-isotopologue) of labeled metabolites is calculated from their isotopic clusters, after correction for naturally occurring isotopes [44].

The most classical approach for MS-based isotopologue analysis is a targeted one and relies on the selective detection of predefined, molecule-specific ions. These ions are selected from preliminary MS analysis of the metabolites of interest. The labeling pattern of the metabolite is extracted from the isotopic cluster measured for its specific ion. For a long period of time, isotopologue analysis has been performed with single quadrupoles. More sensitive and accurate targeted measurement of isotopologues can now be achieved by MS/MS on triple quadrupoles. In that case, the ion (metabolite) of interest is selected in the first quadrupole, fragmented into "daughter" ions in the second one, and a daughter ion chosen for its specificity is selected in the third one. This detection mode offers both improved specificity and sensitivity, although determining the labeling pattern of the metabolite from that of its daughter ions is not straightforward [43]. Besides targeted approaches, untargeted approaches

can be applied. In these approaches, the complete MS spectrum is collected, which potentially allows the measurement of isotopologues for all detectable compounds. Low-resolution MS detectors are not well suited for such untargeted approaches, and until recently, the low sensitivity and quantitative inaccuracy of high-resolution instruments made them poorly suited for (quantitative) isotopic profiling. Modern high-resolution MS instruments have become both more sensitive and more quantitatively accurate, enabling the emergence of powerful untargeted isotopic profiling methods. The first reports of such investigations, which include the study of *T. brucei* PCFs by Creek *et al.* [45], emphasize their huge potential for gaining detailed insights into metabolic networks and their operation.

NMR allows direct determination of isotope position in labeled metabolites, so that this method allows the measurement of isotopomers [46, 47]. For instance, a three-carbon molecule will have three distinct signals in ^{13}C NMR spectra. The intensity of each signal is proportional to the number of ^{13}C atoms, so that the ^{13}C-content of each position can be measured individually. This is usually expressed as "specific" or "positional" enrichment, that is, percentage of ^{13}C atoms in a specific position of the molecule. Specific enrichments can be also measured by ^{1}H NMR by exploiting scalar couplings between ^{1}H and ^{13}C nuclei ("heteronuclear couplings"). Because ^{1}H NMR is much more sensitive than ^{13}C NMR, the indirect detection by ^{1}H NMR is usually preferred to direct detection by ^{13}C NMR, except for quaternary carbons that have no proton coupling. Similarly, the exploitation of scalar couplings between two or more adjacent ^{13}C atoms ("homonuclear couplings") allows for measuring the ^{13}C–^{13}C connectivity in the molecule and distinguishing the various isotopomer species. For instance, by NMR, alanine doubly labeled in positions 1 and 2 can be easily distinguished from alanine doubly labeled in positions 2 and 3. As for the structural resolution of complex metabolite samples, multidimensional NMR experiments have been developed to measure either specific enrichments or isotopomers without need for compound separation. For this reason, NMR provides a rich and highly flexible method to measure positional isotopic information, with the benefit that such positional information very often encodes directly active pathways.

Application of Metabolomics to *T. brucei* – Selected Illustrations

In this section, selected applications of metabolomics to trypanosomatids are given to illustrate the interest of these approaches for getting comprehensive understanding of the unique metabolic traits of these organisms. The examples are taken from *T. brucei*, for which significant metabolic knowledge has recently been gathered using metabolomics. More specifically, considerable progress in the identification of metabolic pathways in *T. brucei* was achieved by applying ^{13}C isotopic profiling strategies to mutants impaired for specific enzymes by means of gene silencing (RNAi mutants) or deletion (KO mutants). Table 13.1

Table 13.1 Applications of isotopic profiling to *T. brucei* procyclic forms.

Metabolic perturbation	Sample and sampling protocol	Analytical platform	(Labeled) carbon source	References
PPDK, PYK	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C NMR	D-[1-^{13}C]glucose	[48]
PPDK, PEPCK, PGKC	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR	D-[U-^{13}C]glucose D-[1-^{13}C]glucose L-Threonine (*NE*)	[49]
FRDg	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR	D-[1-^{13}C]glucose	[50]
FRDg, FRDm1	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR	D-[1-^{13}C]glucose	[51]
FHm, FHc	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C NMR	D-[1-^{13}C]glucose	[52]
FRD(g, m1), FH(c,m), SDH, PEPCK, PDH, PYK, ASCT, ME(c, m), F_0/F_1-ATP synthase	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR	D-[1-^{13}C]glucose L-[4-^{13}C]proline	[53]
CL, AceCS, ASCT	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C NMR	D-[1-^{13}C]glucose	[34]
AAT	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C NMR	L-[4-^{13}C]proline	[54]
ASCT	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR	D-[1-^{13}C]glucose	[55]

(*continued overleaf*)

Table 13.1 (*continued*)

Metabolic perturbation	Sample and sampling protocol	Analytical platform	(Labeled) carbon source	References
PDH, TDH, ACH, ASCT, PEPCK	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_2O	^{13}C, ^{1}H NMR HILIC-MS	D-[U-^{13}C]glucose Threonine (NE) [^{15}N]Threonine	[35]
ASCT, ACH, β subunit of F_0/F_1-ATP synthase	Exometabolome; medium and cells separated by centrifugation, no quenching, NMR analysis of supernatant carried out in D_2O	^{1}H NMR	Glucose (NE)	[36]
TKT	Endometabolome; cells collected by fast filtration, quenching in liquid N2, and extraction in boiling water	IC-MS/MS	Glucose (NE)	[8]
MEc, G6PDH, PGI	Endometabolome; cells collected by fast filtration, quenching in liquid N2, and extraction in boiling water	IC-MS/MS	[U-^{13}C]proline ± glucose (NE)	[26]
PEPCK, SDH, β subunit F_0/F_1-ATP synthase, FAD-GPDH, PDH	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_20	^{13}C NMR	D-[1-^{13}C]glucose	[27]
	Endometabolome; cells collected by fast filtration, quenching in liquid N2, and extraction in boiling water	IC-MS/MS	L-[4-^{13}C]proline	
ACO	Exometabolome; medium collected by centrifugation, no quenching, NMR analysis carried out in D_20	^{13}C NMR Scintillator	D-[U-^{13}C]glucose D-[6-^{14}C]glucose L-[U-^{14}C]proline	[56]
Wild-type	Endometabolome; cells collected by centrifugation, quenching in cold ethanol, and extraction in chloroform: methanol:water (1 : 3 : 1)	IC-MS	D-Glucose L-Proline (separate)	[29]
Wild-type	Endometabolome; cells collected by centrifugation, quenching in cold ethanol, and extraction in chloroform: methanol:water (1 : 3 : 1)	LC-MS	[U-^{13}C]glucose (50%)	[45]

Abbreviations of enzymes: See Figure 13.2. NE, nonenriched carbon source.

provides a list of these investigations, although probably not exhaustive. To help the reader in following the metabolic processes discussed next, a schematic representation of the current view of *T. brucei* central carbon metabolism is given in Figure 13.2. For simplicity and consistency reasons, the examples are taken from the investigation of *T. brucei* PCFs.

Exometabolomics and Succinate Fermentation

The analysis of the exometabolome provides detailed information about the compounds that are either consumed or produced by cells. It gives the global result of cellular metabolic activity since which compounds are used by the cells and into which products they are converted into can be measured. *T. brucei* PCFs convert glucose into succinate and acetate as major end products while alanine, lactate, and malate as the minor ones [27, 35]. Succinate is an intermediate of TCA cycle, and all enzymes of this pathway can be detected in PCFs [57]. Together with the fact that PCFs have a functional ATP synthase coupled to aerobic respiration, this suggested that pyruvate was oxidized in the mitochondrion to fulfill the cells' energetic needs by oxidative phosphorylation. It also suggested a mitochondrial origin of succinate, which is produced from succinyl-CoA in the TCA cycle. However, succinate could also be produced from fumarate via a controversial NADH-dependent fumarate reductase (FRD) activity, described in *T. brucei* PCFs [58, 59]. In 2002, Besteiro *et al.* investigated the metabolic origin of succinate in PCFs by applying isotopic profiling of the exometabolome to the study of relevant mutants [50]. First, these authors established the glycosomal localization of FRD (accordingly called FRDg (fumarate reductase glycosomal)) and generated mutants in which the activity of the enzyme was switched off by using an RNA interference (RNAi) strategy. Then, they incubated PCFs with [1-^{13}C]-labeled glucose and analyzed the nature and labeling patterns of metabolic end products in the medium using ^{13}C-NMR. In FRDg-depleted parasites, succinate production was decreased by 60% compared to wild-type parasites, whereas a significant increase in both fumarate and malate was observed, indicating that most of the succinate was produced via FRDg. Moreover, detailed analysis of the labeling patterns of the metabolic end products measured by ^{13}C-NMR provided evidence that the TCA cycle could not be responsible for the production of the remaining succinate. Briefly, the glycolytic degradation of [1-^{13}C]-glucose generates [3-^{13}C]-pyruvate, which can be further converted into [2-^{13}C]-acetyl-CoA to feed the TCA cycle. The singly labeled acetyl-CoA entering the TCA cycle produces doubly – or more – labeled species after several turns of the cycle. Given the labeling pattern measured for acetyl-CoA, it was calculated that 15% of succinate molecules should be doubly labeled at both C2 and C3 ([2,3-^{13}C2]-succinate) if a complete operation of the TCA cycle was assumed. As mentioned earlier, NMR allows unambiguous discrimination of singly and doubly labeled species. But Besteiro *et al.* could not observe doubly labeled succinate, but only singly labeled forms, in the exometabolome of PCFs incubated with [1-^{13}C]-glucose,

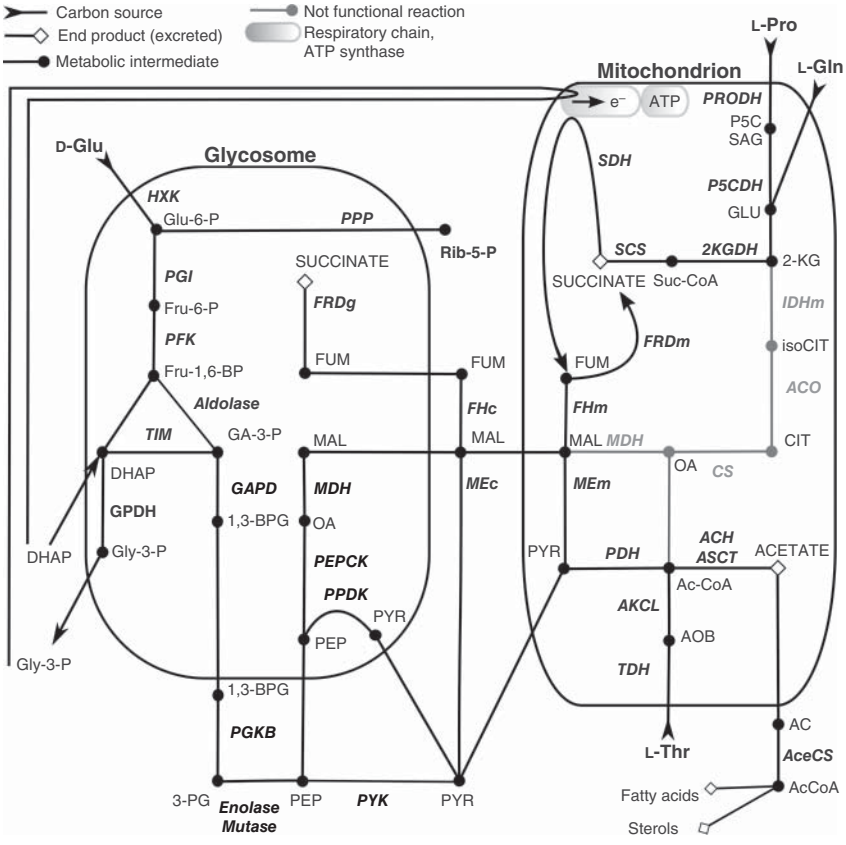


Figure 13.2 Schematic representation of central carbon metabolism in procyclic *T. brucei*. Each node represents a metabolite; direction of reactions in glycolytic or gluconeogenetic flux is not indicated. Abbreviations of enzymes (indicated in bold italic): HXK, hexokinase; PGI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; TIM, triosephosphate isomerase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; PGKB, phosphoglycerol kinase (isoform B); PYK, pyruvate kinase; PPDK, pyruvate phosphate dikinase; PEPCK, phosphoenolpyruvate carboxykinase; MDHg, malate dehydrogenase; FHg/FHc, fumarate hydratase glycosomal/fumarate hydratase cytosolic; FRDg/FRDm, fumarate reductase glycosomal/fumarate reductase mitochondrial; SDH, succinate dehydrogenase; MEc/MEm, malic enzyme cytosolic/malic enzyme mitochondrial; PDH, pyruvate dehydrogenase complex; ACH, acetyl-CoA thioesterase; ASCT, acetyl:succinyl-CoA transferase; CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase, 2KGDH, 2-ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; PRODH, proline dehydrogenase; TDH, threonine 3-dehydrogenase; AKCL, 2-amino-3-ketobutyrate-coenzyme A ligase. Abbreviation of pathway: PPP, pentose phosphate pathway. Abbreviations of compounds: D-Glu, D-glucose; L-Pro, L-proline; L-Thr, L-threonine; L-Gln, L-glutamine; Glu-6-P, glucose-6-phosphate; Rib-5-P, ribose-5-phosphate; Fru-6P, fructose-6-phosphate; Fru-1,6-BP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MAL, malate; FUM, fumarate; SUC, succinate; AC, acetate; CIT, citrate; isoCIT, isocitrate; 2-KG, 2-ketoglutarate; GLU, glutamate; SAG, glutamate semialdehyde; and AOB, amino-oxobutyrate; GPDH, glycerol-3-phosphate dehydrogenase.

showing that the TCA cycle was unlikely to be operating as a complete cycle in *T. brucei* PCFs [50]. One year later, van Weldeen *et al.* confirmed this hypothesis by demonstrating that deletion of the aconitase gene, encoding a TCA cycle enzyme, does not affect the central metabolism of the parasite [56], although several branches of the cycle can be used upon utilization of glucose or proline [52, 60].

The complete pathway by which glucose is converted into succinate via FRDg was further established (Figure 13.2). Glucose is converted into 1,3-biphosphoglycerate in the glycosomes, and then this metabolic intermediate is converted to phosphoenolpyruvate (PEP) in the cytosol. PEP then enters the glycosomes to be converted into oxaloacetate (OAA) by phosphoenolpyruvate carboxykinase (PEPCK) [49]. Subsequently, OAA is converted by a glycosomal malate dehydrogenase (MDHg) into malate, which is dehydrated into fumarate by a cytosolic fumarate hydratase (FHc) [52], and the latter intermediate is reduced to succinate in the glycosomes by FRDg [50]. The entire process is a fermentation, called *glycosomal succinate fermentation*, and enables the reoxidation of glycolytic NADH into NAD^+ within the glycosome by two enzymes, MDHg and FRDg [50]. In addition, the glycosomal succinate fermentation is used to maintain the glycosomal ATP/ADP ratio through the PEPCK reaction, together with the pyruvate phosphate dikinase (PPDK) reaction (conversion of PEP into pyruvate with production of ATP) [49]. Following the work of Besteiro *et al.*, it was later demonstrated that a mitochondrial form of NAD-dependent FRD (FRDm1) was present and active in PCFs in addition to the glycosomal form (FRDg) [51]. These investigations showed that *T. brucei* PCFs possess two alternative processes for succinate production, one in the glycosome and the other one in the mitochondrion. The pathway by which glucose is converted into mitochondrial succinate includes the same initial reactions as in glycosomal succinate fermentation up to the glycosomal formation of malate (Figure 13.2). But instead of being converted into succinate inside, the glycosomes malate (or fumarate not yet experimentally confirmed) enters the mitochondrion to be dehydrated to fumarate by a mitochondrial fumarate hydratase (FHm) and/or reduced into succinate by FRDm1. As discussed for the glycosomal succinate fermentation, the mitochondrial production of succinate is likely to be involved in the maintenance of redox balance by oxidation of NADH via FRDm1. By applying the same approach as Besteiro *et al.*, that is, isotopic profiling of the exometabolome of selected FRD mutants incubated with ^{13}C-enriched glucose, Coustou *et al.* could determine that the two enzymes FRDg and FRDm1 accounted for the total production of succinate, with FRDg contributing to 56–86% and FRDm1 to 14–44% of total excreted succinate [51]. These data show that the contribution of the mitochondrial process is significant. The reaction catalyzed by FRDm1, NADH-dependent reduction of fumarate into succinate, is opposite to the FAD-dependent oxidation of succinate into fumarate catalyzed by SDH in the TCA cycle. Since one may consider that two opposite reactions could not operate simultaneously in the same cellular compartment, the occurrence and significant activity of FRDm1 could be an additional argument against the operation of a complete TCA cycle in PCFs. This is consistent with proline-derived succinate

being excreted in the presence of glucose, suggesting that succinate is not further metabolized into fumarate by SDH [53]. However, Turrens *et al.* proposed that the mitochondrial NADH-dependent FRD and SDH form a cycle transferring electrons from NADH to the quinone pool of the respiratory chain, through the intermediate fumarate, and may constitute a NADH-dehydrogenase-like activity [58]. The occurrence of this hypothetical FRDm1/SDH cycle has not been demonstrated so far in trypanosomatids or any other organism. This example is an early demonstration of the benefit of (exo-)metabolomics for the understanding of *T. brucei* metabolism. First, it has established a valuable experimental strategy for investigating their metabolism by combining metabolomics with the study of mutants impaired for specific enzyme(s). This strategy was further developed and has very significantly improved our knowledge of trypanosomatid metabolism (Table 13.1). Second, this approach was helpful to determine the enzymatic reactions involved in the maintenance of the NAD^+/NADH and ATP/ADP ratios inside the organelle [27, 49, 50] since the glycosomal membrane is not permeable to large molecules, such as nucleotides and cofactors [5]. Being a very unique feature of trypanosomatids, glycosomes have been the subject of intense experimental research since their discovery. All studies initially focused on determining why these organisms encapsulate glycolysis in a dedicated physical compartment [5]. It was initially thought to allow high glycolytic flux by packing the glycolytic enzymes in very small volume [61] or to protect from the particular design of glycolysis, which can cause growth defect [62]. It is now believed that glycosomal glycolytic sequestration is used to provide a high level of metabolic flexibility, by rapidly changing the glycosomal content through autophagy in response to environmental changes [63]. The occurrence of two alternative pathways for succinate production is certainly part of the metabolic flexibility of trypanosomatids.

Endometabolomics, Redox Balances, and Gluconeogenesis

The example given earlier shows the benefit of exometabolomics for the understanding of *T. brucei* metabolism, not only to resolve the metabolic pathways that operate but also to provide valuable insights into the vital redox processes. Indeed, the two succinate fermentation mechanisms occurring in *T. brucei* PCFs are involved in the maintenance of NAD^+/NADH balances. Some valuable insights into the methods of NADPH balancing, but also into gluconeogenesis, were resolved by applying isotopic profiling to the *T. brucei* endometabolome (i.e., intracellular metabolites). The production of NADPH is essential for biosynthetic processes and oxidative stress defense. The possible sources of NADPH in the cytosol of *T. brucei* are the PPP and malic enzymes. The PPP appears to be essential in BSFs [64], but not in PCFs, suggesting different mechanisms for NADPH balancing in the two parasite forms. In 2013, Allmann *et al.* [26] investigated the metabolism of cytosolic NADPH in mutants impaired for glucose-6-phosphate dehydrogenase (G6PDH) activity, the first committed step of PPP, or impaired for the cytosolic malic enzyme (MEc). When PCFs were cultivated in glucose-rich

medium, no significant growth alteration was observed in mutants impaired for only one of the two enzymes. In contrast, the double mutant impaired for both enzymes died after a few days of cultivation. These observations showed that the two NADPH-producing processes have a redundant function in glucose-rich conditions. This redundancy may compensate in trypanosomatids for the lack of a transhydrogenase, which catalyzes the reversible conversion of NADH into NADPH in other organisms. Based on their data, the authors propose the occurrence of a NADH/NADPH converting cycle (or transhydrogenase-like shunt) in *T. brucei* PCFs [26]. In this cycle, electrons are transferred from NADH to malate by MDHg in the glycosomes, and then malate transfers back the electrons to NADPH either in the cytosol via MEc or in the mitochondrion via MEm (malic enzyme mitochondrial). The pyruvate released by the MEs is then recycled to glycosomal PEP via PPDK, and then to OAA via PEPCK (Figure 13.2), to complete the NADH/NADPH cycle. By this process, there is a net transfer of electrons from glycosomal NADH to either cytosolic NADPH or mitochondrial NADPH.

Besides the transhydrogenase-like cycle, an intriguing observation of Allmann *et al.* was that the redundancy of PPP and MEc for NADPH production was also observed when PCFs were grown in glucose-depleted medium that contained proline [26], which is closer to the conditions found in the insect digestive tract [65]. The contribution of malic enzyme to cytosolic NADPH production upon growth on proline was expected, since malate was a known product of proline metabolism in PCFs. The contribution of the PPP to NADPH production in glucose-depleted medium was more surprising. It requires a flux through the PPP in the absence of glucose, suggesting the occurrence of gluconeogenesis from proline, a process that was not reported to occur in *T. brucei.* To investigate such hypothesis, Allmann *et al.* incubated PCF cells with [U-^{13}C]-proline and measured the incorporation of label into intermediates of glycolysis and PPP. As emphasized earlier, MS is a very sensitive analytical method and allows the isotopic profiling of intracellular metabolites. For these experiments, the endometabolome of PCFs was collected in a few seconds by fast filtration of cells, which is appropriate for glycolytic and PPP intermediates that have high turnover rates. The metabolites were separated by ionic-exchange chromatography, a method that is particularly well suited for separation of highly polar compounds such as sugar phosphates and phosphorylated organic acids. The distribution of ^{13}C-isotopologues was measured by MS/MS on a triple quadrupole to ensure accurate quantitative measurement of labeling patterns. Using such approach, Allmann *et al.* could measure that the incorporation of label into glycolytic and PPP intermediates from [U-^{13}C]-proline was very low in the presence of (unlabeled) glucose [26]. In contrast, very significant label was incorporated from [U-^{13}C]-proline into these compounds in the absence of glucose. The fraction of labeled molecules was above 85% for all measured glycolytic intermediates. These data provided direct evidence that proline can be used by gluconeogenesis in PCFs and hence can feed the PPP for NADPH production. The redundancy of cytosolic NADPH-producing processes is a further illustration of the strong metabolic flexibility of *T. brucei*, since they allow adaptation to diverse carbon sources and environmental conditions.

Concluding Remarks

The application of metabolomics to trypanosomes has not only provided novel and valuable insights into their metabolism, it has also emphasized the strong metabolic flexibility of these organisms. So far, investigations have mainly focused on the central carbon metabolism, in order to resolve the highly unusual metabolic features of these organisms. A challenge is now to decipher the complete, that is, cellular wide, metabolic network of *T. brucei* and, more generally, trypanosomatids. In this respect, the untargeted isotopic profiling approach developed by Creek *et al.* is very promising for system-wide elucidation of *T. brucei* metabolism [45]. Based on high-resolution MS, these authors could detect a large number of labeled metabolites after addition of 50% [U-^{13}C]-glucose to cultivations of *T. brucei* PCFs. From the detailed analysis of the metabolites and their labeling patterns, they could assign the biosynthetic routes of many of the detected compounds, thereby building up parts of the PCF metabolic puzzle. They could also identify a new compound, octulose-phosphate, as part of the PPP. Together with the recent availability of the genome-scale metabolic network of *T. brucei* [66], this approach is likely to accelerate significantly the progress on the parasite metabolism, as well as the identification of novel drug targets. Besides metabolic reconstruction, a second challenge for the future is to get more quantitative understanding of the operation of metabolism in trypanosomatids. Measurement of metabolic fluxes can be achieved from isotope-labeling experiments with the help of relevant mathematical models [67]. The strong compartmentation of *T. brucei* metabolism, as well as the occurrence of duplicate reactions, currently makes metabolic flux analysis in this organism a true challenge. Recent mathematical developments as well as the application of time-course labeling experiments might help for this purpose. This approach will allow for getting more detailed information about the operation and regulation of *T. brucei* metabolism. They will also represent a valuable tool for measuring the actual impact of drugs and drug candidates on their metabolism and growth capabilities.

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14
The Role of Metabolomics in Antiparasitic Drug Discovery

*Carlo R. Giannangelo, Katherine M. Ellis, Anna E. Sexton, Daniel Stoessel, and Darren J. Creek**

Abstract

Metabolomics involves the global measurement of small molecule metabolites in a biological system and allows the comprehensive investigation of metabolic pathways in pathogenic parasites. Metabolomics studies have enabled the discovery of novel aspects of parasite metabolism that constitute attractive drug targets, and have elucidated metabolic targets of antiparasitic compounds. This chapter provides an introduction to parasite metabolomics and describes metabolomics methods suitable for kinetoplastid and apicomplexan parasites. Several examples are provided describing applications of metabolomics to understand the mechanisms of action and resistance for a range of antiprotozoal compounds. This unbiased elucidation of drug mechanisms with metabolomics will facilitate the discovery and development of new drugs for parasitic diseases of global importance.

Introduction

Protozoan parasites are a diverse group of eukaryotic pathogens that are responsible for a variety of life-threatening infections in humans, including malaria (*Plasmodium* spp.), African Trypanosomiasis (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and Leishmaniasis (*Leishmania* spp.). These diseases primarily affect tropical and subtropical regions of the world and cause significant morbidity and mortality as well as exacerbate poverty and economic hardship in developing countries. More than 1 million deaths occur annually as a result of protozoan-related diseases [1], and approximately 3.8 billion people are threatened by the risk of infection each year [2].

Control and prevention of these parasitic diseases are heavily reliant on antiprotozoal drug therapy, as there are currently no safe and highly effective vaccines. Unfortunately, the currently used antiprotozoals are severely limited

*Corresponding author.

Comprehensive Analysis of Parasite Biology: From Metabolism to Drug Discovery,
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by liabilities including poor efficacy, drug resistance, toxicity, high cost, and inadequate pharmacokinetic properties. Exacerbating this problem, the majority of antiprotozoal drugs currently in clinical use act by as-yet-unknown modes of action. This impacts our ability to optimize treatment strategies, understand parasite resistance mechanisms, and monitor drug efficacy and toxicity. In the context of these liabilities and the unlikely prospect of effective vaccines for human protozoan parasites in the near future, there is an urgent need for the discovery of new drugs that are safe, effective, and act by novel mechanisms. Despite this necessity, there has been an ongoing failure to invest into basic research and development for these diseases [3, 4]. Consequently, there are significant gaps in our fundamental understanding of the biology of these pathogens [4] and a paucity of new therapeutic products and new chemical entities being approved for the treatment of these tropical diseases [5].

The successful development of tropical medicines faces unique challenges. The ideal drug should be cheap to manufacture and distribute, be orally active, and require only once daily administration or result in a single dose cure. Furthermore, complexities in parasite biology must also be considered, such as latent parasite forms, the location of parasites within host cells, drug efflux mechanisms, and the existence of multiple life-cycle stages. Unfortunately, there is a dearth of validated molecular targets for parasitic diseases due to the lack of detailed information concerning the targets of currently used antiprotozoal drugs. These issues represent major obstacles for the success of drug discovery efforts in parasitic diseases [6].

Thousands of antiparasitic hit compounds have been identified in recent years, following the application of phenotypic screening of large compound libraries against whole organism systems [7–9]. Lead compounds that emerge from these screening programs inherently possess favorable drug-like characteristics, including cell permeability and inhibition of essential targets, which contribute to the success of this approach. The trypanocidal compounds fexinidazole and SCYX-7158 [3] and the antimalarial compounds KAE609 [10], KAF156 [11], and MMV390048 [12] have all advanced to preclinical and clinical trials after emerging from phenotypic screening programs. The phenotypic screening approach, however, forgoes mechanistic information relating to drug mode of action. As such, the elucidation of drug targets and mechanistic information from hits derived from these screens is now a primary goal [13]. An untargeted approach aimed at discerning drug modes of action, which does not rely on *a priori* knowledge of a drug's mechanism of action, will greatly improve the drug discovery process for parasitic diseases by informing rational drug design, and improve our existing understanding of the medicines currently in clinical use [14]. Untargeted metabolomics is an emerging discipline that offers a rapid, unbiased, and system-wide approach for the investigation of parasite metabolic pathways, the elucidation of drug modes of action, and the discovery of likely drug targets.

Principles of Metabolomics

The field of metabolomics is the branch of omics technology concerned with the high-throughput identification and quantification of low-molecular-weight molecules (<1500 Da) in a biological system [14–16]. The complement of small molecules in a system, known as the *metabolome*, includes a range of chemically diverse species, of varying abundance, that are produced through the interactions of larger macromolecules, including DNA, RNA, and proteins. Its close relationship with the phenotype makes metabolomics ideal for assessing the physiological state of an organism or system at a snapshot in time. Consequently, metabolomics has found applications in a diverse range of fields, such as the nutritional sciences [17], personalized medicine [18], toxicology [19], and biomarker discovery for disease diagnosis and monitoring in a variety of cancers [20–22], cardiovascular diseases [23–25], and neurological conditions [26, 27]. Metabolomics is a promising tool for antiparasitic drug discovery, and it has already demonstrated utility in the validation of known targets and the discovery of new drug targets for a range of diseases.

Metabolomics in Drug Discovery

Perturbations to metabolic pathways underpin many disease-state processes, and the identification of enzyme targets within these pathways may represent novel sites for therapeutic intervention. The application of metabolomics to cancer has provided further insight into potential drug targets in energy production pathways within cancer cells, which rely on aerobic glycolysis in a process known as the *Warburg effect* [28].

As untargeted metabolomics provides a global measurement of metabolic alterations within a system, this unbiased methodology can reveal changes to novel or unexplored biochemical pathways, offering a distinct advantage over traditional enzyme inhibition assays. This approach has been applied to neuropathic pain models in rats, which revealed dysregulation of sphingomyelin–ceramide metabolism [29], with the metabolite *N*,*N*-dimethylsphingosine (DMS) highly abundant in rats with neuropathic pain. In this case, metabolomics has revealed a previously unexplored target for therapeutic intervention, as inhibition of DMS production could serve as a potentially attractive therapeutic option for neuropathic pain [29].

In response to viral infection, metabolomics has been used to identify the change in abundance of a number of key metabolites *in vitro*. Human fibroblasts infected with influenza A virus (IVA), herpes simplex virus type-1 (HSV-1), and human cytomegalovirus (HCMV) revealed significant metabolic perturbations that were consistent with the pharmacology of known antivirals and provided insight into possible new antiviral targets [30]. IVA-infected fibroblasts showed a significant upregulation of acetylneuraminic acid, consistent with the mechanism

of action of the anti-influenza drugs such as oseltamivir, which selectively inhibit viral neuraminidase. In the case of HSV-1, changes in the 2′-deoxynucleotides, dTTP and dTMP, were seen, which is consistent with the mechanism of action of HSV-1 treatments such as acyclovir that inhibit viral thymidine kinase. Combined with metabolic flux analysis, metabolomics on HCMV-infected cells revealed changes to tricarboxylic acid (TCA) cycle intermediates and flux through fatty acid biosynthesis, via the enzyme acetyl-coenzyme A carboxylase, as the most significant perturbations [30]. This enzyme is not a current antiviral target but may represent a useful target for future intervention for the treatment of HCMV [30].

Understanding the targets of anti-infective drugs currently in use and those in the developmental pipeline is invaluable for informing rational drug design and overcoming resistance. The antimicrobial triphenylbismuthdichloride was found to inhibit the bacterial pyruvate dehydrogenase complex in a metabolomics investigation in *Staphylococcus aureus* [31]. The identification of this enzyme as an effective drug target now offers new hope in the potential treatment of multidrug-resistant bacterial pathogens [31].

In the field of antiprotozoal drug discovery, metabolomic analysis of sensitive and resistant parasites that have been treated with antiprotozoal compounds can detect drug-induced perturbations to specific parasite metabolites and pathways, offering a global, unbiased approach to discovery of potential drug targets as well as mechanisms of drug action and resistance [14, 28]. It also offers a system-wide approach to analyze parasite biochemistry and host–parasite interactions, leading to the identification of essential parasite pathways and novel enzymes that can be targeted for therapeutic intervention. This review discusses current metabolomics methodology, the application of metabolomics to antiparasitic drug discovery and summarizes the impact of antiprotozoals on kinetoplastid and apicomplexan parasite metabolism.

Metabolomics Methodology

Protozoan parasites contain complex mixtures of thousands of small molecules that vary greatly in their chemical properties and abundance. The nature of the metabolome is highly dependent on both the genome and the environment and is unique for different stages of the parasite life cycle. Therefore, metabolomics analysis requires a validated and highly robust methodology (Figure 14.1) to reproducibly measure the most comprehensive range of metabolites from each sample, in order to obtain the most reliable approximation of the organism's physiology under defined conditions [32, 33].

Study Design

The design of metabolomics experiments can vary widely depending on the aim of the study. A truly untargeted metabolomics approach is suitable for

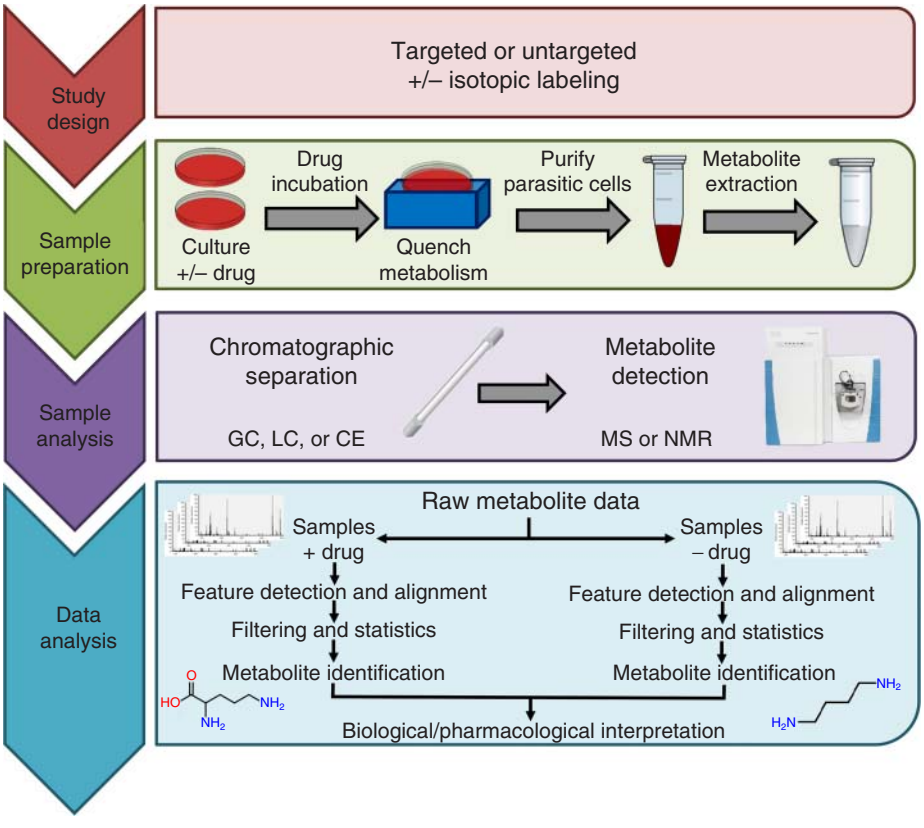


Figure 14.1 General metabolomics workflow for *in vitro* drug mechanism of action studies using protozoan parasites. Parasites are grown in culture and incubated with the test compound for the desired duration. Metabolism is then quenched by rapid cooling, and the parasitic cells may be isolated before metabolite extraction using organic solvent. Metabolite extracts are then analyzed using an appropriate detection method such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) after chromatographic separation with either gas chromatography (GC). liquid chromatography (LC) or capillary electrophoresis (CE) after chromatographic separation. The raw metabolite data must then be processed to determine specific drug-induced metabolic perturbations responsible for parasite death.

hypothesis-free studies, such as the unbiased discovery of drug targets for novel compounds or clinical biomarker discovery. While it is not possible to measure every metabolite in a single experiment, the extraction and analytical conditions can be optimized to detect a wide range of metabolites with diverse chemical properties, and this often involves a combination of analytical techniques and data processing approaches [28, 34]. An alternative approach is to apply targeted metabolomics, whereby a predefined range of metabolites or pathways are analyzed. This approach is suitable for hypothesis-testing experiments, allows

improved sensitivity and quantification accuracy, and may be combined with stable isotope labeling to perform flux calculations [14, 35, 36].

The metabolic response of parasites to drug treatment can be measured in *in vitro* or *in vivo* systems, although host factors may complicate biochemical interpretation in the latter. *In vitro* cell culture systems allow metabolism to be studied in a controlled environment, which is important to delineate pharmacological effects from background variations in parasite metabolism. The choice of growth media is an important consideration, as nutrient availability can interfere with drug action or metabolic response, as demonstrated for the trypanocidal activity of antifolate drugs [37]. Drug incubation times and concentrations can be tuned to investigate primary and secondary effects of drugs on metabolism, and it is generally desirable to obtain samples before a generalized death phenotype is apparent. For optimal statistical power, it is important to have a minimum of four separate biological replicates, and the inclusion of time-series analysis can add further confidence and insight into temporal responses [38].

Sample Preparation

Accurate analysis of intracellular metabolism requires rapid quenching of cell metabolism to minimize the introduction of unwanted variance during sample preparation. Lowering the sample temperature to 0 °C is the most common quenching method for protozoa. Alternative quenching procedures developed in other microbes, such as filtration or freezing in cold methanol, are generally unsuitable for protozoan parasites due to metabolite leakage (DJ Creek and MP Barrett).

Once metabolism has been quenched, parasites may be washed and/or isolated from host cells, followed by extraction of metabolites and removal of macromolecules. Organic solvent extractions are generally effective and compatible with downstream mass spectrometry analysis (Table 14.1).

Sample Analysis

The combination of chromatographic separation paired to mass spectrometry (MS) allows for the most comprehensive analysis of highly complex metabolomics samples. Other techniques including Nuclear Magnetic Resonance (NMR), Fourier Transform Infra-red (FTIR), and Raman spectroscopy may also be applied, albeit with lower metabolite coverage.

Mass spectrometry provides sensitive detection of a wide range of metabolites, and the simultaneous identification of hundreds of metabolites has been facilitated by advances in high-resolution mass spectrometry (Time-of-Flight, Orbitrap, and FT-ICR). Sensitive detection and identification of metabolites are further improved by coupling MS to chromatographic separation techniques, such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE). GC produces high-resolution and reproducible separation

Table 14.1 Metabolomic quenching and extraction techniques for *Plasmodium* and *Trypanosomatid* parasites.

Parasite	Life-cycle stage	Quenching method	Extraction method
Plasmodium spp.	Blood stage	Not quenched	90% methanol [39]
	Blood stage	4 volumes of 100% methanol at −70 °C	80% MeOH on dry ice [40]
	Blood stage	Not quenched	100% MeOH then MeOH:H_2O 8 : 2 plus sonication [41]
	Saponin extracted	Frozen −80 °C	Chloroform:MeOH:ACN 2 : 1 : 1 plus steel beads, then TissueLyser [42]
	Various blood stages	Not quenched	100% MeOH on dry ice, then 80% EtOH with sonication [43]
	Blood stage	Methanol/water/ chloroform 4 : 3 : 1	Biphasic liquid–liquid extraction at pH-adjusted solvents [38]
	Blood stage	Rapid cooling in dry ice ethanol bath	Chloroform:methanol:water 1 : 3 : 1 shaking 1 h [44]
	Blood stage	Ice-cold PBS	80% ACN [45]
Trypanosomatids	Blood stage	Rapid cooling in dry-ice ethanol bath	Chloroform:methanol:water 1 : 3 : 1 shaking 1 h [46, 47]
	Blood stage	Not quenched	80 °C ethanol 2 min [48, 49]

and is ideal for volatile compounds, and has been applied to the sensitive detection of *Plasmodium*-infected blood [50]. However, nonvolatile compounds require derivatization, resulting in low throughput, sensitivity, and reproducibility [51].

LC methods are readily coupled to high-resolution MS and are ideal for the analysis of a wide range of soluble metabolites. The availability of diverse LC column chemistries allows the analytical method to be optimized for certain metabolite classes [14, 51].

Reversed-phase chromatography is the most common form of chromatography applied to global metabolite profiling studies. This method is highly robust and provides extensive coverage of lipids and nonpolar secondary metabolites, but is not appropriate for the analysis of polar compounds, which constitute the majority of metabolic intermediates in the essential central metabolic pathways.

The addition of ion-pairing reagents can improve retention and resolution of polar metabolites; however, these are often not compatible with MS detection. Hydrophilic interaction chromatography (HILIC), ion chromatography (IC), and CE are well suited for the profiling of primary metabolites, and HILIC has been extensively applied to parasite metabolomics [14].

Method validation is a key consideration when selecting the ideal method or combination of methods for a metabolomics study. While metabolite coverage is of primary concern, method reproducibility is of critical importance to allow label-free semiquantitative comparisons of treated and untreated samples in a statistically robust manner. The inclusion of multiple internal standards, and periodic analysis of matched quality control samples, is essential to monitor analytical performance and potentially correct for signal drift.

Metabolite Identification

The identification of metabolites in untargeted metabolomics studies is a key bottleneck that must be overcome to infer biological or pharmacological meaning. The Metabolomics Standards Initiative has defined four different confidence levels by which a metabolite is identified [52]. The highest level of identification confidence (level 1) requires the comparison of two or more orthogonal properties with authentic chemical standards, which have been analyzed under identical analytical conditions. In practice, this is achieved by the combination of MSMS or retention time data with high-resolution mass spectrometry. However, it is important to note that successful dereplication of isomeric species often requires optimization of the chromatographic or fragmentation parameters. In the absence of authentic chemical standards for every parasite-derived metabolite, external databases or predictive algorithms for mass, MSMS, and/or retention time may be used to provide putative identification (level 2). Unidentified metabolite features from known (level 3) or unknown (level 4) metabolite classes should also be reported, as these may represent novel endogenous or drug-derived metabolites [53, 54].

Data Analysis

Any metabolomics analysis will produce large datasets; therefore, it is essential that the data are analyzed in an efficient and meaningful way to measure the relative abundance of hundreds of metabolites [28]. Several commercial and open-source software packages are available for the detection and alignment of metabolite features (peaks) and downstream artifact filtering, metabolite identification, statistical comparison, and pathway or network modeling [55].

Metabolomics for Drug Discovery in Kinetoplastid Parasites

Kinetoplastida comprise a phylogenetically ancient group of protozoa, many of which can cause significant diseases in humans and animals. They are responsible for extensive morbidity and mortality in the developing world and also place a substantial economic burden on the countries that are affected [56, 57]. These protozoa include the South American trypanosome, *T. cruzi*, which causes

Chagas disease; the *T. brucei* subspecies, which causes human African trypanosomiasis (HAT); and multiple *Leishmania* species, which cause various forms of Leishmaniasis.

Human African Trypanosomiasis

HAT, also known as *sleeping sickness*, is caused by infection of the host with *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense* [58, 59] following a bite by the tsetse fly vector. In the first stage of the disease, known as the hemolymphatic phase, nonspecific symptoms such as headache and fever are most common. Stage 2 is considered the neurological phase and occurs when the parasite crosses the blood–brain barrier, inevitably resulting in death in the absence of effective treatment [60]. Although reported HAT mortality has significantly declined in recent years [2], there are serious concerns regarding drug resistance [49], cost, and toxicity for the currently available arsenal of trypanocidal medicines. Treatment options for stage 1 of the disease include suramin and pentamidine, both of which must be administered by injection [61, 62], while stage 2 treatment utilizes melarsoprol or eflornithine, either as monotherapy or in combination with nifurtimox [63–65].

A metabolomics approach has been used to explore the mechanisms of action of several trypanocidal compounds in *T. b. brucei* [66, 67]. Untargeted metabolomic analysis of eflornithine has confirmed its mode of action. Eflornithine treatment induced predictable changes in the levels of polyamine pathway intermediates, resulting in significant accumulation of ornithine and depletion of putrescine, mediated by the inhibition of ornithine decarboxylase. Due to the untargeted nature of the method, the analysis also revealed the absence of arginase activity as well as drug-induced changes in N-acetylated ornithine and putrescine and depletion of the downstream polyamine pathway product spermidine [67]. Polyamines serve an important role in trypanosomes, combining with glutathione to form trypanothione, the major intracellular antioxidant molecule [68–70]. Oxidative stress was also thought to be involved in nifurtimox action [65, 71]. However, metabolomic analysis revealed additive, but not synergistic, metabolic perturbations for eflornithine and nifurtimox, which supports the lack of trypanocidal synergism observed *in vitro* [67]. The untargeted metabolomics analysis of nifurtimox treatment confirmed metabolic activation by nitroreductase to form a reactive nitrile intermediate [72, 73] and revealed changes in endogenous metabolites involved in nucleotide and carbohydrate metabolism [67].

Untargeted metabolomics applied to pentamidine-treated parasites showed nonspecific changes in metabolite levels that could not be directly related to a specific metabolic pathway [37]. Rather, the drug may impact nonmetabolic parasite targets, by interacting with nucleic acids or may even disrupt the mitochondrial membrane potential [74, 75].

Metabolomics has also been utilized for drug target discovery for novel antitrypanosomal compounds. Halogenated pyrimidines have demonstrated high affinity for the trypanosomal uracil uptake transporter, leading to incorporation of 5-fluorouracil into pyrimidine nucleotides and RNA [46]. The analogs 5-fluoro-2′-deoxyuridine and 5-fluoro-2′-deoxycytidine demonstrated prodrug activity, with the active metabolite 5F-dUMP inhibiting thymidylate synthase activity. Overall, these studies showed that halogenated pyrimidines interfere with pyrimidine salvage in *T. b. brucei* by distinct mechanisms [46]. Curcumin analogs bearing a monoenone linker motif show highly potent trypanocidal activity. Untargeted metabolomics revealed significant depletion of intracellular thiols, such as trypanothione and glutathione. This was most likely mediated through the rapid formation of drug-thiol adducts and was not apparent in drug-resistant parasites, confirming the link between thiol depletion and trypanocidal activity [53].

Chagas Disease

Chagas disease, or American trypanosomiasis, is caused by infection with the parasite *T. cruzi*, following the bite of an infected triatomine bug. Up to 10 million people are infected [2] with the disease manifested by low-grade fever, lymphocytosis, and hyperthermia [76]. Although the acute phase may remain asymptomatic, it can progress to a chronic phase and cause death by cardiac failure [76]. Benznidazole and nifurtimox are the only licensed medicines with proven efficacy against Chagas disease; however, drug resistance has been identified [77, 78].

Multiple drug-specific metabolomic perturbations were observed in *T. cruzi* following benznidazole treatment [54]. Benznidazole is believed to be activated by an unusual type I nitroreductase, leading to the formation of a dihydroxy-dihydroimidazole derivative, which decomposes to give toxic glyoxal [73, 79]. A metabolomics study identified depletion of low-molecular-weight thiols by covalent binding of activated benznidazole, which is believed to be responsible for the drug's toxicity [54]. This is consistent with the drug's proposed mechanism, involving covalent modification of lipids, DNA, and proteins [80, 81]. However, key oxidation products of nucleotides could not be detected [54].

Leishmaniasis

Infection with *Leishmania* spp. can cause a complex disease state that may manifest as visceral, cutaneous, or mucocutaneous clinical forms. The parasite is transmitted by the bite of a sandfly and, once inside the host, largely resides in macrophages [82]. Drugs currently available for the treatment of Leishmaniasis include miltefosine, amphotericin B [83], and pentavalent antimonials (Sb^V) [84]. Unfortunately, they are restricted by less than ideal pharmacokinetics, toxicity, and poor efficacy. The mode of action for miltefosine has been proposed to involve inhibition of protein kinase B [85], influencing lipid metabolism, GPI anchor

synthesis, and signal transduction [86]. It has also been suggested to interact with lipid monolayers [87], membrane phospholipids [88], and choline transport [89] and induce apoptotic responses including DNA fragmentation and chromatin condensation [90]. Untargeted metabolomic analysis identified increased alkene fragment release, which is proposed to be synchronized with the production of reactive oxygen species [91]. This study also identified signs of DNA damage by detecting changes in thiols and polyamines, loss of cell membrane integrity, and intact native membrane phospholipids [91]. Amphotericin B is believed to inhibit glucose uptake and compromises the permeability barrier to small metabolites in *L. donovani* promastigotes by affecting membrane sterols [92]. Sb^V is believed to inhibit trypanothione reductase activity in the parasite [93] and stimulate macrophages to kill their intracellular invaders [94]. An untargeted metabolomic approach has also been used to investigate the mechanisms of resistance to antimonial drug combinations in *L. donovani.* Experimentally generated resistant lines demonstrate metabolic adaptations that protect against drug-induced and external oxidative stress, in addition to changes in membrane fluidity and drug uptake [84].

Metabolomics for Drug Discovery in Apicomplexan Parasites

The *Apicomplexa* phylum contains several human pathogens that are responsible for significant morbidity and mortality worldwide, including five species of *Plasmodium* that cause human malaria. The *Plasmodium* parasite has a complex life cycle and is transmitted by the bite of the female *Anopheles* mosquito during a blood feed. Patients experience symptoms during the parasite's asexual replication within host red blood cells, and current treatments act on this stage. However, resistance has now emerged to the first- and last-line therapeutic agents, the artemisinins, which threatens to make the disease untreatable [95]. Use of other antimalarials, such as quinolines and antifolates, is already limited by widespread resistance, highlighting the urgent need for new antimalarials with novel modes of action.

Antimalarial Drug Mechanisms

Several approved antimalarial medicines act by perturbing parasite metabolism. Metabolomics, as an approach to detect drug-induced changes in parasite metabolism, has been validated with studies of the antimalarial atovaquone [96]. Atovaquone acts by inhibiting the cytochrome bc_1 complex in the mitochondrial electron transport chain, consequently leading to the dysfunction of dihydroorotate dehydrogenase (DHODH). DHODH dysfunction perturbs pyrimidine synthesis, which is ultimately fatal to the parasite [97, 98]. As expected, metabolomics analysis of atovaquone-treated *Plasmodium falciparum*–infected red blood cells (iRBCs) showed an accumulation of the substrate of DHODH,

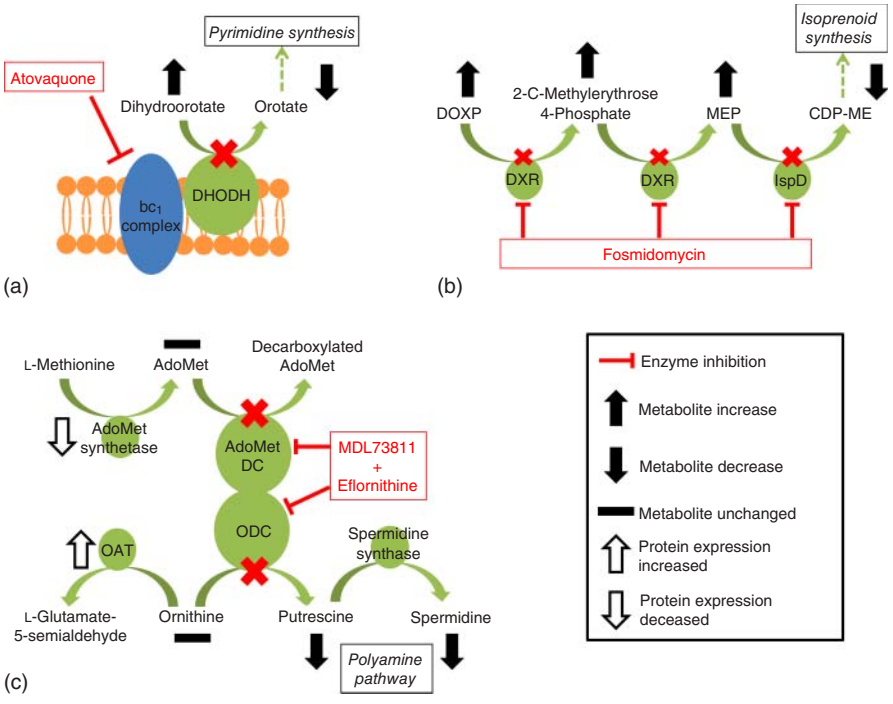


Figure 14.2 Schematic of metabolic mechanisms of action of three anti-apicomplexan compounds. (a) Atovaquone inhibits the cyctochrome bc1 complex in the mitochondria. This inhibits oxidation of the ubiquinone cofactor resulting in dysfunction of dihydroorotate dehydrogenase (DHODH) such that dihydroorotate accumulates and pyrimidine synthesis is disrupted [96]. (b) Fosmidomycin inhibits deoxyxylulose phosphate reductoisomerase (DXR) causing accumulation of 1-D-deoxyxylulose 5-phosphate (DOXP). It also inhibits methylerythritol phosphate cytidylyltransferase (IspD) causing the accumulation of 2-*C*-methylerythrose and methylerythritol phosphate (MEP) and the depletion of cytidine diphosphate methylerythritol (CDP-ME). This disrupts isoprenoid biosynthesis [42]. (c) Together, eflornithine and MDL73811 inhibit the bifunctional enzyme, *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (AdoMetDC/ODC), which leads to the depletion of polyamines and is ultimately fatal to the parasite. The substrate of AdoMetDC, AdoMet, does not accumulate as expected upon AdoMetDC inhibition. This is due to the decreased expression of AdoMet synthetase, as revealed by transcriptomics and proteomics, such that less AdoMet is synthesized. Similarly, the substrate of ODC, ornithine, does not accumulate as expected upon ODC inhibition. This is due to the increase in ornithine aminotransferase (OAT) expression, which degrades ornithine [41].

dihydroorotate, and its precursor, carbamoyl-L-aspartate (Figure 14.2a). Complementarily, the products of DHODH activity, downstream pyrimidine nucleotides, were depleted [45, 96]. Antifolate drugs kill malaria parasites by inhibition of either dihydrofolate reductase (DHFR) or dihydropteroate synthase (DHPS), perturbing folate metabolism and consequently depriving the pyrimidine synthesis pathway of essential intermediates [99]. This depletion of thymidine nucleotides

by cycloguanil has been confirmed using metabolomics methodology [45]. Another antifolate, proguanil, mediated dysregulation of arginine metabolism, suggesting that a novel mechanism may be involved in the antimalarial mechanism of this drug. Other antimalarials including dihydroartemisinin and chloroquine induced perturbations in hemoglobin-derived peptides, consistent with their proposed mechanisms of action involving the parasite digestive vacuole [45].

Plasmodium Drug-Resistance Mechanisms

Resistance has emerged to all available antimalarial drugs, and resistance mechanisms must be considered during the development of new antimalarials. Genomic sequencing is effective at detecting the genetic mutations associated with drug resistance, but does not always elucidate the mechanisms responsible for resistance. Chloroquine (CQ) resistance is primarily associated with mutations in *P. falciparum* CQ-resistance transporter (*pfcrt*) [100]. However, the molecular mechanisms by which point mutations in PfCRT facilitate CQ resistance remain unclear. Metabolomics revealed an accumulation of many hemoglobin-derived peptides in lines of *P. falciparum* expressing the CQ-resistant form of PfCRT compared to lines expressing the CQ-sensitive form [39]. This suggests impaired hemoglobin digestion in CQ-resistant parasites. Indeed, there was a fitness cost attributed to the expression of the CQ-resistant form of PfCRT [39], which could explain the reemergence of CQ-sensitive parasites in the field following cessation of widespread CQ use.

Novel Anti-Apicomplexan Compounds

Based on the success of atovaquone, differences in human and parasite electron transport chains have been investigated as potential drug targets for malaria. It was found that *P. falciparum* lacks a canonical NADH dehydrogenase and instead possesses a bacterial-like type II NADH:ubiquinone reductase (PfNDH2) situated upstream of the bc_1 complex in the electron transport chain [101]. Biagini *et al.* [96] led a drug discovery initiative to find drugs with activity against PfNDH2 that could kill atovaquone-resistant parasites. Upon treatment of iRBCs with the novel quinolone compound, CK-2-68, metabolomics detected perturbations consistent with DHODH dysfunction. This confirmed that the mechanism of action of CK-2-68 was via disruption of the electron transport chain [96].

Investigation of host and parasite metabolism led to the discovery that, unlike humans, malaria parasites possess a nonmevalonate isoprenoid biosynthesis pathway [102]. Several antibiotics target this pathway in bacteria, and when tested, it was found that these antibiotics had antimalarial activity [103]. Fosmidomycin was one such drug, which blocks isoprenoid synthesis in *Escherichia coli* via inhibiting deoxyxylulose phosphate reductoisomerase (DXR) [104]. Targeted metabolomics of fosmidomycin-treated *P. falciparum* showed the anticipated

accumulation of DXR's substrate, 1-D-deoxyxylulose 5-phosphate (DOXP), but did not detect the expected depletion of its product, methylerythritol phosphate (MEP). In fact, MEP levels increased. There was depletion in metabolites further downstream in the isoprenoid pathway, including cytidine diphosphate methylerythritol (CDP-ME), the product of the enzyme methylerythritol phosphate cytidylyltransferase (IspD) (Figure 14.2b). This suggests that fosmidomycin may also target IspD, which was supported by *in vitro* inhibition of recombinant IspD. Supplementation of fosmidomycin-treated parasites with a downstream isoprenoid was able to rescue *P. falciparum*, suggesting that fosmidomycin exclusively targets the isoprenoid pathway [42]. Fosmidomycin is currently in clinical trials in combination with clindamycin [105, 106] for uncomplicated malaria.

Eflornithine, when coadministered with a bis(benzyl)polyamine analog, can cure mice of the rodent malaria strain, *Plasmodium berghei* [107]. Together, eflornithine and the bis(benzyl)polyamine analog, MDL73811, inhibit the bifunctional *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (AdoMetDC/ODC) enzyme, blocking polyamine synthesis [108, 109]. To investigate polyamine depletion as an antimalarial strategy, a combined transcriptomics, proteomics, and metabolomics study was performed [41]. Metabolomics confirmed that polyamine depletion is induced by eflornithine-MDL73811 (Figure 14.2c). Surprisingly, the substrate of AdoMetDC AdoMet (*S*-adenosylmethionine), did not accumulate as expected, upon inhibition of the enzyme. Proteomics and transcriptomics detected a decrease in AdoMet synthetase such that less AdoMet would be synthesized, revealing a compensatory feedback mechanism in *P. falciparum.* Furthermore, the substrate of ornithine decarboxylase (ODC), ornithine, did not accumulate upon drug treatment as occurs in trypanosomes. This can be explained by the increase in ornithine aminotransferase (OAT) expression, which degrades ornithine.

Summary

Due to the lack of information on the mechanisms of action of current drugs, there are very few well-defined clinically relevant drug targets for protozoan parasites. Therefore, recent high-throughput screens of large compound libraries belonging to pharmaceutical companies [7, 110] have been invaluable for identifying novel antiparasitic compounds from a diverse range of chemical classes. However, there is a significant bottleneck in the development of these compounds for clinical use due to a lack of information regarding their mechanism of action [13]. Metabolomics represents a novel approach for understanding parasite biology and provides a valuable way to capture a global, untargeted perspective on drug-induced changes to parasite metabolism, which can then generate hypotheses regarding mechanisms of drug action. This will support the rational design of novel antiparasitic compounds that overcome issues relating to drug toxicity, resistance, and poor pharmacokinetic properties. It will also aid in the optimal utilization of the current repertoire of drugs by better guiding combination

therapies and the monitoring of efficacy and resistance. Metabolomics has already revealed the modes of action of multiple antiparasitic drugs [53, 67, 91, 96] and has uncovered many fundamental aspects of parasite biology such as isoprenoid synthesis in *P. falciparum* [42] and pyrimidine metabolism in *T. brucei* [46] that could be targeted for therapeutic intervention.

Metabolomics has been driven forward by the recent advances in analytical technologies that allow for greater metabolite coverage from complex biological matrices with increased sensitivity and resolution [111]. This will continue to improve in coming years, and the ability to efficiently analyze and biochemically interpret omics scale data will likely be the limiting steps in the progression of metabolomics in the drug discovery field. However, the improvement and accessibility of a variety of freely available data analysis packages are beginning to overcome this hurdle. The interpretation of metabolomics data, and the ability to link changes in metabolite levels with drug mechanisms, largely relies on our knowledge of parasite biochemical pathways and their interactions with host cell metabolism, which is unfortunately not completely understood [14]. Metabolomics can facilitate traditional biochemical approaches to more comprehensively annotate and characterize parasite metabolites and pathways, thus enhancing our ability to interpret drug-induced metabolic perturbations.

The integration of metabolomics data across multiple levels of systems biology, including the proteome, transcriptome, and genome, also offers huge potential for drug discovery. It will allow mechanisms of drug action to be discerned in more detail and allow researchers to better understand modes of resistance from a system-wide perspective, across multiple molecular levels. The advent of metabolomic ^{13}C-flux analysis also provides further in-depth investigations of drug mechanisms and potential biochemical targets by identifying choke points in parasite metabolism [112]. When combined with computational approaches, network analysis can help explain therapeutic effects and aid in the development of predictive *in silico* models for drug action, which may offer extensive financial savings during the drug discovery process [113, 114].

Metabolomics is not only limited to the investigation of drug mechanisms of action and the identification of drug targets, but also has applications across the entire drug discovery pipeline, including identification of biomarkers and the analysis of off-target actions that could explain toxicity and adverse effects. Metabolomics has already demonstrated utility in drug discovery for parasitic diseases and will play an important role in its future, fostering the development of novel drugs to combat these life-threatening infections.

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15
The Importance of Targeting Lipid Metabolism in Parasites for Drug Discovery

*Simon A. Young, Matthew D. Roberts, and Terry K. Smith**

Abstract

In this chapter, we highlight that lipid metabolism contains a plethora of targets that continue to be exploited for drug discovery purposes against protozoan and apicomplexan parasitic diseases. As parasitic cells divide relatively fast compared to host cells, their requirement for lipids is far greater than that of the host. They have to balance their specific lipid precursor scavenging and biosynthetic requirements to maintain their lipid composition homeostasis as they divide and differentiate through the cell-cycle and life-cycle stages, respectively. This includes the mevalonate and nonmevalonate pathways with their wide variety of essential downstream metabolites that influence/control numerous essential cellular processes, governed by posttranslational modifications. In the past 10 years, facilitated by the use of mass spectrometry/lipidomics/metabolomics, it has become increasingly easier to phenotype subtle changes going on within the parasite, not only the whole cell, but increasingly within specific organelles. Collectively, this gives us a better understanding on the complexities of lipid metabolism associated with various cellular functions and even drug resistance.

Introduction

The protozoan parasites that cause African sleeping sickness in sub-Saharan Africa (*Trypanosoma brucei*), Chagas disease in Central and South America (*Trypanosoma cruzi*), "Old World" cutaneous leishmaniasis in the Middle East, and "New World" visceral leishmaniasis (*kala-azar*) in East Africa, South America, and Southeast Asia (various *Leishmania* species) are among the most neglected diseases on the planet. Together with malaria caused by *Plasmodium* spp. and related apicomplexans *Toxoplasma* and *Cryptosporidium*, millions of people are affected representing a huge percentage of the world's disease burden. Drugs that exist for the treatment of these diseases have low efficacy, high toxicity

*Corresponding author.

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(at levels that would be unacceptable in the developed world), and fall well below the standards of modern medicine; increasing levels of drug resistance also pose a progressively serious problem. There is clearly an urgent need to identify new drugs and new drug targets. The current status of these drug discovery efforts for some of these diseases have recently been reviewed [1–4].

In the drug discovery process, selecting the best drug targets is crucial for maximizing chances of success. Lipid metabolism is an essential process for cell types whose evolutionary success is based upon rapid proliferation and/or differentiation. In this regard, there are similarities in the rapid proliferation and high metabolic rate of both tumor cells and protozoan parasites. Lipids are crucial to parasites as they are used as structural components of membranes, energy stores, pathogenic factors, and signaling molecules. As such, they depend upon a wide variety of systems for the uptake and metabolism of these molecules to satisfy this demand, which can dramatically vary during life cycle and morphological changes. The notable dependence on lipid resources for parasite survival in their hosts has and will continue to create prospects to restrict parasite growth and cure infections. Identified lipid metabolism in these parasites reveals that they are auxotrophic for many lipid precursors, which force them to meet their high demand for lipids through networks of both *de novo* synthesis and scavenging. This provides the scientific community with opportunities to exploit these "Achilles heels" for effective drug discovery purposes.

Parasites Nutrient Requirements from the Host for Lipid Metabolism

As exclusively extracellular parasites in the mammalian bloodstream, the causative agent of human African Trypanosomiasis, the kinetoplastid protozoan *T. brucei* must acquire essential nutrients from this environment to proliferate and maintain a significant parasitemia. For example, the mammalian bloodstream form is entirely dependent on the uptake of glucose to generate ATP through glycolysis [5] and plasma proteins including albumin and transferrin (supplying iron) by a receptor-mediated process [6]. Up to 20% of the dry weight of *T. brucei* is comprised of lipids, the majority being phospholipids in addition to neutral lipids, plasmalogens, fatty acids (FAs), isoprenoids, and sterols [7]. The *T. brucei* bloodstream form is uncommon relative to other trypanosomatids in that the indispensible abundant sterol in the membranes is cholesterol sequestered directly from host serum lipoproteins [8]. The phospholipid composition of mammalian bloodstream-form *T. brucei* is typical of eukaryotes (Table 15.1) with phosphatidylcholine (PC) at approximately 45% (moles% lipid phosphorous), the most abundant, followed by approximately 20% phosphatidylethanolamine (PE), approximately 6% phosphatidylinositol (PI), approximately 5% phosphatidylserine (PS), approximately 2% lysophosphatidylcholine (lyso-PC), and approximately 2% phosphatidylglycerol (PG) with another 5% of minor lipids such as cardiolipin, phosphatidic acid (PA), and PI phosphates [9]. The remaining 15% is made up of sphingolipids, such as sphingomyelin (SM), and ethanolamine

Table 15.1 Comparison of lipid composition of parasites.

	T. brucei BSF [9–13]	*T. brucei* PCF [9–13]	*T. cruzi* epim [14–17]	*L. major* prom [18]	*P. falciparum* trophozoite [19, 20]	*T. gondii* tachyzoites [21, 22]
Total phospholipids	80	80	34.7	70	–	–
PC[a)]	45	60	45.2	33	50	60.83
PE[a)]	20	10	26.8	10	20	20.57
PI[a)]	6	12	11.9	10	15	3.6
PS[a)]	4	3	–	–	5	4.151
Others[a)]	8	10	12.5	7	5	0.7
Sphingolipids	15[a)]	5[a)]	3.7	10	20	5.2
SM	✓	✓	✓	–	✓	✓
IPC	–	✓	✓	✓	–	–
EPC	✓	–	–	–	–	✓
Complex sphingolipids	GlcCer, LacCer	–	GlcCer, GalCer, LacCer	–	CMH, CDH, CTH	CMH, CDH
Neutral lipids[b)]	16.1	–	65[c)]	30[c)]	–	0.05
Sterols	96[d)]	50[d)]	65[c)]	30[c)]	–	0.05[e)]
Major class	Cholest	Cholesta-5,7,24-trienol + Ergost	Ergost	Ergost	Cholest	Cholest

Unless listed otherwise, data is expressed as percentage of total lipids.
Glucosyl ceramide, GlcCer; galactosylceramide, GalCer; lactosylceramide, LacCer; ceramide monohexoside, CMH; ceramide dihexoside, CDH; ceramide trihexoside, CTH; Cholest, Cholesterol; and Ergost, Ergosterol.
– Data not reported.
a) Data expressed as percentage of phospholipid composition. Other PL species include phosphoglycerol (PG), phosphatidic acid (PA), and cardiolipin (CL).
b) Neutral lipids include monoacyl glycerols (MAGs), diacyl glycerols (DAGs), and triacyl glycerols (TAGs)
c) Taken as single result.
d) As percentage of total sterols.
e) Taken as mol/mg protein.

phosphorylceramide (EPC). Unusually, there are large amounts of ether-linked FAs in the phospholipids, particularly in PE with alkyl-acyl (plasmanyl) and alkenyl-acyl (plasmalogen or plasmenyl) glycerol backbone configurations [9].

T. brucei lack all the components to *de novo* synthesize their phospholipids [13], so require the uptake of lipids from the host bloodstream, predominantly through the receptor-mediated endocytosis of circulating low-density lipoprotein (LDL) particles [8]. These endocytosed FA and lipids are not incorporated into membranes intact but rather catabolized in the trypanosome lysosome into precursors for *de novo* phospholipid biosynthesis. In *T. brucei*, FA biosynthesis proceeds by the prokaryotic type II pathway (FASII) in the mitochondria, although an unusual ER-localized fatty acid elongation (FAE) mechanism also exists, which can also *de novo* synthesize FA [23].

Particularly important to *T. brucei* is the availability of the phospholipid head groups, choline, ethanolamine, serine, and *myo*-inositol, which must be acquired free or in substrates in sufficient amounts to maintain the parasites' required membrane lipid composition [10]. For example, in the mammalian bloodstream, the demand for choline is met in part by choline uptake and the uptake and catabolism of lyso-PC by *T. brucei* [8, 24]. Unusually, *myo*-inositol is used from two distinct sources; extracellularly for bulk PI synthesis in the Golgi and *de novo* in the endoplasmic reticulum specifically for PI in the glycosylphosphatidylinositol (GPI) anchors of *T. brucei* surface proteins [25–28].

Related kinetoplastid parasites, *Leishmania* spp. infections in humans have a range of clinical consequences between a potentially fatal visceral diseases and relatively minor restricted cutaneous lesions [29]. Upon infecting a mammalian host, *Leishmania* metacyclics invade phagocytic cells such as macrophages, transforming the phagolysosomal vacuole they reside in. The metacyclics undergo significant developmental and morphological changes, differentiate into amastigotes, and rapidly replicate before bursting out of the macrophages to disseminate the infection. It is clear that these processes in some way will require dynamic changes in lipid acquisition and metabolism [18].

Similar to other blood-borne parasites, *Leishmania* spp. have a lipid composition distinct from their human host. Phospholipids make up approximately 70% of the cellular lipid content (the remainder consisting of sterols and neutral lipids), and again PC is the most abundant (~50%) followed by PE and PI (each ~15%), and approximately 10% minor phospholipids (PA, CL, PG, and lyso-PLs; Table 15.1) . PS is almost absent, with metabolic labeling and mass spectrometry confirming a lack of synthesis in *Leishmania major* promastigotes [30, 31]. As in *T. brucei*, plasmanyl and plasmalogen PE species are elevated in *Leishmania* [18]. Unlike bloodstream-form *T. brucei*, however, *Leishmania* exclusively synthesize inositol phosphorylceramide (IPC) rather than SM as their sphingolipid. In addition to an array of common eukaryotic FA, *Leishmania* have more atypical acyl chains in their phospholipids, the purpose of which is not understood. Many of the abundant PC species in *Leishmania* have long unsaturated FA, and similarly, a small percentage of PE (~10%) can have a cyclopropyl FA (specifically *cis*-9,10-methyleneoctadecanoic acid) chain [32].

Both *Leishmania* spp. and *T. cruzi* have genetic components of the FASII synthetic pathway for FA and, although not studied, are assumed to be analogous to the *T. brucei* pathway [23]. Similar to fungi, *Leishmania* parasite membranes contain the vital ergosterol (and ergosterol-related sterols) rather than cholesterol. These sterols are *de novo* synthesized from acetyl-CoA via mevalonate in the isoprenoid pathway, followed by the later steps of sterol synthesis with a key enzyme, the essential sterol 14α-demethylase (CYP51), removing a 14 alpha-methyl group from lanosterol to ultimately produce ergosterol [33]. Due to the demand for new membranes in the transformation and replication of the parasites in the phagolysosome of macrophages, *Leishmania* spp. parasites take up a range of lipid precursors from their human host and transport both choline and ethanolamine for *de novo* lipid synthesis [34]. In the case of ethanolamine, it is also necessarily incorporated into GPI anchors and necessary for the ethanolamine phosphoglycerol modification of eukaryotic elongation factor 1A [35]. While the genetic origin of these transporters is unknown, the *myo*-inositol uptake mechanism has been well characterized [36].

As an obligate intracellular parasite of a variety of mammalian cells, the causative agent of Chagas disease, *T. cruzi* is similar in its metabolic requirements to other kinetoplastid parasites. The success of a *T. cruzi* infection is dependent on efficient exchange of nutrients such as lipid precursors via the parasitophorous vacuole, where the parasites reside and develop following invasion. Phospholipid analysis of *T. cruzi* epimastigotes showed similarities to *T. brucei*, with the most abundant phospholipid PC (44%), then PE (28%), PI (12%), SM (4%), and the remaining 12% comprised of CL, PA, lyso-PC and PS [14], and the sphingolipid IPC [37] (Table 15.1). Similar to *Leishmania* spp., *T. cruzi* predominantly contain and synthesize ergosterol (and ergosterol-like sterols), although up to 10% of their sterol content is cholesterol acquired from the extracellular environment [38]. Analogous to lipid bodies, *T. cruzi* epimastigotes have reservosomes, unique storage organelles containing large amounts of cholesterol and cholesteryl esters. In nutrient-depleted conditions, these stores are consumed preferentially for the production of ergosterol, and it is clear that the parasites are able to manipulate these cholesterol levels according to demand [39]. It is expected as for other kinetoplastids that *T. cruzi* scavenges a range of lipid head group precursors, but this is only experimentally proven for *myo*-inositol [40].

For the causative agent of malaria, *Plasmodium* spp., lipids are essential components needed for critical cellular processes such as membrane formation and trafficking, hemozoin formation, and cell signaling, as reviewed by Deschamps *et al.* [41]. In the human life-cycle stages of the malaria parasite, there are multiple steps of cell growth and division, requiring the acquisition of lipid components for rapid membrane synthesis. Upon invasion of host erythrocytes by *P. falciparum*, there is extensive remodeling and creation of cellular structures such as a bilayered parasitophorous vacuole membrane (PVM) surrounding the parasite and a tubulovesicular network (TVN) extending from the PVM into the host cytoplasm [42]. In this manner, the parasites creates an ideal environment to allow the acquisition of extracellular nutrients such as lipid precursors while simultaneously

avoiding and manipulating the host immune system, generating the acute symptoms experienced by an infected individual.

Glycerophospholipids are the main constituents of *P. falciparum* membranes in the erythrocyte infecting stages and are synthesized predominantly by the parasite using a complex metabolic network rarely found in a single organism. In addition to standard glycerophospholipid synthesis, both by the eukaryotic *de novo* process and by an ancestral prokaryotic CDP-diacylglycerol-dependent pathway, the parasite has additional enzymatic reactions (classically restricted to plants) that connect some of the pathways for greater flexibility [43]. Compared with other eukaryotes, the phospholipid content of *Plasmodium* purified from RBCs is uncommon with an unusually high amount of PE (35–45%), alongside PC (40–50%), PI (4–11%), and SM and PS (<5%) (Table 15.1). Coincident with the maturation of the parasite in the RBC is an essential increase in neutral lipids, particularly triacylglycerols (TAGs) in lipid bodies [44], which are ultimately catabolized to free FAs as the parasite merozoites egress from the RBCs [45]. Surprisingly, cholesterol is barely detectable in the intraerythrocytic stages of the parasites, and a lack of *de novo* synthetic pathways means that the minimal amount present is likely to be acquired from the host through the PVM [46].

Initially, it was postulated that at the key erythrocyte-invading stage, *Plasmodium* spp. lacked the ability for *de novo* FA synthesis, relying entirely on the host for its complement of FAs for lipid biosynthesis, as reviewed by Vial *et al.* [47, 48]. Genome sequencing, however, revealed a complete prokaryotic type II fatty acid synthesis (FASII) pathway, putatively localized to the apicoplast, a plastid-like organelle [49, 50]. Significantly, it was shown that FASII fatty acid synthesis was redundant for most *Plasmodium* developmental stages in the human life cycle and is only critical during the late intrahepatocytic stage [51]. It is the extracellular availability of FAs, specifically palmitic acid (C16:0) and oleic acid (C18:1, *n*−9) associated with serum albumin, rather than PL or neutral lipids, that is essential for the parasites' survival and appears to be ample for phospholipid synthesis during other life-cycle stages in the human host. In a physiological situation, albumin-bound lyso-PC is additionally internalized and deacylated by *Plasmodium*-infected RBC to generate more free FAs [52]. While FA acquisition is essential to the intraerythrocytic stage of *Plasmodium* spp., it is unclear whether these serum-derived components are taken up in an energy-dependent manner or simply by passive diffusion [46]. Less is known regarding the nonerythrocytic stages of the *Plasmodium* infection, but a recent study confirmed that the intrahepatocytic stages similarly import lipids [53]. Further research is required in different life-cycle stages of *Plasmodium* spp. to clarify the importance of the complex and varied processes of lipid acquisition and biosynthesis that contain many promising therapeutic targets.

Similarly to *Plasmodium* spp., the causative agent of toxoplasmosis, the apicomplexan *Toxoplasma gondii* constructs its own intracellular niche in the form of a parasitophorous vacuole in almost all nucleated mammalian cell types. The

Toxoplasma parasite has membranes composed of both polar and neutral lipids and as an actively dividing organism needs to have an efficient lipid metabolism. PC is the most abundant lipid (75% of the total) of *Toxoplasma*, followed by PE (10%), PI (7.5%), and PS (6%) in addition to minor lipid species, as reviewed by Coppens [54]. There are three sources of lipids within the membranes of *T. gondii*, intact lipids scavenged from the host, lipids synthesized by the parasite independent of the host, and *de novo* synthesized lipids that predominantly require precursors acquired from the extracellular environment. These critical precursors are transported, through manipulation of the host cell machinery, into the parasite from the extracellular environment.

In addition to an apicoplast-localized FASII FA synthetic pathway, *T. gondii* has a complementary mitochondrial FASI pathway and can also take up selected extracellular free FAs and elongate them, as reviewed by Ramakrishnan *et al.* [23]. Using FAs, *T. gondii* also has the ability to synthesize TAG stored in lipid bodies and also galactosylglycerolipids. The parasite also has fully functioning Kennedy pathways to *de novo* synthesize its amino glycerophospholipids (PC and PE and PS) but additionally acquires the head group precursors (choline, ethanolamine, and serine) from the host [54]. *T. gondii* possesses an unusual glycosphingolipid (absent from mammalian cells) IPC, which is synthesized *de novo* from serine and palmitoyl-CoA through ceramide. GPIs, a major class of lipids essential as membrane anchors of surface proteins in *Toxoplasma*, are glycolipids synthesized by the transfer of *N*-acetylglucosamine to the phospholipid PI. Similarly to many parasites, *Toxoplasma* has no identifiable synthetic pathway for sterols, but cholesterol (from LDL) is detectable in the membranes and is converted into cholesteryl esters abundant in lipid bodies [54].

The acquisition and synthesis of lipids are equally important in many other types of protozoan parasites. The so-called *mucosal parasites* such as *Entamoeba* and *Giardia* are amitochondriate protozoans that infect the human intestinal tract and cause diarrhea and other digestive abnormalities in millions of people each year [55]. These enteric pathogens are unable to *de novo* synthesize sufficient amounts of lipids and sterols and thus survive both by scavenging exogenous lipids and by having specialized metabolic pathways to ensure their proliferation and survival, as reviewed by Das *et al.* [56, 57]. Clearly, mechanisms of lipid uptake and synthesis are important processes to investigate, and evidence has shown that amitochondriates take up intact lipids and cholesterol both from host lipoprotein particles and as individual molecules through transporters. Scavenged phospholipids can undergo FA remodeling through deacylation and reacylation to alter the lipid's properties, thus avoiding the energetically draining *de novo* synthetic route. Similarly, amitochondriate parasites can both elongate and desaturate FAs to generate new phospholipids. Again, further research on these pathways will identify key enzymes and activities that will provide candidates for the design of antiprotozoal drugs with a potentially wide therapeutic range against mucosal parasites.

Drugs Affecting Phospholipid Metabolism in Protozoan Parasites

Due to their reliance on the lipid synthesis for new membranes required for proliferation, kinetoplastid parasites can be targeted by inhibitors of sterol and phospholipid biosynthesis, chemotherapeutic agents with proven selective potent activity *in vitro* and *in vivo.* In actuality, many host-derived amino acids, carbohydrates, and lipids are required for *de novo* synthesis of lipids in *T. brucei* and other parasites, and as such, there are a significant number of essential enzymes as described by Smith and Butikofer [10] that could be targeted by inhibitory compounds.

Perhaps, the most prominent compounds relating to the disruption of lipid metabolism in kinetoplastid parasites is the alkyl lysophospholipid (ALP) series. These lipid analogs are readily taken up by parasites and have antiproliferative properties involving perturbation of membranes, resulting in cell death. In addition to their long-studied anticancer effects, a number of ALP analogs were found to show antiprotozoal activity against *Leishmania donovani*, the causative agent of visceral leishmaniasis [58].

Different ALPs have varying efficacy for specific kinetoplastid species and life-cycle stage, but a range of *Leishmania*, *T. brucei*, and *T. cruzi* parasites have shown significant susceptibility with effective dose killing responses in the low micromolar range, as reviewed by Croft *et al.* [59]. ALPs have been tested clinically with miltefosine preventing *L. donovani* infection in mice with very few side effects resulting in the formulation Impavido, since 2000, the only approved oral drug for leishmaniasis [60, 61]. Impavido is essential for the first-line treatment of endemic visceral leishmaniasis in Asia and is notably effective in treating patients infected with antimony-resistant *L. donovani* [62]. The specific mode of action of miltefosine is still under debate, but most ALP-treated parasites show damaged plasma membranes, indicative of changes to the lipid content [59]. Detailed analysis using high-performance liquid chromatography (HPLC) of miltefosine-treated *L. donovani* promastigotes revealed an increase in the PE-to-PC ratio and a significant increase of cholesterol was detected using gas chromatography–mass spectrometry (GC-MS) [63]. More sensitive HPLC electrospray ionization–mass spectrometry (ESI-MS) revealed complex changes in some members of the various lipid classes of miltefosine-treated *L. donovani* promastigotes [64]. More modern metabolomic approaches defined obvious cellular changes in *L. donovani* treated promastigotes and indicated, although membrane phospholipids were not changed, disruption of internal metabolites involved in lipid synthesis [65]. Ultimately, it remains to be seen whether these observed changes in lipid content are the direct mode of action of ALPs or simply an adaptation of the parasites to this type of drug. Clearly, the ALPs have good therapeutic potential, however, with antiparasitic synergism determined against *T. cruzi* epimastigotes of ET-18-OCH_3 (inhibiting *de novo* PC biosynthesis) with ketoconazole, a sterol biosynthesis inhibitor that has an alternative mode of action inhibiting PC biosynthesis [66].

A more unusual compound affecting lipid metabolism is ajoene [(*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide] (Figure 15.1), a stable constituent of garlic trypanocidal to both epimastigotes and amastigotes of *T. cruzi* [67]. In culture, ajoene (80 μM) causes the growth arrest of the epimastigote form while 100 μM induces cell lysis within a day. Similarly, 40 μM ajoene was sufficient to successfully eliminate *T. cruzi* amastigotes proliferating within Vero cells within 4 days. Significant alterations in the phospholipid composition of the growth-inhibited epimastigotes were observed with a decrease in PC, from the most abundant phospholipid in wild-type cells to the least abundant. Concurrently, a predominance of PE, the biosynthetic precursor to PC, in these cells was observed. In addition to these changes in phospholipid head groups, there were clear alterations in the FA chains of the lipids, with a notable increase in saturated species and associated decrease in C18:2 linoleic acid, normally the most abundant FA. A molecular target of ajoene was not identified, but the mode of action in *T. cruzi* was suggested to involve the specific alteration of the phospholipid composition of the mitochondrial and endoplasmic reticular membranes, thus affecting parasite proliferation. Further changes in the composition of sterols and neutral lipids indicated that ajoene may have multiple targets in its trypanocidal effect, further illustrated by the more recent publication demonstrating an *in vitro* covalent

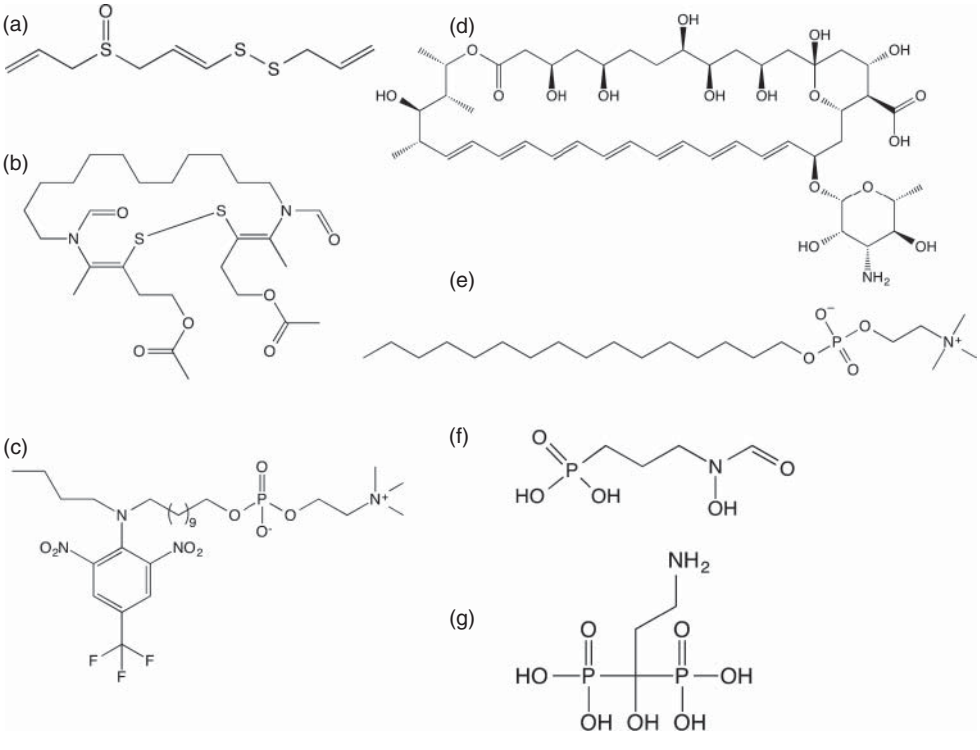


Figure 15.1 Structures of compounds with antiparasitic activity. (a) Ajoene; (b) albitiazolium; (c) TC95; (d) amphotericin B; (e) miltefosine; (f) fosmidomycin; and (g) pamidronate.

inhibition of the *T. cruzi* trypanothione reductase, a key enzyme of the intracellular thiol metabolism [68]. Ajoene has shown antileishmanial activity *in vitro* against both amastigotes and promastigotes of various strains of *Leishmania mexicana* and *L. donovani* [69]. Greater potency than that for *T. cruzi* was observed with leishmanial growth inhibited at concentrations over 0.3 μM and 100% lysis with 10 μM treatment for 4 days. Although an effect on phospholipid composition was not assessed, ultrastructural studies showed similar aberrant morphologies of the mitochondrial and nuclear membranes and the presence of large autophagic vacuoles [70]. To date, the effect of ajoene on any of the life-cycle stages of *T. brucei* is unpublished if not unknown. Ajoene has proved surprisingly potent in a murine model of malaria, where a single dose of 50 mg/kg given the same day as the infection could suppress the development of the parasites with no obvious side effects to the mice [70]. Furthermore, a combinatorial treatment of ajoene (50 mg/kg) and chloroquine (4.5 mg/kg) on the day of infection correspondingly abolished infections in mice.

In designing compounds for the inhibition of phospholipid synthesis, an obvious strategy is focusing on the dependence of parasites on the synthesis of significant amounts of PC and PE. With most parasites, the synthesis of these lipids (as discussed in Section Parasites Nutrient Requirements fromthe Host for LipidMetabolism) requires predominantly the uptake of the precursor materials for the specific phospholipid head groups. In *P. falciparum*-infected erythrocytes, the transport of choline is a limiting step in the *de novo* biosynthesis of PC and it is clear that targeting this by using choline analogs has promise as a novel malaria treatment, as reviewed by Peyrottes *et al.* [71]. A wide range of choline analogs have been designed with an ability to cure malaria infections in nonhuman primates and rodents, inhibiting the asexual *P. falciparum* blood stages at low nanomolar concentrations. These bisthiazolium compounds show both specificity and potency by accumulating in the parasite, inhibiting choline transport and thus PC synthesis, and simultaneously disturbing hemoglobin degradation in the digestive vacuole [72]. Developing an antiparasitic compound of this type with multiple modes of action would be beneficial in delaying the possibility of drug resistance. One choline analog in particular, Albitiazolium (Figure 15.1), has moved into phase II clinical trials with hope as an orally deliverable cure for uncomplicated malaria. Albitiazolium inhibits parasite growth ($EC_{50} = 2$ nM) and has the rare ability to cure mice in a single intraperitoneal dose at an $ED_{50} = 1$ mg/kg [73]. The primary mode of action of this is the potent competitive inhibition of choline uptake into the intraerythrocytic parasite, while at higher concentrations, activity of the three enzymes in the PC biosynthesis pathway are affected, again aiding in interrupting the development of drug resistance [74]. Further insight revealed a massive irreversible accumulation in *P. falciparum*-infected red blood cells, due to a proton motive force driven uptake by a parasitophorous vacuole specific high-affinity transporter, potentially making this novel antimalarial agent evade parasite resistance mechanisms [75].

Development of these compounds is not as advanced as in malaria therapy, but choline analogs are also proving to be effective in the inhibition of kinetoplastid

parasites. Testing effective antimalarial choline analogs against *Trypanosoma* and *Leishmania* spp. showed promising antikinetoplastid activity, particularly against *T. brucei* with four compounds having submicromolar EC_{50} values [76]. Similarly to *P. falciparum,* in *Leishmania* spp., these analogs are known to inhibit a choline transporter [34], but as *T. brucei* seemingly has no choline uptake mechanism, the mode of action is unclear and requires further research. This is more apparent as the compounds showed no effect on the phospholipid content of *T. brucei* but instead disturbed the mitochondrial membrane potential and thus mitochondrial function [76]. Analogs based on the common head group of PC and SM have also shown promise against kinetoplastids, with one alkyl phosphocholine dinitroaniline hybrid TC95 particularly effective against *Leishmania amazonensis*, a causative agent of cutaneous leishmaniasis [77]. TC95 is potent against both intracellular amastigotes and promastigotes (EC_{50} 1.2 and 2.6 μM, respectively) and more selective than the ALP miltefosine. Cells treated with TC95 (Figure 15.1) showed significant cytoskeletal alterations, affecting the morphology of the plasma membrane and flagellum and arresting the cell cycle mainly in G1. To date, any effect on the lipid profile of *L. amazonensis* is unpublished, but transmission electron microscopy of treated cells showed deformation of organellar membranes and an accumulation of lipid bodies, indicators of disrupted lipid metabolism.

In summary, targeting the Kennedy pathways of human-infective parasites, creating compounds to disrupt the essential *de novo* synthesis of PC and PE is clearly a valid and promising therapeutic approach. It is important to note, nevertheless, that endeavors to find molecular inhibitors of the essential processes of sphingolipid acquisition and degradation should also bear fruit both in kinetoplastids and in apicomplexans, as reviewed by Young *et al.* [78] and Coppens [79], respectively. Finally, the lack of an obvious antiparasitic mechanism for many of the potent compounds discussed in this section illustrates the need for more comprehensive molecular investigations into identifying their cellular targets.

Drugs Affecting Sterol Metabolism in Protozoan Parasites

Isoprenoids are ubiquitous in nature and comprise over 23 000 different compounds. They play a plethora of vital roles and are involved in, for example, electron transport (heme A and ubiquinone), play regulatory roles in cell growth and development (cytokines, prenylated proteins) and in the regulation of metabolism (sterols), and also act as coenzymes (dolichols). Isoprenoids are components of larger molecules, such as prenyl groups attached to proteins (farnesyl or geranylgeranyl groups), which are essential for the membrane anchorage of proteins involved in signal transduction and crucial for regulation of cell growth. Isopentenyl groups on some tRNAs are essential for maintaining the binding to the ribosome and for codon interaction with their cognate anticodons [80].

The synthesis of all isoprenoids occurs by successive condensations of the two isomers of the universal precursors 5-carbon isoprene unit: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Two different pathways exist to synthesize these basic building blocks, namely the mevalonate pathway, which is targeted by statins affecting human HMG-CoA reductase to reduce hypercholesterolemia, which is used by most eukaryotes including humans and trypanosomatids [81–84], and the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway [85, 86], which has gained great interest as a potential drug target against apicomplexan diseases such as malaria and toxoplasmosis. *Cryptosporidium* does not appear to have its own *de novo* synthesis of isoprene units and relies upon scavenging them from its host for its own downstream needs [87].

Drugs targeting the mevalonate pathway, specifically the HMG-CoA reductase inhibitors, statins, have also shown antiparasitic activities. Compactin is toxic in high concentrations and cytostatic at lower concentrations against procyclic *T. brucei.* Synvinolin arrests procyclic growth and simvastatin inhibits the multiplication of *T. brucei* [88–90]. The antiproliferative effects of lovastatin against *T. cruzi* epimastigotes and amastigotes has also been demonstrated, albeit with moderate efficacy, but they have also been shown to potentiate the antiproliferative action of other ergosterol inhibitors in *T. cruzi* [91].

In *P. falciparum*, one of the enzymes of the nonmevalonate pathway has been proven essential for survival [92]. Fosmidomycin (Figure 15.1), a potent inhibitor of the enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase, the second enzyme in the nonmevalonate pathway, has been used in the clinic as an antibacterial and displays antimalarial properties [93]. Unfortunately, fosmidomycin is not taken up by *T. gondii* and thus is ineffective; however, the herbicide lomazone does inhibit the DOXP synthase in *T. gondii* and its development [94].

However, the apicomplexan enzymes are still poorly characterized, partly due to difficulties in obtaining recombinant expression of soluble enzymes from apicomplexans and this is limiting progress in this promising area. *T. gondii* produces abscisic acid, an isoprenoid plant hormone, which induces calcium signaling that drives the egress of the parasite from tachyzoites to chronic cyst forms [95].

There are several promising drugs targets downstream of the mevalonate pathways that are being developed against trypanosomatids. The well-known antifungal Amphotericin B (Figure 15.1), a polyene antibiotic, is selective and effective due to the high affinity for ergosterol, the major sterol in *T. brucei (*insect form), *T. cruzi*, and *Leishmania* spp. [96]. Several drugs that interfere with the biosynthesis of ergosterol are being developed, and the inhibition of the farnesylpyrophosphate synthase and transferase enzymes has wide appeal as antiparasitic agents, as reviewed recently [2–4].

Bisphosphonate inhibitors of farnesyl diphosphate (FPP) synthase, an essential enzyme in *T. brucei*, inhibit the proliferation of *T. brucei.* Furthermore, extensive studies on protein prenylation, mediated by farnesyltransferase and two geranylgeranyltransferases, have shown that protein prenylation is essential in *T. brucei* and other kinetoplastids [97, 98].

Pamidronate (Figure 15.1) a nitrogen-containing bisphosphonate inhibitor of the FPP synthase causes a decrease in parasitemia in infected mice and inhibits the *in vitro* intracellular replication of amastigotes, while several repurpose human cancer transferase inhibitors have been investigated; however, none have shown adequate *in vitro* activity [99]. The utilization of "piggyback medicinal chemistry" of anticancer research in this area may lead to the identification of promising lead compounds against all three kinetoplastids.

Other enzymes of the ergosterol biosynthesis pathway have been targeted as potential valid drug targets for Chagas disease, as is the case of squalene synthase. This enzyme is responsible for the first committed step of ergosterol biosynthesis and was suggested as a viable target in these parasites *Leishmania* spp. and *T. cruzi.* Several mammalian squalene synthase inhibitors have demonstrated good *in vitro* activity in *T. cruzi* epimastigotes, affecting their growth and ultrastructure, and they show moderate *in vivo* efficacy in a *T. cruzi* mouse model [100]. Design and synthesis of aryloxyethyl thiocyanate derivatives as potent squalene synthase inhibitors result in decreased *T. cruzi* proliferation. 4-Phenoxyphenoxyethyl thiocyanate is highly promising as its mode of action has been elucidated, thus showing chemical validation [101]. Allylamine terbinafine, a squalene epoxidase inhibitor, is effective against *T. cruzi* and could be used in a combinational therapy [89].

Ergosterol biosynthesis inhibitors are among the most common drugs used to treat fungal infections, and after the validation of this pathway in different kinetoplastids, compounds that were originally developed as antifungals were tested against these parasites. While some of the early generations of imidazoles (e.g., miconazole, ketoconazole) and triazole (e.g., itraconazole, fluconazole) sterol biosynthesis inhibitors have some attenuating effect on the infections, they failed in achieving parasitological cures [102]. However, as newer azoles to treat fungi infections are still an ongoing interest for pharmaceutical companies, latest generations of drugs have also been tested for against *T. cruzi* and *Leishmania* spp., as recently reviewed [2, 3]. Several inhibitors of sterol 14 α-demethylase (CYP51) including posaconazole are also being pursued through the drug discovery pipeline against Chagas disease [103].

Drug Resistances and Its Association with Altered Lipid Composition

The alkyl-phospholipid drug miltefosine (hexadecylphosphocholine) (Figure 15.1) is the main current drug for the treatment of visceral leishmaniasis especially in India. The exact mechanism of drug action remains uncertain, and it has been suggested that the drug acts upon the CDP–choline pathway [34, 63, 104, 105], causing the cells to exhibit a reduction in membrane PC content with a synergistic increase of membrane PE. Other proposed mechanisms indicate the induction of apoptosis-like cell death [106–109] and mitochondrial dysfunction by the inhibition of cytochrome C oxidase [110]. The varied nature of these targets suggests more than one mode of action.

Resistance to miltefosine has yet to be characterized *in vivo*, but resistance has been generated in *in vitro* cultured parasites [104], and it is presumed that it is attributable to mutations resulting in a defect of a transporter. The Leishmania donovani miltefosine transporter (*Ld*MT) has been characterized as a P4 ATPase [111] and is aided by the membrane protein LdRos3, which belongs to the Cdc50 family [112]. Similar mutations were confirmed in miltefosine-resistant *L. major* [113] and thought to be the cause of innate resistance against miltefosine in *Leishmania braziliensis* [114]. Parasites containing inactive *Ld*MT have been shown to remain infective, and it was shown that resistance persists *in vivo* [115, 116].

These resistant strains were also shown to possess partially inactivated fatty acyl elongases and a reduction of sterol methylation, as indicated by altered membrane lipid composition and fluidity [117]. This has since been contradicted by metabolomic data, suggesting the resistant parasites were exhibiting an adaptive response to reduced drug concentration [118]. Drug efflux is also thought to play a role in miltefosine resistance due to increased resistance upon the overexpression of an ABC P-glycoprotein-like transporter: *Ltr*MDR1 [119–122].

Apicomplexans have been shown to alter their lipid composition/metabolism upon drug resistance. In *P. falciparum*, increased activity of PfPI3K has been shown as a marker of artemisinin resistance [123], while alterations in lipid composition have been associated with quinolone resistance [124] and the associated ABC transporters *Tg*ABCG2 [125] and *Pf*MDR [126]

Modern Technological Methods to Phenotype Changes in Lipid Content

The application of modern instrumental methods to the analysis of biomolecules won the Nobel Prize in 2002 and has since been embraced across all areas of the biosciences. Herein, we discuss the merits of mass spectrometry and NMR as key analytical tools for the study of numerous classes of lipids.

Electrospray Ionization–Mass Spectrometry (ESI-MS)

Since its application to proteins through the work of Fenn *et al.* [127] in the late 1980s, ESI-MS (thoroughly reviewed by Fenn *et al.* [128]) has rapidly become an indispensible technique in the analysis of large intact biomolecules. As a soft ionization technique, ESI-MS is highly versatile in the study of biological materials, particularly the analysis of whole cellular products ranging from metabolites and genetic material to membrane lipids and intact protein complexes [129–131]. While the precise ionization mechanism of ESI-MS will not be discussed, it should be emphasized here that material analyzed under electrospray conditions is formed from charged molecules in a solution and undergoes little fragmentation. Additionally, ESI is capable of producing multiply charged species, which can be utilized to overcome the mass-range limitation of the mass spectrometer. A review of ionization techniques and their application to lipid

analyses can be found in the literature [132, 133]. ESI-MS confers the advantage of working with solution-based extracts, allowing the analysis of cell extracts by directly infusing into the mass spectrometer. This "shotgun-"like analysis, first demonstrated by Han and Goss [134], provides a consistent representation of most lipid classes within a sample, enhancing sensitivity and ensuring reproducibility. With the advances and application of microfluidics to ESI, nanoflow ionization sources are commercially available offering enhanced sensitivity of low abundance lipid species. Identification of lipid species is commonly achieved through a tandem MS approach. With the advantage of multiple analyzers, such as a triple quadrupole, lipids in the sample can be selectively fragmented into ions characteristic of distinct lipid classes and acyl composition, enabling the differentiation of lipids from within each class alongside their specific acyl composition (Figure 15.2).

By the use of parent ion scanning, all species containing a class-specific head group can be identified in a single acquisition, as demonstrated by Schwudke *et al.*

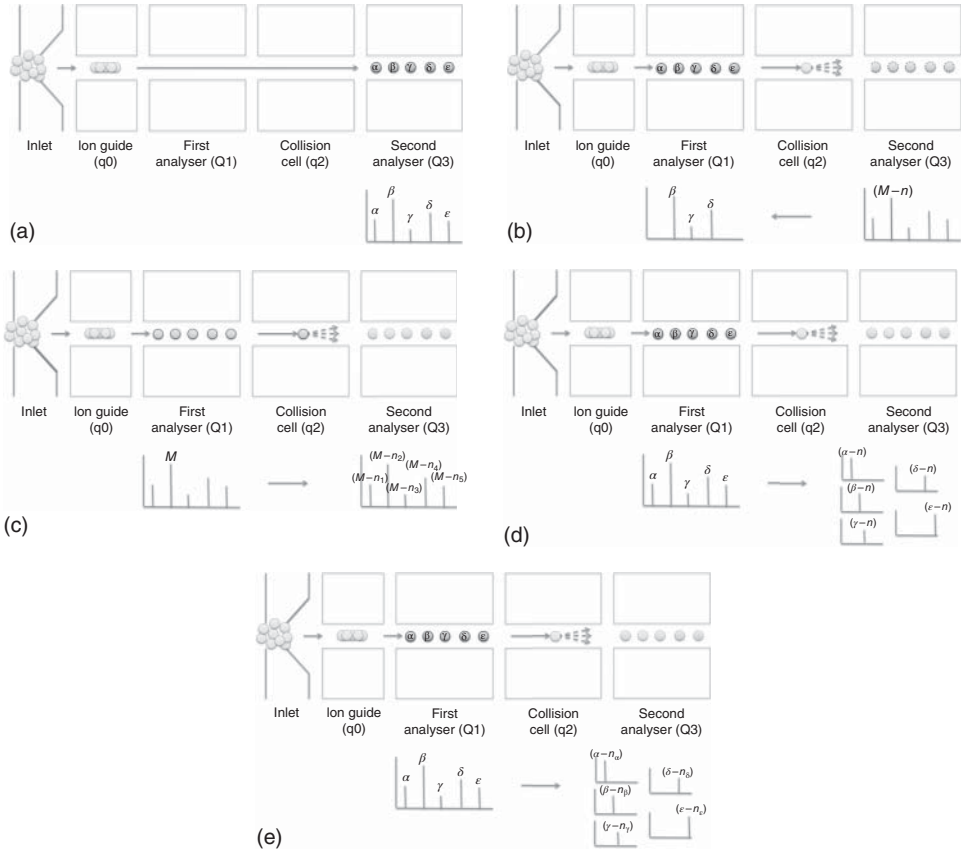


Figure 15.2 Mass spectrometer scanning modes: (a) survey scan, (b) parent ion scanning, (c) daughter product scanning, (d) neutral loss, and (e) single/multiple reaction monitoring.

[135]. The previously mentioned consistency of shotgun analysis allows quantitation of lipids through the comparison of ion intensities to an internal standard of known concentration [136]. Using a system such as an orbitrap, it is possible to perform MS^n analyses, generating a fragmentation series for detailed structural analysis.

ESI-MS can be directly coupled to chromatographic techniques such as HPLC, allowing the separation of products prior to analysis. This comes at the cost of an increased sample volume to overcome column losses and a reduction in reproducibility through nonuniform distribution of species within an eluant peak. The choice of solvent system for LC-MS is also a critical consideration, as lack of volatility or reliance on ionic additives can suppress sample ionization. Quantitation is achievable through the use of internal standards, requiring comparison to a standard curve of species within an individual lipid class. Both normal-phase (silica-based stationary phase or hydrophilic interaction columns) and reversed-phase chromatography (alkyl stationary phase, i.e., C18) are applicable to the separation of lipids and can be utilized to perform a two-dimensional separation [136]. Further information on LC-MS can be found in the two reviews by Brouwers [137] and Han *et al.* [138]. In addition to the structural resolution of individual lipid species, ESI-MS presents an attractive tool for studying lipids in parasitic organisms. Combining the wealth of information obtained from fragmentation-based scanning methods and high-resolution analyses provides a significant insight into phenotyping the lipid content of an organism. An example of this approach is in the study of *T. brucei*, whereby over 500 individual lipid species were identified from total lipid extracts from the parasite's major life-cycle forms [9]. Other studies have employed stable isotope-labeled substrates to elucidate pathway mechanisms in lipid biosynthesis, highlighting enzymes that would form attractive drug targets [139–141]. Alternately, lipidomics can be employed to examine drug activity as any change in the lipidome can be quantitated and used to elucidate mechanistic action [76, 142].

Gas Chromatography–Mass Spectrometry (GC-MS)

Differing from ESI, GC-MS is best-suited to the identification and quantification of FAs [143] and sterols. Much like LC, GC columns can be obtained with a variety of different chemistries and polarities, but for the purposes of FA analysis described here, the use of a polar column is implied unless otherwise stated. As the technique involves interactions in the gas phase, volatile solvents/compounds are utilized. To this end, FAs are commonly derivatized to enhance volatility by the formation of fatty acid methyl esters (FAMEs) [144, 145] or silyl esters [146]. The technique achieves resolution of FA species by the strength of their varied interaction to the polar stationary phase of the column, eluting FAs in the order of chain length, saturation, and branching. Unlike ESI, GC-MS utilizes either chemical ionization, which gives $[M+H]^+$, or EI, a "hard" ionization process that induces fragmentation. In this way, the by-products of fragmentation

aid in the identification of the species including information on chain length, points of unsaturation, and branching. For example, FAMEs can undergo a rearrangement upon ionization, as first described by McLafferty [147], producing a characteristic fragment of m/z 74 for straight-chain methyl esters. For further information on fragmentation of FAs via GC-MS, see the work of Jensen *et al.* [148], which covers gas-phase ion decomposition. Through the use of an internal standard added prior to derivatization, it is possible to directly quantitate FAMEs based on relative areas of chromatogram peaks. This makes GC-MS amenable to the quantitation of total FAs from biological samples, such as the FAMEs characterization of human blood [149], quantitation of bacterial FAs [150], and the characterization of a novel cyclopropyl FA in *Leishmania* [151].

Nuclear Magnetic Resonance

Typically used for in-depth structural analyses of a wide variety of molecules, NMR can also be applied to the analysis of phospholipids. While the ^{1}H spectra of a lipid mixture would be prohibitively complex, the individual phospholipid classes can be separated upon a ^{31}P spectrum [152]. The differences in the phospholipid head groups impact upon the phosphorous environment and thus the ^{31}P shift (Figure 15.3). The acquisition needs to be performed with proton decoupling to remove the nuclear Overhauser effect to ensure a single peak for an individual phospholipid class. Although analysis can be performed directly from cell lipid extracts, improved peak resolution and separation can be achieved by alteration of the solvent conditions [153]. With the addition of an internal standard, quantitation can be achieved by comparison of integration traces. This direct NMR represents a rapid route toward quantitating total phospholipid content of cell extracts, removing the need for lengthy chromatographic separation and subsequent multiple steps while giving comparative results [154]. For a more thorough separation of species, a two-dimensional ^{31}P, ^{1}H approach has been

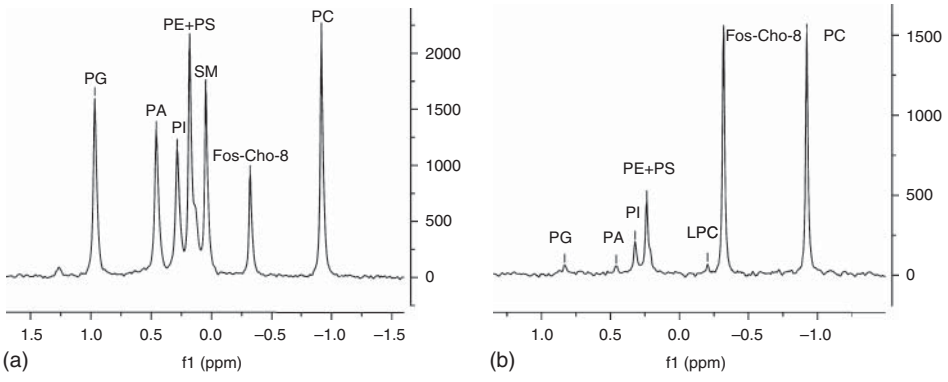


Figure 15.3 NMR spectra of (a) a mixture of lipid standards and (b) lipid extract from *Crithidia fasciculata* (density 1×10^{8} cells/ml). Fos-Cho 8 is used as an internal standard.

utilized, allowing the assignment of ether-linked PE species [155]. The main disadvantage to the analysis of lipids is the lack of sensitivity comparative to MS, as milligram quantities are required for detection, which in turn equate to large culture volumes of parasites. Additionally, while lipids are distinguishable in ^{31}P NMR by head group, no distinguishing information can be gained upon the individual composition of lipids. As seen with phospholipids, sterols can also be quantified by ^{31}P NMR, by first undergoing derivatization with a phosphorylating agent [156, 157], thus allowing direct quantification of lipid classes, sterols, and so on, from bulk lipid extracts.

Perspectives

Since the discovery of the etiological agents of these parasitic diseases, some more than 100 years ago, a cure has been pursued. Specific drugs to treat these diseases is a matter of urgency, due to the rising incidence of resistance and the general lack of efficacy of the current drugs, many of which are at least 50 years old. Extensive research in the past decade has clearly shown that rapidly dividing parasites rely heavily upon lipid metabolism, both *de novo* synthesis and scavenging, both of which contain viable and selectable targets. These potential drug targets require validation both genetically and chemically, and their suitability with respect to selectivity should be carefully considered. Structure-based approaches to inhibitor discovery are highly complementary to high-throughput screening of compound libraries. However, many of the enzymes involved are membrane-bound, this slowing down the progress on both structural studies and development of suitable assays. Despite this, lipid metabolism provides an exciting and rich seam of highly promising drug targets that can be exploited to provide future generations of drugs to tackle some of the most devastating diseases in the Third and developing world.

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16
Carbon Metabolism of *Plasmodium falciparum*

*Marco Biddau and Sylke Müller**

Abstract

Carbon metabolism of *Plasmodium* has attracted renewed interest. Advanced reverse genetics and analytical technologies allowed dissecting intermediary carbon metabolism of malaria parasites and assessing and validating its suitability for drug discovery. It was corroborated that anaerobic glycolysis is the major energy-generating pathway during intraerythrocytic life of the parasites while sexual development appears to rely much more on mitochondrial activity. The roles of parasite-specific metabolic adaptations such as carbon dioxide fixation via phosphoenolpyruvate carboxylase will be discussed especially as this diversion from glycolysis is indispensable for the human malaria parasite *Plasmodium falciparum* at least *in vitro*. It is well established that maintaining the electrochemical gradient across the inner mitochondrial membrane is vital for parasite survival, and atovaquone in combination with proguanil is the antimalarial in clinical use that takes advantage of the essential function of the mitochondrial electron transport chain. Other essential pathways suitable for drug discovery linked to carbon metabolism are those found in the parasite's plastid-like organelle, the apicoplast. The most promising is the biosynthesis of isopentenyl-pyrophosphate, which has been identified as the *raison d'être* of the organelle. Other targets, which affect liver-stage development of the parasites when inhibited, are the type II fatty acid biosynthesis (FASII) and the pyruvate dehydrogenase complex (PDC) present in the organelle, and it has been suggested that sexual development in the mosquito is affected by the loss of FAS II and PDC in the human malaria species *P. falciparum*.

An important aspect of drug discovery against human malaria that needs to be taken into account is that different *Plasmodium* species have distinct metabolic requirements, which depend on their host cell environment, and this may impact on the suitability of a potential drug target.

*Corresponding author.

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Introduction

Protozoan parasites of the genus *Plasmodium* cause malaria in a wide variety of vertebrates [1], but only five species namely *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* infect humans [2]. *P. falciparum* causes severe malaria, and the red blood cell (RBC) stages of this human malaria parasite have been studied extensively because they were adapted to *in vitro* culturing in the 1970s by Trager and Jensen [3]. The only other human-infective *Plasmodium* species that has successfully been adapted to *in vitro* culturing is *P. knowlesi* [4, 5], while *P. vivax* can only be maintained in culture with difficulty because it relies on reticulocytes as host cells [6]. Before the *in vitro* culture of *P. falciparum* was established, researchers focused on the investigation of *Plasmodium* species infecting birds (e.g., *P. lophurae*) and monkeys (e.g., *P. knowlesi*) and also those infecting rodents (*P. yoelii*, *P. berghei*, *P. chabaudi*, *P. vinckei*, etc.), all also being used as animal models for human malaria [7–13].

Early biochemical studies established that *Plasmodium* during their intraerythrocytic development generate ATP primarily through substrate-level phosphorylation via anaerobic glycolysis while they apparently do not use their single mitochondrion to generate ATP via oxidative phosphorylation in the same way as we know it from aerobic mitochondria of other organisms (Figure 16.1, Table 16.1) [24, 33–35]. This generalization is not without problems, and it was found that the metabolic diversity between *Plasmodium* species and life-cycle stages is substantial and depends at least in parts on the host cell preference of the respective parasite species [27]. Very low flux of glucose into tricarboxylic acid cycle (TCA) metabolites was determined in the rodent malaria species *P. berghei* [36, 37], while in the avian malaria species *P. lophurae* and the primate malaria species *P. knowlesi*, glucose flux into TCA cycle appears to be more significant (Table 16.1) [12, 31]. In addition, evidence was provided that *P. lophurae* and *P. knowlesi* perform CO_2 fixation as part of their carbon metabolism, and more recently, this was also shown to be the case in *P. falciparum* and *P. berghei* (Table 16.1) [7, 19, 27, 38–40]. The *Plasmodium* genomes contain genes encoding phosphoenolpyruvate carboxylase (PEPC; PF3D7_1426700) and phosphoenolpyruvate carboxykinase (PEPCK; PF3D7_1342800), respectively, both potentially involved in this process. However, employing a reverse genetics approach linked to metabolomics using heavy isotope metabolic labeling, it was shown unambiguously that *P. falciparum* PEPC fixes CO_2 [19]. The study also demonstrated that PEPCK is not involved in CO_2 fixation during RBC development of the parasites, and this is in agreement with the expression pattern of the gene encoding PEPCK, which is strictly associated with gametocyte development [39]. In other organisms, PEPCK is usually linked to gluconeogenesis, but this may not be the protein's function in *Plasmodium* given that genes encoding key components of this anabolic process are absent from the parasite genome [41].

It is now widely accepted that during life in the RBC, *Plasmodium* utilizes glucose as major energy source; the hexose is efficiently converted into lactate

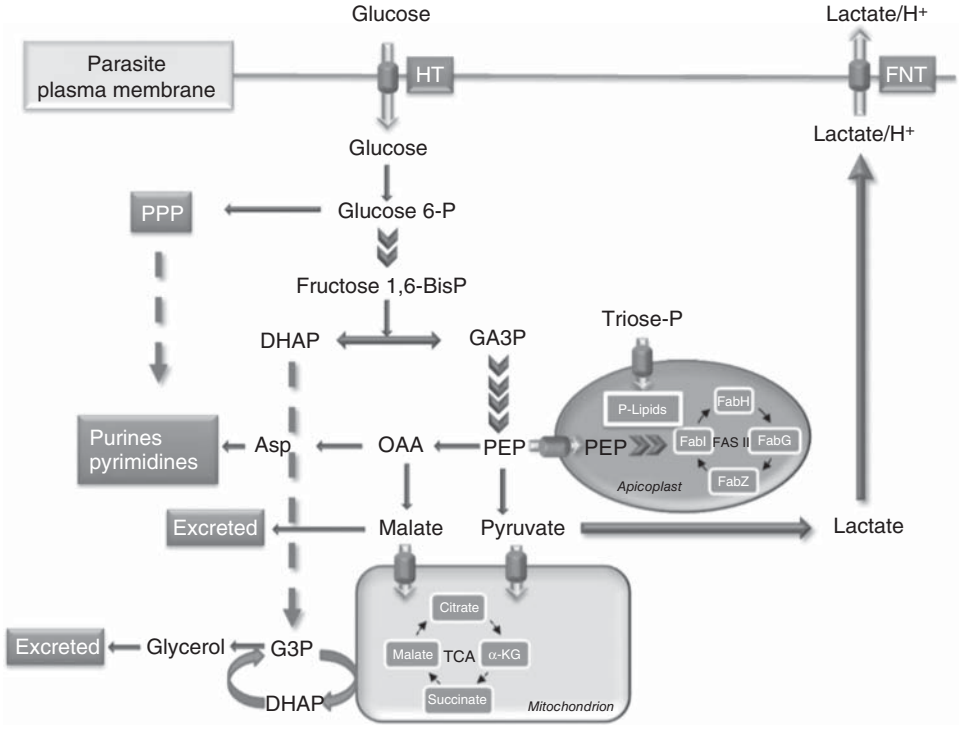


Figure 16.1 Glucose metabolism in the cytosol of *Plasmodium*. Glucose transport is increased approximately 75-fold upon the infection of RBC with *Plasmodium*. Transport is mediated through the hexose transporter (HT) in the plasma membrane of the parasites [14]. Glucose is catabolized via glycolysis with lactate being the major metabolic end product [15] that is excreted from the parasite cell via the formate–nitrite transporter (FNT, PF3D7_0316600) [16, 17]. Several metabolic pathways branch off glycolysis such as the pentose phosphate pathway (PPP) [18], the carboxylation of phosphoenolpyruvate (PEP) via PEPC to generate aspartate and malate [19]. Aspartate (Asp) is an important precursor for the salvage of purines or the biosynthesis of pyrimidines [20], while malate is imported into the mitochondrion through an α-ketoglutarate (α-KG)-malate carrier (PF3D7_0823900) [21] or excreted from the parasites potentially via FNT [16, 17, 19]. Pyruvate enters the mitochondrion by a mitochondrial pyruvate carrier (MPC 2, PF3D7_1470400) where it feeds into TCA metabolism and is involved in maintenance of intramitochondrial redox balance. PEP and triose phosphates (triose-P) are transported into the apicoplast by a triose phosphate/PEP–inorganic phosphate translocator (PF3D7_0530200) [22], which provides metabolic intermediates required for downstream synthetic pathways such as type II fatty acid biosynthesis (FASII), phospholipid biosynthesis (P-lipids), and the synthesis of isoprenoid precursors via the non-mevalonate pathway (not shown) operating in the organelle.

Table 16.1 Sources of energy in different *Plasmodium* species.

Plasmodium species	Stage	ATP generation	TCA activity	CO_2-fixation	Host cell	References
P. falciparum	Blood stage	Glycolysis	Entry points: 1. Glu/αKG 2. Pyruvate 3. Malate Not essential	Yes; essential	Erythrocytes	[19, 23]
	Gametocytes	Glycolysis Mitochondrion (cristate)	Entry points 1. Pyruvate 2. Glu/αKG 3. Malate Not essential	Yes; essentiality Not studied	Erythrocytes	[23, 24]
	Sexual development	Glycolysis Mitochondrion (cristate)	Active TCA; essential	Not studied		[25]
P. knowlesi	Blood stage	Glycolysis Use of lactate in presence of O_2 Mitochondrion	Active TCA	Yes; essentiality not studied	Erythrocytes	[26]
P. berghei	Blood stage	Glycolysis Mitochondrion (acristate)	Entry points 1. Glu/αKG 2. Pyruvate 3. Malate Not essential	Yes; not essential	Reticulocytes/ erythrocytes	[27, 28]

	Gametocytes	Glycolysis Mitochondrion (cristate)	Entry points: 1. Pyruvate 2. Glu/αKG 3. Malate	Yes; essential		[27, 28]
	Sexual development	Glycolysis and mitochondrion (cristate)	Entry points: 1. Pyruvate 2. Glu/αKG 3. Malate Essential	Yes; essential		[27–30]
P. lophurae	Blood stage	Glycolysis Mitochondrion	Active TCA	Yes; essentiality not studied	Erythrocytes (nucleated)	[31, 32]

as primary metabolic end product and in the process generating ATP and regenerating NAD^+ necessary for maintaining cytosolic redox balance [15, 19, 23, 25]. Accordingly, glucose consumption increases significantly in RBC infected with *Plasmodium* [12, 26, 42]. In *P. knowlesi*, it was found that lactate is further metabolized in the presence of oxygen (Table 16.1) [26, 43]. Otherwise, lactate, the metabolic end product of most *Plasmodium* species, is excreted into the medium/environment, and recently, the transporter responsible for this process was identified [16, 17]. Given the prominent and important role of lactate dehydrogenase (LDH; PF3D7_1324900) to uphold flux through glycolysis and to maintaining cytosolic redox balance, the enzyme was considered a suitable target for drug development. Apart from its essential role in the parasite's homolactic glucose fermentation, it has parasite-specific structural features and biochemical peculiarities that were believed to be exploitable for drug discovery [44–50]. However, this was not followed up after initial promising progress because the chemical compounds inhibiting the enzyme were deemed unfavorable for future drug development [51]. Recent advances in analytical methodologies allowed employing targeted metabolomics combined with heavy-isotope-labeling of metabolic intermediates, and this corroborated that the human malaria parasite *P. falciparum* converts most of the glucose into lactate; however, the parasites also fully oxidize a small proportion of glucose by mitochondrial, oxidative TCA metabolism during their RBC development [19, 23, 25].

This review summarizes the recent advances in our understanding of *Plasmodium* carbon metabolism, focusing primarily on *P. falciparum.* It also uses some information from rodent and other malaria species to emphasize that the specific requirements of different *Plasmodium* species that are used to assess and validate drug targets in the parasite's intermediary metabolism may differ considerably from each other, thus asking the following question: how suitable *in vitro* cultures and animal models are to investigate these highly adaptable and flexible processes in these parasites?

Cytosolic Glucose Metabolism

As outlined earlier, glucose is the major energy source during the intraerythrocytic life of *Plasmodium.* The uptake of this vital nutrient via a parasite-specific, plasma-membrane-located hexose transporter (PF3D7_0204700) [14] is therefore essential, and consequently, a knockout of the gene encoding the transporter in both *P. falciparum* and *P. berghei* was only possible when a functional episomal copy of the gene was present in the parasites [52–54]. Glucose uptake into the *Plasmodium*-infected RBC increases approximately 75-fold compared to uninfected RBCs [12, 42], and this results in a significant increase in lactate production; the metabolic end product is excreted from the infected RBC to prevent acidification of the parasite cell by a parasite-specific transporter that belongs to the microbial formate–nitrite transporter (FNT) family (PF3D7_0316600) [16, 17]. The expression pattern of the gene in *P. falciparum* matches that of the

parasite hexose transporter and the unusual primary structure, usually only found in microorganisms that transport anions such as formate, nitrite, and hydrosulfide, make this an interesting target for future drug discovery projects [55–59]. *Plasmodium* FNT was found to cotransport lactate and protons throughout the neutral to acidic pH range and its localizations in the parasite plasma membrane and the parasite digestive vacuole are consistent with its role to expel lactate and potentially other monocarboxylates and to prevent acidification of the parasite cytoplasm [16, 17].

About 75–85% of glucose is catabolized through anaerobic glycolysis [15, 42]. The residual 15% of metabolic intermediates generated through glycolysis branch off to feed other pathways such as the pentose phosphate pathway (PPP), which uses glucose 6-phosphate to convert it into ribose 5-phophate for nucleotide biosynthesis and salvage and that generates NADPH, which is required for redox reactions (Figure 16.1) [18, 60]. Other reactions that branch off the glycolytic pathway are those that use triose phosphates to form, for instance, glycerol 3-phosphate and glycerol [61]. This unusual activity may have two roles in the parasites – one to provide NAD^+, so to contribute to maintain cytosolic redox balance, and the other may be important to transfer reducing equivalents into the mitochondrial mETC via mitochondrial glycerol 3-phosphate dehydrogenase represented by one of two gene products in *P. falciparum* (PF3D7_1216200; PF3D7_1114800) [19, 61, 62].

Another unusual reaction found in *Plasmodium* is the carboxylation of phosphoenolpyruvate (PEP) through the activity of PEPC [7, 19, 38, 40]. PEPC is well characterized in plants and bacteria, and it has a key function for photosynthesis in C4 and crassulacean acid metabolism (CAM) plants, where it fixes CO_2 and the oxaloacetate (OAA) produced is reduced to malate by NAD(P)H-dependent malate dehydrogenase (MDH) [63–65]. In bacteria, PEPC has an anaplerotic role and replenishes C4-dicarboxylic acids (for instance malate) that are used for energy and biosynthetic metabolism [66]. Possible functions of this reaction for *Plasmodium* are discussed in the next section.

PEP and dihydroxyacetone phosphate (DHAP) are also transferred into the apicoplast, so contributing to the organelle's ADP/ATP balance and providing intermediates for downstream biosynthetic reactions operating in the apicoplast (Figure 16.1) [67]. Pyruvate is (apart from being converted into lactate) also transferred into the mitochondrion, where it is decarboxylated by the branched-chain α-keto acid dehydrogenase complex (BCKDH) [28, 68], an enzyme complex related to the pyruvate dehydrogenase complex (PDC). However, PDC operates exclusively in the apicoplast of *Plasmodium* [69, 70].

Mitochondrial Metabolism

The mitochondrion is known to be the power house of the aerobic cell [71]. TCA cycle activity fuels mETC, which is instrumental for the generation of ATP in aerobic organisms [72]. In *Plasmodium*, it was uncertain until recently whether a

link between cytosolic glycolysis and mitochondrial TCA metabolism exists during their intraerythrocytic development [73]. One reason for the presumption that glycolysis is not linked to TCA metabolism was that the PDC of the parasites was exclusively found in the apicoplast [35, 69, 70], and no obvious alternative was present in the mitochondrion that would allow the decarboxylation of pyruvate and the generation of acetyl-CoA to feed TCA metabolism. On the other hand, it was somewhat puzzling that the genes encoding all TCA cycle enzymes are present in the *Plasmodium* genome and that they are expressed throughout the RBC life cycle of *P. falciparum*, implying that they are functional and operating [41, 74–76]. Therefore, it was deduced that the mitochondrion has functions other than energy generation, and it was proposed that the organelle's primary role is to function as electron sink for dihydroorotate dehydrogenase (DHODH), a vital enzyme of pyrimidine biosynthesis [77].

However, several decades ago, it was shown by ^{14}C-glucose metabolic labeling experiments of avian and simian *Plasmodium* species (*P. lophurae* and *P. knowlesi*, respectively) that the carbon skeleton of glucose was not exclusively converted into lactate and excreted from the parasites but that an appreciable percentage was incorporated into TCA cycle intermediates (Table 16.1) [7, 26, 31, 32, 38]. This activity was much less pronounced in *P. berghei* and no incorporation of glucose into TCA metabolites was detectable in *P. falciparum* [35, 36]. Recent technological advances using metabolic profiling by linking liquid or gas chromatographic separations of metabolites with highly sensitive high-resolution mass spectrometry in metabolomics approaches allowed for scrutinizing the metabolic activities operating in *P. falciparum* and also in *P. berghei* more closely [19, 23]. This highlighted and confirmed that the majority of glucose (~75–85%) is catabolized to lactate through anaerobic glycolysis during RBC growth (Figure 16.1). However, as outlined earlier, the hexose is also fed into TCA metabolism in the form of acetyl-CoA resulting from pyruvate decarboxylation via BCKDH, a PDC-like enzyme complex working in the parasite mitochondrion (Figures 16.1 and 16.2) [19, 23, 25, 28]. Based on a combination of genetic and targeted metabolomics approaches, evidence was provided that BCKDH is responsible for generation of acetyl-CoA although enzymatic studies are lacking that describe the biochemical features of the enzyme complex due to problems with regenerating the parasite enzyme complex *in vitro* [28]. Surprisingly, the BCKDH subunit E1α was found to be dispensable in *P. berghei*, although mutants lacking *bckdh-e1* appear to be restricted to life in reticulocytes. This strongly supports the idea that these parasite mutants depend on host cell metabolic functions/metabolites that are absent in mature erythrocytes [27, 28]. Supplementation with acetate of the culture medium of *P. berghei bckdh-e1* knockout mutants during their intra erythrocytic maturation cycle significantly improved parasite growth, suggesting that it is acetyl-CoA or acetate that needs to be supplied by the host cell to allow normal growth in the absence of BCKDH activity [28]. The host cell preference of *P. falciparum* is apparently promiscuous

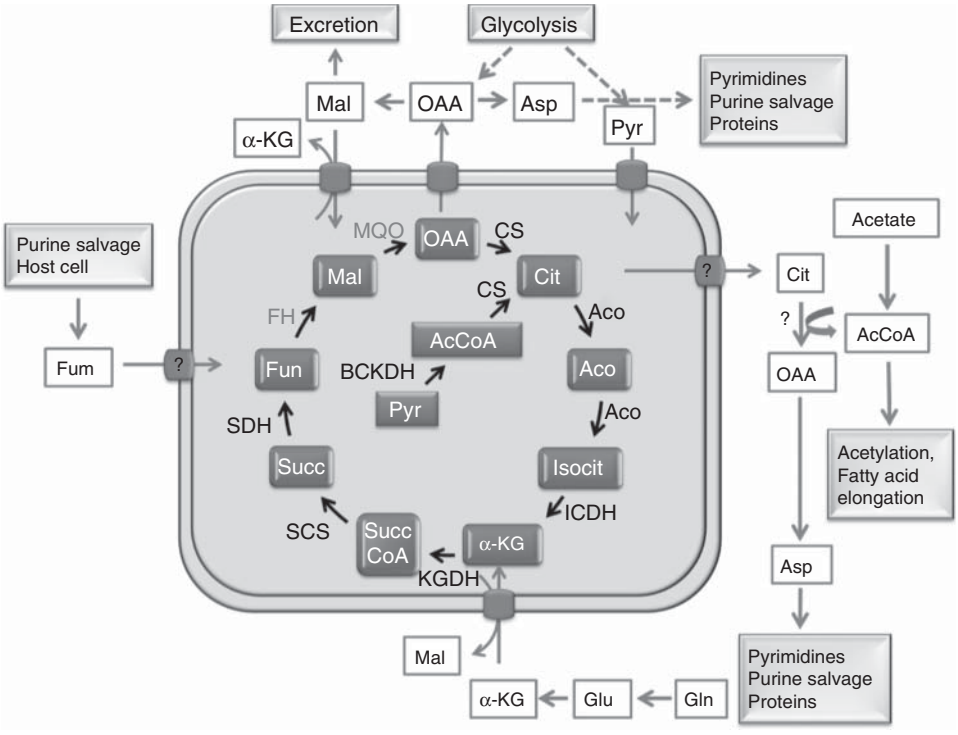


Figure 16.2 Mitochondrial TCA cycle and its links to intermediary metabolism. There are three major entry points into *Plasmodium* TCA metabolism: acetyl-CoA (AcCoA) generated from pyruvate via BCKDH, member of the α-ketoacid dehydrogenase complexes [28], malate [19], and α-ketoglutarate (α-KG) derived from glutamine [19, 23]. It is not clear whether it is oxaloacetate (OAA) or citrate (Cit) that leaves the mitochondrion to maintain the malate shuttle and to feed pyrimidine biosynthesis. In addition, it is possible that Cit contributes to the transfer of AcCoA from the mitochondrion to the cytosol; however, no experimental evidence has so far been provided to support this proposal [25, 28]. Bulusu and colleagues showed that fumarate (Fum), either derived from purine salvage or provided by the host cell, enters the mitochondrion via an unidentified transporter [20]. All TCA cycle enzymes are dispensable during blood-stage development of *P. falciparum* apart from fumarate hydratase (FH) and malate:quinone oxidoreductase (MQO) [78] emphasizing the importance of maintaining a functional malate shuttle. Abbreviations: AcCoA, acetyl coenzyme A; Aco, aconitase (Pf3D7_1342100) or aconitate; Asp, aspartate; α-KG, α-ketoglutarate; BCKDH, branched-chain α-ketoacid dehydrogenase (PF3D7_1311800; PF3D7_1312600; PF3D7_0504600; PF3D7_0303700); CS, citrate synthase (Pf3D7_1022500); FH, fumarate hydratase (Pf3D7_0927400); Cit, citrate; Fum, fumarate; Isocit, isocitrate; ICDH, isocitrate dehydrogenase (Pf3D7_1345700); Mal, malate; MQO, malate:quinone oxidoreductase (PF3D7_0616800); OAA, oxaloacetate; Pyr, pyruvate; SCS, succinyl CoA synthetase (PF3D7_1108500; PF3D7_1431600); SDH, succinate dehydrogenase (PF3D7_1033800; PF3D7_1034400; PF3D7_1212800; PF3D7_1010300); Succ, succinate; and Succ CoA, succinyl coenzyme A.

and *bckdh-e1* knockout or inhibition may also restrict the human parasite species to reticulocytes as host cells and thus may control parasitemia in an *in vivo* situation. Since parasites such as *P. vivax* are known to infect reticulocytes preferentially, the enzyme complex may, however, not be a suitable target for drug development against these human parasites [27]. A recent study by Srivastava and colleagues supports this concept and strongly suggests that reticulocytes provide a metabolically richer host cell environment for *Plasmodium* than mature erythrocytes, which clearly need to be taken into account in future drug discovery strategies [27].

Identification of the link between glycolysis and mitochondrial TCA cycle was an important and exciting step to understanding *Plasmodium* intermediary carbon metabolism, although it also demonstrated that metabolic flux from glucose into TCA cycle intermediates is only marginal during the intraerythrocytic life of the human malaria parasites *P. falciparum.* In fact, it was revealed that it is not glucose but the amino acids glutamine and glutamate that are the major source of the carbon skeleton required for the TCA activity during growth of *P. falciparum* in RBCs [19, 23, 78]. Apart from these prominent entry points into TCA metabolism, malate and fumarate were also shown to feed the TCA cycle [19, 20].

The carbon skeleton of glutamate is readily incorporated into α-KG (α-ketoglutarate), which is then metabolized largely into succinate [23, 28]. Interestingly, incorporation into fumarate and malate and further conversion into citrate are not as pronounced as would be expected. This suggests that succinate may be one of the major end products of the parasite's TCA cycle accumulating in the organelle (or being excreted) rather than being efficiently metabolized into the downstream products (Figure 16.2). Another explanation for the apparent reduced flux of heavy-isotope-labeled carbon from glutamine into fumarate and malate is a dilution of the overall label incorporation into these products through metabolites provided by the host cell. Overall, it is therefore difficult to judge the efficiency of the metabolic flux through TCA metabolism with glutamate as entry point [19, 23, 78]. This is a feasible explanation because uninfected RBCs contain appreciable amounts of fumarate and malate and also possess the catalytic machinery to convert these two metabolites into each other efficiently by erythrocytic fumarate hydratase [79]. Complete TCA cycle activity was only determined upon prolonged incubation with ^{13}C-glucose, clearly showing cyclic activity but at the same time supporting that metabolic flux through the whole cycle is only marginal [19, 23, 78]. These data may also pose the question whether the TCA cycle in *P. falciparum* really functions as a cycle at all.

Heavy-isotope-labeling studies with ^{13}C-glucose revealed that malate is another entry point into TCA metabolism (malate is a downstream product of the PEPC and MDH (PF3D7_0618500)) (Figures 16.1 and 16.2). Malate is converted into OAA and citrate via enzymes operating in the mitochondrion (malate:quinone oxidoreductase, MQO: PF3D7_0616800, and citrate synthase, CS: PF3D7_1022500) [19, 23]. The precise role of this entry point is not fully understood, but malate transports reducing equivalents across the inner mitochondrial membrane and thus may be a key player for maintenance of cytosolic

and mitochondrial redox balance, and at the same time, malate oxidation feeds electrons into the mETC and generates OAA [19]. This is reminiscent of the malate shuttle operating in other organisms; however, components that are part of the malate shuttle in other organisms are not present in *Plasmodium*, suggesting an alternative function of this shuttle in parasite metabolism [19]. Evidence suggests that OAA that is formed through the oxidation of malate by MQO in the mitochondrion is required as a substrate for CS in oxidative TCA metabolism. Citrate either is further oxidized via TCA activity or is a source for cytosolic acetyl-CoA as suggested in recent reports (Figure 16.2) [25, 28]. An alternative scenario is that OAA leaves the mitochondrion via a dicarboxylate–tricarboxylate transporter [80] and is the substrate for aspartate amino transferase (AAT; PF3D7_0204500) that generates aspartate, which feeds into pyrimidine biosynthesis [19, 20]. Interestingly, it appears that a portion of malate generated through the PEPC reaction or the malate shuttle is also excreted from the parasite [19, 78]. This *Plasmodium*-specific malate cycle may have regulatory functions and OAA appears to be the "metabolic node" that orchestrates the destination of metabolic intermediates. The importance of this reaction is supported by the finding that the deletion of *pepc* from the parasite genome resulted in a severe growth defect of *P. falciparum* RBC stages, which was also apparent in *P. berghei* lacking the gene [19, 27]. However, in the murine parasite species, the loss of PEPC function had no effect on the virulence of the parasites while sexual development was completely inhibited [27]. Together, these data suggest that PEPC has important prerequisites that make it a suitable target for future drug discovery. It is important for the survival of various stages of *Plasmodium,* and it is not present in the human host. However, it remains to be assessed whether the protein is druggable, and unfortunately, so far, it has been impossible to recombinantly express PEPC from any *Plasmodium* species – certainly something worthwhile pursuing. In the late 1980s, plant PEPC was considered a target of herbicides [81], and a recent study describes how this approach is being pursued and has identified "drug-like" compounds that can be used in crop management even distinguishing between C4 plant PEPCs and those enzyme that are not photosynthetic enzymes and therefore less likely to be good targets in plants [82].

Genetic manipulation of *P. falciparum* has now provided evidence that most enzymes of the TCA cycle such as CS, aconitase (ACO; PF3D7_1342100), isocitrate dehydrogenase (ICDH; PF3D7_1345700), the decarboxylase subunit of the α-ketoglutarate dehydrogenase (α-KGDH-E1; Pf3D7_0820700), succinate CoA synthetase (SCS; PF3D7_1437700), and succinate dehydrogenase (SDH; PF3D7_1034400) are not essential for *P. falciparum* intraerythrocytic development; the knockout of the genes resulted in no effects on *P. falciparum* viability [78]. This was particularly surprising because the main entry into the TCA cycle is via α-KG and that therefore the reaction of α-KGDH was proposed to be essential for parasite survival [35]. Instead, the parasites respond to the loss of KGDH function by adjusting their metabolic activities to compensate. Malate is no longer excreted but is now fed into the TCA cycle [19, 78].

Several investigators have deleted genes encoding TCA cycle and mETC enzymes from *P. berghei*, and they found that these were important for sexual development but only marginally affected asexual development [29, 30]. This is in agreement with the finding that asexual RBC stages only have a moderate flux of glucose into TCA metabolism while gametocytes appear to adjust their metabolic activities and have a much more pronounced flux through their TCA cycle [23].

Two genes of the TCA cycle were refractory to genetic deletion. These are encoding fumarate hydratase (FH) and MQO suggesting that they have important if not essential roles for parasite survival during RBC maturation [78]. These findings tie in well with the suggestion that the malate shuttle is essential for *Plasmodium* and that enzymes apparently part of TCA metabolism play a pivotal role in maintaining this shuttle, and it is proposed that this shuttle is crucial for redox control and pyrimidine biosynthesis (Figure 16.2) [19, 20].

Mitochondrial Electron Transport – A Validated Drug Target

Apart from TCA activity, the mitochondrion houses the mETC. In *Plasmodium,* five proteins are known to transfer electrons into mETC, namely non-proton pumping NADH:ubiquinone oxidoreductase (NDH2, complex I), succinate:ubiquinone oxidoreductase (SDH, complex II), glycerol 3-phosphate dehydrogenase, MQO, and DHODH; their individual contribution to the reduction of the ubiquinone pool is still not fully understood [83]. Other components of the mETC are ubiquinol:cytochrome *c* oxidoreductase (Cyt*bc1*, complex III), cytochrome *c* oxidase (Cyt*ox*, complex IV), and ATP synthase (complex V). The electron carriers between the complexes of the mETC are either ubiquinone or cytochrome *c* and complex III and complex IV transfer protons from the inner mitochondrial matrix into the intermembrane space, generating a proton motive force. The function of ATP synthase is not entirely clear, especially as two key subunits of the enzyme complex are missing from the parasite genome [41]. This is also the case in other Apicomplexa suggesting that the whole phylum has lost the ability to synthesize ATP through oxidative phosphorylation. On the other hand, it is possible that the genes encoding the two missing subunits of complex V are extremely divergent from those of other organisms [84] and that in life-cycle stages where ATP synthase becomes essential, the complex operates to generate ATP [85]. One important role of complex V is likely to be the maintenance of the electrochemical gradient across the inner mitochondrial membrane, a process that appears to be affected by the antimalarial drug proguanil [35, 77].

It has been demonstrated that the mETC is essential for parasite survival and has been exploited for drug development. The hydroxynaphthoquinone atovaquone inactivates Cyt*bc1*, and it was shown that the inhibitor binds to the ubiquinone oxidation site (Q_o-site) of the protein [86]. Other very promising chemicals such as acridinediones and pyridones have been identified in recent years as potent inhibitors of Cyt*bc1* and are being taken forward for future drug development [35, 87–89]. This is important as resistance against atovaquone

has arisen due to mutations of *cytbc1* [89–91]. This corroborates that the mETC in the parasites has divergent functions from those of the human host, and it has been shown that the oxidized ubiquinone pool acts as an electron sink for DHODH, an essential step of pyrimidine biosynthesis, a pathway the parasites rely upon for their pyrimidine supply because they lack the ability to salvage pyrimidines [35, 92–94]. Atovaquone apparently also interferes with the activity of other ubiquinone-dependent oxidoreductases such as MQO and SDH, which was exploited when dissecting the roles of these proteins for mitochondrial metabolism in the parasites [78].

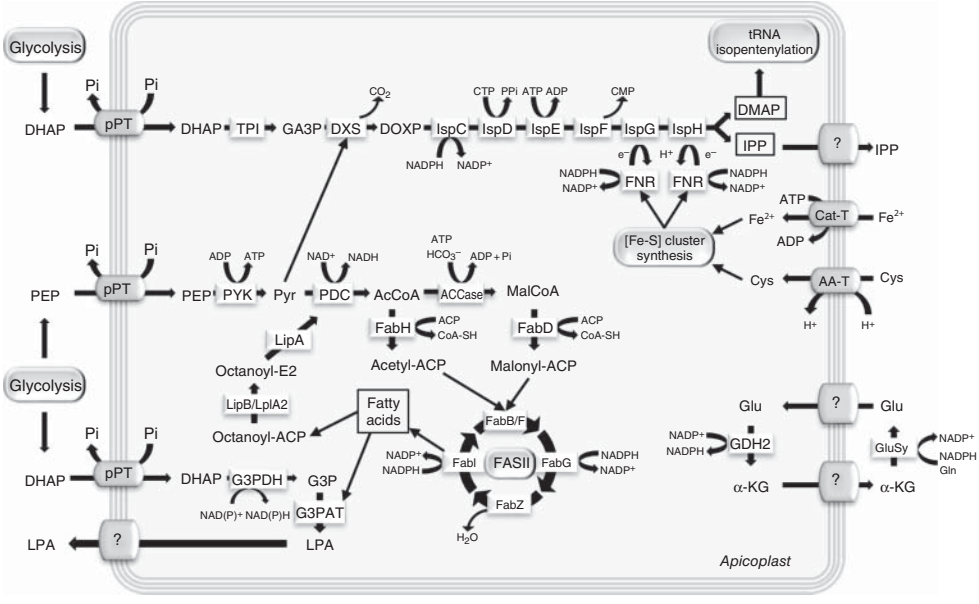
Apicoplast Metabolism

Almost all Apicomplexa possess a plastid-like organelle that is derived from an ancestral secondary endosymbiotic event [67, 95, 96]. The so-called *apicoplast* is a four-membrane-bound, plastid-like organelle that has lost its photosynthetic activity, and most of its DNA was translocated to the parasite's nucleus; only 35 kb of circular DNA are maintained in the organelle that encode less than 50 proteins, which are important for the replication of the organelle's DNA [67, 96, 97]. It is likely that apicoplast metabolism resembles that of a chloroplast in the absence of light. However, the apicoplast is unable to generate reducing equivalents lacking the photosystems usually present in a plastid organelle, and thus, it depends entirely on the parasite cell to supply reduced carbon compounds to maintain its suggested metabolic roles [22, 41]. The hypothesis that the apicoplast is a passive relict compartment acting like a "parasite in a parasite" was rapidly rejected when it was found that some drugs that directly act on the organelle's DNA replication were lethal for the parasites and so demonstrated that the organelle is essential for blood-stage development of *Plasmodium* [98–100] and suitable target for intervention strategies. Structural and bioinformatics analyses of apicoplast-targeted proteins revealed that a bipartite leader sequence comprising a signal peptide and a transit peptide is necessary for the entry of proteins into the organelle [101–103]. Based on this information, predictions suggested that more than 500 proteins may be targeted to the apicoplast, initiating the analyses of their metabolic roles [97]. Using bioinformatics approaches, the presence of metabolic pathways was proposed including the non-mevalonate biosynthesis of isoprenoid precursors (IPPs), type II fatty acid biosynthesis (FASII), and the biosynthesis of phospholipids and iron–sulfur [Fe–S] clusters. It appears that part of the heme biosynthesis pathway is also present in the organelle [41, 97, 104–106]. All of these pathways are catalyzed by enzymes homologous to prokaryotic proteins, which either are absent from the mammalian host or differ considerably from the mammalian host counterparts and thus are formidable targets for chemotherapeutic interventions [95, 107–109].

Being a synthetic and energy-dependent organelle, the apicoplast requires carbon skeleton, ATP, and NAD(P)H to maintain its anabolic roles [22]. A series of membrane translocators are required to provide these components

to the organelle. Comparing the genomes of *P. falciparum* and plants led to the identification of two genes that encode the chloroplast-like phosphate transporters in the outer and inner membranes of the organelle (pPT (plastid phosphate transporter); PF3D7_0530200; PF3D7_0508300) [110–112]. This is currently the only fully characterized apicoplast carbon antiporter providing

Figure 16.3 Carbon metabolism in *Plasmodium* apicoplast. Triose phosphates are the principal carbon sources in the apicoplast, transported into the organelle by plant-derived pPT [110, 112]. PEP and DHAP play an important role in FASII and isoprenoid biosynthesis pathways, respectively [97]. Synthesis of [Fe–S] clusters is necessary to sustain numerous of the metabolic pathways in the organelle [115]; therefore, Cys and Fe^{2+} are required and are possibly transported into the apicoplast by putative, chloroplast-like transporters; these have been identified in the parasite genome but still need to be characterized. Enzymes involved in phosphatidic acid synthesis are essential for liver-stage development of rodent malaria parasites [116]; whether the lysophosphatidic acid (LPA) synthesized is transported into the cytosol or used in the apicoplast is still unclear [116]. FASII is not essential for the intraerythrocytic stages of the parasite [117, 118]; fatty acids synthesized at different life-cycle stages can be used as substrates for phosphatidic acid synthesis, phospholipid biosynthesis, or octanoic acid is used by enzymes of the lipoylation system (LipA, LipB, and LplA2) essential for the function of apicoplast PDC [119–121]. How the apicoplast maintains its ADP/ATP and $NAD(P)^+/NAD(P)H + H^+$ balance is not fully understood, and this figure provides some information about cofactor requirements of different pathways. GDH2 is shown as a possible source for NADPH in the apicoplast [122, 123]. Abbreviations: AA-T, amino acid transporter (PF3D7_1231400); ACCase, acetyl-coenzyme A carboxylase (PF3D7_1026900; PF3D7_1469600); AcCoA, acetyl-coenzyme A; ACP, acyl carrier protein (PF3D7_0208500); Cat-T, cation transporter (PF3D7_0516100; PF3D7_0727800); DHAP, dihydroxyacetone phosphate; DMAP, dimethylallyl-pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase (PF3D7_1337200); E2, dihydrolipoamide acyltransferase (PF3D7_1020800); Fab B/F, 3-oxoacyl-acyl-carrier protein synthase I/II (PF3D7_0626300); FabD, malonyl coenzyme A-ACP transacylase precursor (PF3D7_1312000); FabG, beta-ketoacyl-ACP reductase (PF3D7_0922900); FabH, beta-ketoacyl-ACP synthase III (PF3D7_0211400); FabI, enoyl-ACP reductase (PF3D7_0615100); FabZ, beta-hydroxyacyl-ACP dehydratase (PF3D7_1323000); FNR, ferredoxin–NADP reductase (PF3D7_0623200); FASII, fatty acid synthesis II; G3P, glycerol 3-phosphate; G3PAT, glycerol-3-phosphate 1-*O*-acyltransferase (PF3D7_1318200); G3PDH, glycerol 3-phosphate dehydrogenase (PF3D7_1216200); GA3P, glyceraldehyde 3-phosphate; GDH2, NADP-specific glutamate dehydrogenase (PF3D7_1430700); GluSy, NAD(P)H-dependent glutamate synthase (PF3D7_1435300); IPP, isopentenyl-pyrophosphate; IspC, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (PF3D7_1467300); IspD, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (PF3D7_0106900); IspE, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (PF3D7_0503100); IspF, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (PF3D7_0209300); IspG, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (PF3D7_1022800); IspH, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (PF3D7_0104400); LipA, lipoyl synthase (PF3D7_1344600); LipB, lipoate–protein ligase B (PF3D7_0823600); LPA, lysophosphatidic acid; LplA2, lipoate–protein ligase 2 (PF3D7_0923600); MalCoA, malonyl-coenzyme A; PDC, pyruvate dehydrogenase complex (PF3D7_1124500; PF3D7_1446400; PF3D7_1020800; PF3D7_1232200); PEP, phosphoenolpyruvate; pPT, plastid phosphate transporter (PF3D7_0530200; PF3D7_0508300); PYK, pyruvate kinase; and Pyr, pyruvate.



PEP, DHAP, and 3-phosphoglycerate (3PGA) to the organelle while exporting inorganic phosphate (Pi). It was shown that this translocator is essential for the survival of *P. berghei* as well as *Toxoplasma gondii*, an apicomplexan parasite closely related to *Plasmodium* [113, 114]. Once in the organelle, pPT-transported metabolites have different destinies: DHAP is converted into glyceraldehyde 3-phosphate (GA3P) and used for IPP biosynthesis; it is also used to generate glycerol 3-phosphate by a plastid-specific glycerol 3-phosphate dehydrogenase and utilized for phospholipid biosynthesis (Figure 16.3) [97, 116].

Apicoplast pyruvate kinase (PyK; PF3D7_1037100) uses PEP to generate ATP and pyruvate. Pyruvate is then shared between non-mevalonate IPP and FASII while the role of 3PGA is still unclear [112]. In order to feed FASII, PDC, a key protein complex comprising four enzyme subunits (E1α, E1β, E2, and E3), decarboxylates pyruvate and generates acetyl-CoA and NADH + H^+. PDC has been extensively studied by chemical and reverse genetics approaches to evaluate and assess its importance for the survival of *Plasmodium* intraerythrocytic and other life-cycle stages, and thus, its suitability as a drug target. Combining reverse genetics and chemical inhibition suggested that PDC may be a potential drug target during intraerythrocytic life [124]. However, deletions of the genes encoding pyruvate dehydrogenase α (E1α; PF3D7_1124500) and apicoplast dihydrolipoamide dehydrogenase (aE3; PF3D7_0815900) in *P. yoelii* and *P. falciparum* revealed unambiguously that the activity of the enzyme complex is dispensable for intraerythrocytic survival of the parasites [25, 125, 126]. While not essential for intraerythrocytic growth, the *P. falciparum pdc e1α* mutants showed aberrant

oocyst development during sexual development [25], and *P. yoelii pdc e1α* and *e3* mutants displayed arrested late-liver-stage development [125]. *P. falciparum e3* mutants were only analyzed for a phenotypic response during intraerythrocytic development, and they were characterized by a persistent synchronicity *in vitro*, which is unusual when compared to the wild-type strain 3D7. In addition, their antioxidant defense appears to be upregulated to some extent [126]. Overall, the data accumulated so far clearly show that PDC is not suitable for the development of drugs that target the intraerythrocytic growth of *Plasmodium*, but the enzyme complex may be exploitable for the design of transmission blocking agents as well as for inhibitors that affect liver-stage development. The latter has some interesting prospects as the late liver stages of *Plasmodium* potentially could trigger a protective host immune response as discussed by van Schaijk *et al*. [127]. It is surprising that the *e2* subunit of *Plasmodium* PDC (PF3D7_1020800) has not been analyzed in any of the attempts to assess the function of the protein complex in the various parasite species. Our own attempts to delete the gene encoding PDC E2 were so far unsuccessful, and we are currently pursuing a conditional knockout approach to assess the function of this PDC subunit. The reason for pursuing this approach is that this protein may have moonlighting functions as has been revealed for plant plastid PDC E2. Plant plastid PDC E2 is a regulator of gene expression, and the enzyme is crucial for the control of metabolic functions of the organelle [128–130]. If *Plasmodium* PDC E2 has similar functions, which are distinct from those associated with PDC activity, it is possible that these are vital to maintaining organelle integrity and thus may be indispensable for parasite survival explaining as to why it was so far impossible to delete the *pdc e2* gene from the *P. falciparum* genome (Marco Biddau *et al.*, unpublished observations).

Acetyl-CoA produced by PDC is the substrate of the well-characterized apicoplast-located FAS II [107, 131, 132]. For almost a decade, this pathway was considered one of the chief new drug targets against intraerythrocytic development of *Plasmodium*, but in 2008, it was demonstrated by several research teams that enzymes comprising FASII in *P. falciparum* and other *Plasmodium* species were not essential for parasite survival during their intraerythrocytic growth, and as outlined earlier, this pathway is only essential for late-liver-stage development of the parasites [117, 118]. Similarly to the *pdc* gene deletions, it was found that in *P. falciparum*, FASII arrests sexual development of the parasites in the mosquito host [25, 127], suggesting that the metabolic requirements of the rodent malaria models and those of *P. falciparum* may differ considerably [27, 107, 127].

Unlike FASII, IPP biosynthesis is an essential apicoplast pathway during intraerythrocytic development and is subject to extensive studies aimed at developing antimalarial drugs [104, 106, 133, 134]. Fosmidomycin inhibits 1-deoxy-D-xylulose-5-phosphate reductoisomerase, and, in combination with clindamycin, showed promising antimalarial activity in clinical studies [135]. However, resistance was observed rapidly against this drug combination, and therefore, new chemicals targeting this pathway that will not lead to drug resistance so fast are urgently needed [136, 137]. The essentiality of isoprenoids can be explained by their crucial roles as precursors for prosthetic groups

in various enzymes (such as the prenylation of proteins, [138]), by providing dolichol precursors involved in protein glycosylation and by being precursors of ubiquinones and plastoquinones, which are typical electron carriers in the mETC [97, 139]. The apicoplast houses the nonmevalonate IPP synthetic pathway, which is only found in plants and microorganisms but not in humans (Figure 16.3) [97, 104, 139]. It is suggested that the end products of the pathway IPP and DMAP (dimethylallyl-pyrophosphate) are exported from the apicoplast – although a specific transporter has not yet been identified [97]. DMAP may also have a role in gene regulation by conferring the isopentenylation of tRNAs (Figure 16.3). These modified tRNAs may be important for the suppression of frameshift mutations and early stop codons by modifying codon–anticodon interactions [97]. The essential role of IPP biosynthesis is further emphasized by Yeh and DeRisi, who concluded that the only essential role of the organelle is the generation and provision of IPP based on an inhibition and chemical rescue study [106]. In fact, IPP biosynthesis alone needs one molecule of ATP, one molecule of GTP, and three molecules of NADPH to generate one molecule IPP and one DMAP molecule [140]. Moreover, maintaining the enzyme activities requires a constant supply of [Fe–S] clusters [115, 141] generating an additional necessity for ATP in the organelle. Further, a source for the amino acid L-cysteine is obligatory, and this is possibly achieved via an amino acid transporter (AA-T) in the apicoplast membrane although no experimental evidence exists to support this (Figure 16.3).

The relevance of [Fe–S]-cluster biosynthesis has highlighted the potential of this pathway as a suitable drug target, seemingly even acting at different parasite stages. Therefore, it has attracted some attention in the recent past and are hopefully providing new insights that can inform future drug discovery programmes against malaria [115, 142, 143].

In addition, the apparent ATP and NAD(P)H requirement may well exceed what is provided by PyK and by PDC (providing NADH) and a putative apicoplast-targeted pyridine nucleotide transhydrogenase (converting NADH into NADPH) – (PF3D7_1453500) [41]. Other Apicomplexa such as *T. gondii* retain an electron shuttle system across the apicoplast membrane where DHAP-derived GA3P is converted into 1,3-diphosphoglycerate (1,3-DPGA) by glyceraldehyde-3-phosphate dehydrogenase (GAPDH; PF3D7_1462800), then excreted as 3PGA by the action of a phosphoglycerate kinase (PGK; PF3D7_0922500) and pPT transporters, generating NADPH and ATP in the apicoplast [97]. This shuttle operates in plants in reverse and *Plasmodium* appears to lack the plastid GAPDH and PGK system, excluding this option for the malaria parasites to maintain ADP/ATP ratio and redox balance in the organelle [97]. An example for an enzyme that may provide an additional source for reducing equivalents to sustain organelle redox homeostasis in *Plasmodium* is the apicoplast glutamate dehydrogenase (GDH2; PF3D7_1430700), which reduces $NADP^+$ to $NADPH + H^+$ and converts L-glutamate to αKG (Figure 16.3) [122]. αKG, if excreted into the cytosol, can then enter the mitochondrion where it feeds into the TCA cycle, or it is used as substrate for the putative glutamate synthase

(PF3D7_1435300) as a nitrogen source for downstream reactions operating in the parasites [122, 123].

Conclusions

In this review, it was outlined that *Plasmodium* carbon metabolism offers numerous choke points that may be suitable drug targets. Several of these have been investigated in detail, and initial inhibitor screening projects have identified compounds that interfere with different parts of the parasites' carbon metabolism. Clearly, the dependence on glycolytic function has made LDH an attractive target but initial enthusiasm vanished when it was found that despite the parasite-specific features of *Plasmodium*, LDH, the protein, could not be targeted by chemicals suitable for future drug development.

The past decade has also revisited the investigation of intermediary carbon metabolism in *Plasmodium*, and the combination of genetic manipulation with focused metabolomics has been very informative and proved to be a powerful approach that helped to dissect mitochondrial TCA activity in particular and led to a better understanding of some of its functions especially during intraerythrocytic development of *P. falciparum* and also *P. berghei.* These approaches revealed that mitochondrial TCA activity is largely dispensable for parasite viability through their asexual development in the RBCs and that these reactions become much more significant when the parasites develop into gametocytes and further for their sexual development [19, 23, 27, 28, 30, 78]. However, most of the studies in the mosquito vector were performed using the animal model parasite *P. berghei*, and it remains to be established how transferable metabolomics datasets are between mouse model parasite species and human malaria species. The reported findings clearly reflect different metabolic requirements of the developing parasites in their differing host environments and provide valuable information about the flexibility of parasite metabolism and their ability to adapt their metabolic activities to varying environmental and nutritional conditions.

A well-known compound, which is indeed used as a drug against malaria, is atovaquone (used in combination with proguanil, Malarone). Atovaquone targets Cyt*bc1* in the mETC of the parasites, and unfortunately, resistance against the drug is now spreading. Surprisingly, other mitochondrial activities comprising the TCA cycle appear to be dispensable for intraerythrocytic growth but become important during gametocytogenesis and thus may be suitable for transmission blocking intervention. Only fumarate hydratase, MQO in the mitochondrion, and PEPC in the cytosol have additional potential for drug discovery probably because they form an unusual malate shuttle that has regulatory functions in intermediary carbon metabolism. In this context, it has recently been postulated that, for instance, host cell preference of different *Plasmodium* species dramatically impacts on the metabolic requirements of the respective parasites and that the apparent versatility of host cell preference of *P. falciparum* greatly influences the

potential to combat this devastating parasite during its life in the human host. Similarly, it was suggested that the strict reticulocyte host-cell preference of *P. vivax* may pose some therapeutic hurdles that need to be carefully considered [27].

Metabolic activities occurring in the apicoplast are mostly parasite-specific and were initially considered to be highly promising antimalarial drug targets. Particular emphasis was put on assessing parasite FASII after initial studies suggested its essentiality for parasite growth *in vitro* and *in vivo.* This was, however, found to be flawed, and instead, it was demonstrated that FASII is essential for late-liver-stage development, thus changing the emphasis of the drug discovery process entirely. Another promising target pathway resident in the apicoplast is IPP biosynthesis, but so far, no clinical viable drug has become available despite encouraging results with fosmidomycin/clindamycin in clinical trials. Although there are promising and encouraging reports that propagate the organelle and its specific pathways as drug target, there are still many questions that need to be addressed to fully understand the function of the apicoplast and the pathways it contains. It is, for instance, an enigma how the organelle obtains sufficient ATP, carbon skeletons, reducing equivalents, and other building blocks required for the various anabolic activities occurring within it, given that *Plasmodium* appears to not contain a plastid glycolytic pathway and hexoses are unlikely to be transported into the organelle by pPTs [22, 112]. Recent advances in metabolomics and development in whole and intact organelle isolation methods [144] will be useful in future endeavors to provide valuable information on how the apicoplast copes with the apparent energy deficit derived from its activities. Only when we fully understand the functions of these metabolic activities and how these contribute to parasite survival, will it be possible to define, identify, and evaluate new drug targets acting on an organelle that has already been shown to be essential for parasite survival and is in principle a highly exploitable chemotherapeutic target.

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Abbreviations

α-KG	α-Ketoglutarate
AA-T	Amino acid transporter
ACCase	Acetyl-CoA carboxylase
AcCoA	Acetyl-CoA

Aco	Aconitase
ACP	Acyl carrier protein
BCKDH	Branched-chain ketoacid dehydrogenase
Cat-T	Cation transporter
Cit	Citrate
CS	Citrate synthase
DHAP	Dihydroxyacetone phosphate
DMAP	Dimethylallyl-pyrophosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
FabD	Malonyl CoA-ACP transacylase precursor
FabG	Beta-ketoacyl-ACP reductase
FabH	Beta-ketoacyl-ACP synthase III
FabI	Enoyl-ACP reductase
FabZ	Beta-hydroxyacyl-ACP dehydratase
FNR	Ferredoxin – NADP reductase
FH	Fumarate hydratase
FNT	Formate – nitrite transporter
FASII	Fatty acid synthesis II
Fum	Fumarate
G3P	Glycerol 3-phosphate
G3PAT	Glycerol-3-phosphate 1-*O*-acyltransferase
G3PDH	Glycerol 3-phosphate dehydrogenase
GA3P	Glyceraldehyde 3-phosphate
GDH2	NADP-specific glutamate dehydrogenase
GluSy	NAD(P)H-dependent glutamate synthase
HT	Hexose transporter
ICDH	Isocitrate dehydrogenase
IPP	Isopentenyl-pyrophosphate
Isocit	Isocitrate
IspC	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
IspD	2-*C*-Methyl-D-erythritol 4-phosphate cytidylyltransferase
IspE	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol kinase
IspF	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase
IspG	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
IspH	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase
KGDH	α-Ketoglutarate dehydrogenase
LipA	Lipoyl synthase
LipB	Lipoate – protein ligase B
LPA	Lisophosphatidic acid
LplA2	Lipoate – protein ligase 2
Mal	Malate
MQO	Malate:quinone oxydoreductase
OAA	Oxaloacetate
PDC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate

PPP	Pentose phosphate pathway
pPT	Plastid phosphate transporter
PyK	Pyruvate kinase
Pyr	Pyruvate
SCS	Succinate CoA synthetase
SDH	Succinate dehydrogenase
Succ	Succinate
Succ CoA	Succinyl-CoA
TCA	Tricarboxylic acid cycle
TPI	Triosephosphate isomerase

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Part Three
Gene Expression and Its Regulation – A Promising Research Area for Drug Discovery

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17
Epigenetic Gene Regulation: Key to Development and Survival of Malaria Parasites

*Sabine Anne-Kristin Fraschka and Richárd Bártfai**

Abstract

Malaria parasites exhibit a very complex, multistage life cycle that requires extensive and accurate regulation of gene expression. Over the past decade, it has become increasingly clear that epigenetic mechanisms are a prerequisite for tightly controlled gene expression regulation by orchestrating chromatin composition, structure, and dynamics and, hence, play a key role in the development and survival of the human malaria parasite *Plasmodium falciparum*. This chapter provides an overview of *P. falciparum*'s epigenetic machinery and its role in various biological processes. It summarizes the current knowledge on *P. falciparum*'s chromatin landscape and illustrates the complexity and importance of epigenetic regulation of gene expression in *P. falciparum* by means of two examples: the pathogenic process of antigenic variation and the transmission-ensuring process of gametocyte conversion. Although, until now, the precise involvement of epigenetic mechanisms in these processes remains to be unraveled, it becomes evident that deciphering these mechanisms will contribute to understanding parasite pathogenicity and could help to find new strategies to fight this challenging parasite.

Introduction

Plasmodium is the unicellular parasite that causes malaria. It has a complex life cycle involving two hosts: the mosquito vector and a vertebrate host. Within both hosts, *Plasmodium* undergoes rapid transitions into several morphologically and functionally distinct forms. Unlike in multicellular eukaryotes, where the majority of cells differentiate into certain cell types with stable patterns of gene expression, *Plasmodium* constantly reestablishes its gene expression pattern for every single form within its life cycle. However, *Plasmodium* is also capable to maintain expression patterns of certain genes over several generations; for instance, the expression

*Corresponding author.

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of a single antigenic variation gene is conserved during multiplication of asexual blood-stage parasites. To achieve this, *Plasmodium* not only needs to control its gene expression in a perfectly timed manner but also needs to "remember" patterns of gene expression over generations of parasites.

Over the past decades, scientists investigated the role of epigenetic mechanisms in controlling the regulation and memory of gene expression of this perplexing eukaryote. In this chapter, we provide an overview of the various epigenetic mechanisms and discuss why they are key to parasite survival. We mostly refer to findings obtained from the intraerythrocytic cycle of the human malaria parasite *Plasmodium falciparum*, unless indicated otherwise.

Epigenetics – An Overview

"Epigenetics" in its classical term refers to "causal interactions between genes and their products, which bring the phenotype into being" [1]. In other words, cells with an identical genome are able to transmit different phenotypic traits to their descendants. Nowadays, the term *epigenetics* is more commonly used to refer to any chromatin-based regulatory processes, for example, posttranslational modifications (PTMs) of histones or DNA methylation. Therefore, the classical definition is often paraphrased as "epigenetic memory," meaning that genetically identical cells give rise to different phenotypes that are propagated over several generations.

The epigenetic machinery includes a wide range of components, including DNA, noncoding RNA, and numerous proteins. The well-known histones form the building block of chromatin, the nucleosome. A single nucleosome consists of a histone octamer composed of two of each canonical histone (H2A, H2B, H3, and H4) and approximately 147 bp of DNA wrapped around it. Canonical histones can be exchanged by histone variants, which may alter the biophysical properties of nucleosomes and provide distinct sites for PTMs. All histones and their variants can be modified posttranslationally with different chemical groups (e.g., acetylation, mono-, di-, or trimethylation, and phosphorylation), or they can be conjugated to small proteins (ubiquitination, sumoylation). Particularly, serine, arginine, and tyrosine residues located in the N-terminal histone tail, protruding from the octamer core, are subject to these modifications. In addition, DNA itself can be modified with chemical groups (e.g., methylation or hydroxymethylation) [2]. Proteins that place ("writers"), remove ("erasers"), or bind to ("readers") modifications are also considered prime components of the epigenetic machinery and have key functions in dynamic modifications and interpretation of the epigenetic code [3]. "Writers," for instance, attach acetyl groups to lysine residues (histone acetyltransferases – HATs) or methyl groups to lysine (histone lysine methyltransferases – HKMT) or arginine residues (histone arginine methyltransferase – HRMT). "Erasers" reverse these modifications by removing acetyl (histone deacetylases – HDACs) or methyl groups (histone demethylases). "Readers" contain domains that recognize and bind to

specific modifications. These proteins often reside in multiprotein complexes or contain multiple distinct domains themselves, thereby exhibiting combinations of "reader," "writer," or "eraser" activities. One well-investigated "reader," for example, is heterochromatin protein 1 (HP1) that binds trimethylated lysine 9 of histone 3 (H3K9me3) directly through its chromodomain. Interestingly, HP1 also recruits the enzyme SU(VAR)39, a HKMT, which "writes" the H3K9me3 modification providing a prime example of how epigenetic processes are often interconnected [2].

All components of the epigenetic machinery together orchestrate the composition, the structure, and the dynamics of chromatin. This in turn contributes to the regulation of chromatin structure dependent processes such as chromosome segregation and transcriptional regulation of gene expression. For instance, "open" chromatin can facilitate the binding of transcriptional activators and thus transcription of the underlying gene (loosely packed and active chromatin state = euchromatin), whereas denser "closed" chromatin has the opposite effect and silences gene expression (compact and inactive chromatin state = heterochromatin). Chromatin is also subjected to a "higher order," 3D organization within the nucleus. Heterochromatic regions, for instance, often cluster and are localized close to the nuclear periphery (e.g., lamina) while euchromatin is more commonly associated with the nuclear lumen.

The Unique Genome and Chromatin Landscape of *Plasmodium falciparum*

P. falciparum has a haploid genome organized into 14 chromosomes consisting of approximately 23 million bp. More than half of the genome is covered by coding sequences that encode about 5300 proteins, and almost 80% of the genome is transcriptionally active during blood-stage development [4, 5]. Remarkably, the *P. falciparum* genome is extremely AT-rich with an overall A + T content of 80.6%. Interestingly, the base composition differs in different chromosome regions. The centromeres are most A + T-rich (~97%), followed by intergenic regions (~86%) and coding sequences (~76%). Telomeric and subtelomeric regions, on the other hand, contain the most GC-rich sequences (~73% A + T) [5].

The "chromatome" of *P. falciparum* is composed of the four canonical histones (H2A, H2B, H3, and H4), four histone variants (H2A.Z, H2B.Z, H3.3, and CenH3), at least 70 different PTMs including methylation, acetylation, phosphorylation, sumoylation, and ubiquitinylation of certain amino acid residues [6–10], and numerous "reader," "writer," and "eraser" proteins of which very little is known. Nonetheless, 4 HATs, 10 HKMTs, 3 HRMTs, 1 DNA (cytosine-5)-methyltransferase (DNMT), 5 HDACs, 3 lysine demethylases, and at least 18 "readers" have been predicted based on domain and homology searches (Table 17.1) [11, 12]. Specific combinations of (variant) histones and their modifications "divide" the genome into functionally distinct chromatin domains (centromere, telomere, eu- and heterochromatin) [13–15]. The epigenetic makeup of these domains is discussed in the following sections.

Table 17.1 The "writers," "erasers," and "readers" of *P. falciparum*'s epigenetic machinery [11].

	Enzyme or domain/PTM mark	Gene ID/annotation/PTM mark[a)]
"Writers"	Histone acetyltransferase (HAT)	PF3D7_0823300/PfGCN5 PF3D7_1118600/PfMYST PF3D7_1227800/PfElp3? PF3D7_0416400/PfHAT1
	Histone lysine methyltransferase (HKMT)	PF3D7_0629700/PfSET1 PF3D7_1322100/PfSET2 PF3D7_0827800/PfSET3 PF3D7_0910000/PfSET4 PF3D7_1214200/PfSET5 PF3D7_1355300/PfSET6 PF3D7_1115200/PfSET7 PF3D7_0403900/PfSET8 PF3D7_0508100/PfSET9 PF3D7_1221000/PfSET10
	Histone arginine methyltransferase (HRMT)	PF3D7_1426200/PfPRMT1 PF3D7_1361000/PfPRMT5 PF3D7_0811500/PfCARM1
	DNA (cytosine-5)-methyltransferase (DNMT)	PF3D7_0727300/PfDNMT
"Erasers"	Histone deacetylase (HDAC)	PF3D7_0925700/PfHDAC1 PF3D7_1472200/PfHDA1 PF3D7_1008000/PfHDA2 PF3D7_1328800/PfSir2A PF3D7_1451400/PfSir2B
	Lysine demethylase	PF3D7_0809900/PfJHDM1 PF3D7_0602800/PfJHDM2 PF3D7_1211600/PfLSD1
"Readers"	Bromodomain/Kac	PF3D7_0823300/PfGCN5 PF3D7_0629700/PfSET1 PF3D7_0110500 PF3D7_1212900 PF3D7_1234100 PF3D7_1033700 PF3D7_1475600

Table 17.1 (*continued*)

Enzyme or domain/PTM mark	Gene ID/annotation/PTM mark[a)]
Chromodomain/Kme	PF3D7_1220900/PfHP1/H3K9me3 PF3D7_1118600/PfMYST PF3D7_1140700
Double chromodomain/Kme	PF3D7_1023900
Tudor domain/Kme	PF3D7_1136300/PfTSN PF3D7_0323500
PHD fingers/Kme	PF3D7_0629700/PfSET1 PF3D7_1322100/PfSET2 PF3D7_1221000/PfSET10/H3/H3K4me PF3D7_1360700/SUMO ligase PF3D7_1211600/PfLSD1 PF3D7_0310200 PF3D7_1008100 PF3D7_1141800 PF3D7_1433400
14-3-3 proteins/Sph	PF3D7_0818200/H3S28ph/H3S28pS32h PF3D7_1362100 PF3D7_1422900

a) Only for "readers," if known.

Centromeres

The smallest and most AT-rich chromatin domains, the centromeres, are essential for the proper segregation of the chromosomes during cell division. They are demarcated by the histone variant PfCenH3 throughout the entire intraerythrocytic cycle [16] (Figure 17.1). On each chromosome, PfCenH3 covers a single region of 4–4.5 kb that encompasses a 2–2.5 kb repeat-rich core with an A + T content of approximately 97%. In addition to PfCenH3, centromeric nucleosomes may also contain the histone variants PfH2A.Z and PfH2B.Z [16]. Next to these histone variants, small non-coding RNAs transcribed from bidirectional promoters flanking the AT-rich centromere cores associate with the centromeric DNA [17, 18] and were suggested to play an important role in centromeric chromatin formation, for instance, as a tether to recruit PfCenH3 [13, 17, 18]. Interestingly, while centromeres are delineated by pericentromeric heterochromatin in many – including evolutionary-related – organisms [19], heterochromatic marks are apparently absent near *P. falciparum* centromeres [16].

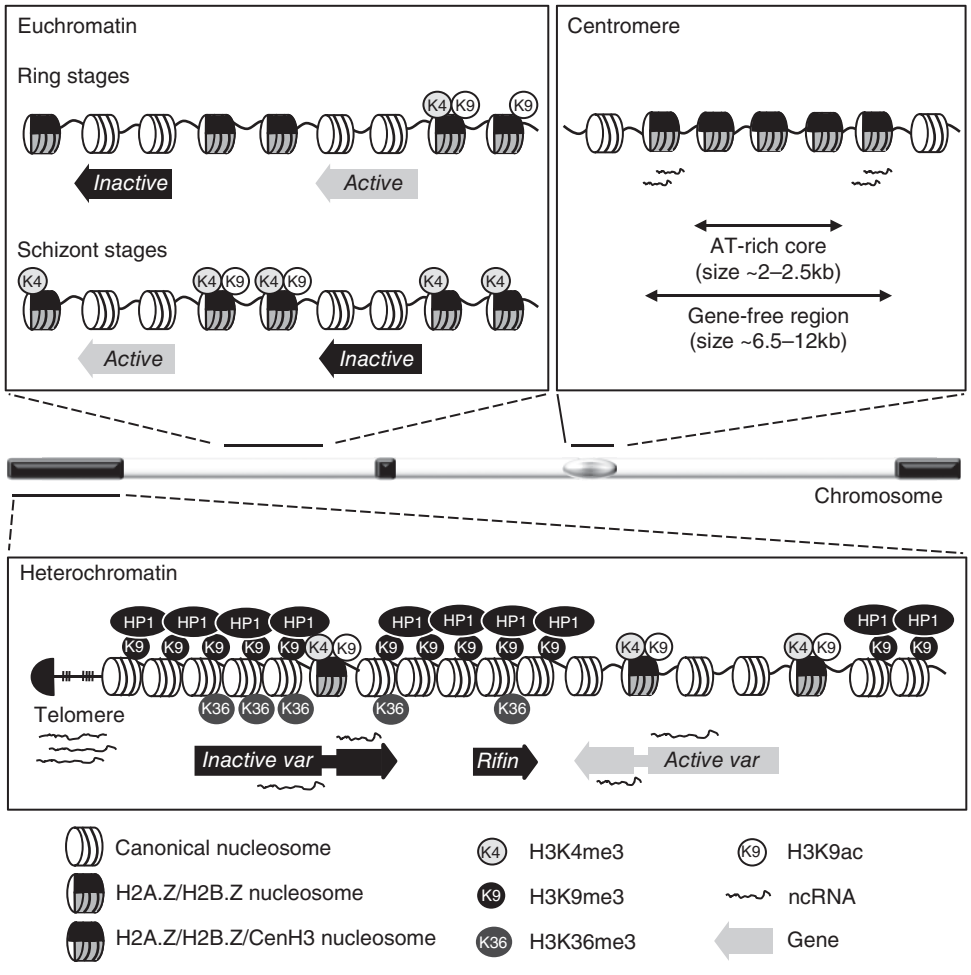


Figure 17.1 Schematic representation of *P. falciparum*'s chromatin landscape and stage-specific or transcription-coupled chromatin changes. Euchromatin (white parts of the schematic chromosome): Euchromatic intergenic regions are demarcated by PfH2A.Z/PfH2B.Z double-variant nucleosomes, H3K4me3 and H3K9ac. H3K4me3 marks euchromatic intergenic regions in a stage-specific manner: low in early blood stages, high in late blood stages. H3K9ac is placed in a transcription-coupled manner: high in 5′ upstream regions of active genes, low in 5′ upstream regions of inactive genes. Centromere (silver ellipse): The centromere is a 4–4.5 kb region demarked by PfH2A.Z/PfH2B.Z/PfCenH3 triple-variant nucleosomes; its 2–2.5 kb AT-rich core is flanked by bidirectional promoters that give rise to small ncRNAs. Heterochromatin (black blocks): Subtelomeres and intrachromosomal islands are marked by H3K9me3, PfHP1, and H3K36me3. lncRNAs are transcribed in sense and antisense orientation from bidirectional intronic *var* gene promoters. 5′ upstream and bidirectional intronic *var* gene promoter of an active *var* gene are marked with H2A.Z/H2B.Z double-variant nucleosomes, H3K9ac and H3K4me3. In the subtelomeric non-coding region TAREs 1–6 express lncRNAs.

Telomeres

P. falciparum's GC-richest sequences are the telomeres, consisting of degenerated G-rich repeats of which GGGTT(T/C)A is the most frequent [20]. Their primary function is to protect the chromosome ends, and they are organized into two parts: an internal region associated with three to four nucleosomes and an outer nucleosome-free region [20]. Telomeres of different chromosomes usually cluster together at the nuclear periphery forming four to seven heterologous chromosome-end clusters [21]. Furthermore, several findings support the idea that the outer nucleosome-free region in *P. falciparum* is similarly organized as in yeast. In yeast, the nucleosome-free region of the telomere, the telosome, is occupied by multiprotein complexes, which protect the ends of linear chromosomes from degradation by exonucleases and play a role in anchoring telomeres to the nuclear periphery [22]. Ortholog proteins of these telosome complexes in *P. falciparum* are, for instance, PfSir2A, a member of the NAD^+-dependent/class III HDAC family [23], "*P. falciparum* origin recognition complex 1 protein" (PfORC1) [24], and "*P. falciparum* telomerase reverse-transcriptase"(PfTERT) [25]. What the exact composition of this complex is, how it is recruited to the telomere, and whether it plays a role in subtelomeric heterochromatin formation in *P. falciparum* remain to be investigated.

Euchromatin

The largest part of *P. falciparum*'s epigenome is found in an open and transcriptional permissive euchromatic state. Throughout the intraerythrocytic cycle, AT-richer euchromatic intergenic regions are demarcated by PfH2A.Z/PfH2B.Z double-variant nucleosomes [26–28] and the classical "active" marks H3K4me3 and H3K9ac [26, 29–31] (Figure 17.1). H3K4me3 marks euchromatic intergenic regions in a stage-specific manner: early blood stages show only minor enrichment of H3K4me3, whereas at later stages, intergenic regions of active genes are clearly marked [26]. H3K9ac, however, follows the expression pattern of most genes and seems to be placed in a transcription-coupled manner [26, 29]. Furthermore, the following marks were found to associate with euchromatic regions: H3K14ac, H3K56ac, H3K79me3, H4K5ac, H4K8ac, H4K12ac, H4K16ac, H4K20me, and H4R3me2 [29, 30, 32, 33]. Intriguingly, "readers," "writers," or "erasers" of these modifications are still poorly investigated. However, two HATs, PfGCN5 and PfMYST, and one HKMT, PfSET8, have been characterized to some extent [34–37] (Table 17.1). PfGCN5 preferentially acetylates H3 *in vitro*, particularly on H3K9 and H3K14, and interacts with the adaptor protein PfADA2; probably within the context of a large multiprotein complex similar to other eukaryotes [35, 38–40]. Its inhibition reduced H3K9ac and H3K14ac – but not H4ac levels – and negatively affected parasite growth, suggesting an important role in genome-wide gene activation [41–43]. PfMYST, in contrast, preferentially acetylates H4 *in vitro*, especially H4K5ac, H4K8ac, H4K12ac, and H4K16ac,

and likely plays a role in transcriptional regulation of gene expression, cell cycle progression, and DNA damage repair [34]. Finally, PfSET8, which is highly homologous to *Toxoplasma gondii*'s TgSET8, may mono-, di-, and trimethylate H4K20 in *P. falciparum* [36, 37].

Heterochromatin : Subtelomeric Regions and Intrachromosomal Islands

Heterochromatin in *P. falciparum* can be found on all subtelomeric regions, some chromosome internal islands, as well as few singular genes, and encompasses approximately 400 genes, which account for approximately 8% of the parasite's coding genome [30, 31, 44–46]. The heterochromatic subtelomeres can be subdivided into two regions: (i) non-coding repeat-rich regions directly bordering the telomere and (ii) protein-coding regions. The non-coding region is composed of a mosaic of six different blocks of repetitive sequences, the "telomere associated repetitive elements" (TAREs 1–6). These are positioned in the same order on all chromosomes and span around 20–40 kb [20, 47]. Interestingly, TAREs 1–6 give rise to long non-coding RNAs (lncRNAs) with yet unknown function, which were proposed to be involved in heterochromatin establishment [24, 48–50] (see below). Strikingly, both the protein-coding subtelomeres and the intrachromosomal heterochromatic islands encode primarily for *Plasmodium*-specific multigene families, for example, *var*, *rifin*, *stevor*, and *pfmc-2tm*, which are partly involved in antigenic variation and subject to mutually exclusive gene expression [51] (see 17.4.1).

Heterochromatic regions are defined by hypoacetylation of histones and reduced accessibility to MNase digestion, suggesting more compact nucleosomal packaging [30, 31, 45]. As a result, genes underlying heterochromatic regions are generally silenced. Other hallmarks of (constitutive) heterochromatin are H3K9me3 [30, 31] and heterochromatin protein PfHP1 [44, 45] (Figure 17.1). Orthologs of PfHP1 in *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and human contain a chromodomain that mediates H3K9me2 and H3K9me3 binding and a chromo shadow domain that enables oligomerization of HP1 proteins leading to nucleosome aggregation and hence the formation of densely packed heterochromatin [52, 53]. PfHP1 also contains both domains and localizes to H3K9me3-marked heterochromatin. Furthermore, a recombinant PfHP1 chromodomain binds both H3K9me2 and H3K9me3 *in vitro*, and PfHP1 depletion results in reduced H3K9me3 levels in blood-stage parasites. Hence, it is likely that PfHP1 and H3K9me3 may have a similar role in heterochromatin formation as shown for other organisms [44, 50, 54] (see the following text). A functional homolog of SU(VAR)39, the enzyme that places H3K9me3 in most organisms, could not be confirmed in *P. falciparum*. However, based on homology searches, PfSet3 (the only *Plasmodium* protein with a pre-SET domain), appears to be the most likely candidate to deposit this mark [30, 37, 55]. Recently, also unexpected enrichment of H3K36me3 was observed in heterochromatic subtelomeres and intrachromosomal islands [56–58]. Knockdown of PfSET2, the HKMT

predicted to deposit H3K36me2/me3 in *P. falciparum*, results in decreased H3K36me3 levels and upregulation of *var* gene expression [37, 56, 57]. However, H3K36me2/3 levels were only reduced in ring parasites, suggesting that alternative enzyme(s) might perform this methyltransferase activity in other stages [56]. Additionally, H4K20me3 was reported to be present in heterochromatic regions [29, 30, 36], but the repressive H3K27me3 mark, which hallmarks facultative heterochromatin in many multicellular organisms, has so far not been detected in *P. falciparum* [9].

Formation of the silent heterochromatic state is crucial for the regulation of several clonally variant gene families and single heterochromatized genes (see the following text). Thus, it is of great interest to understand the mechanisms of heterochromatin formation in *P. falciparum*. In model organisms, assembly of heterochromatic domains is thought to occur in three different steps: (i) establishment of heterochromatin at nucleation sites due to lncRNAs or DNA-binding proteins that recruit an initial HKMT to deposit H3K9me2/3, (ii) subsequent spreading of its silencing state in a self-perpetuating manner into neighboring regions via HP1 and HKMT interactions, and (iii) its maintenance over several generations due to identical reestablishment of its state on newly replicated chromatin after each cell division, all as concerted actions of different histone-modifying "writer," "eraser," and "reader" proteins [59–64]. In *P. falciparum*, heterochromatin establishment may involve PfHDA2, a member of the class II HDAC family, and the two NAD^+-dependent/class III HDAC family members, PfSir2A and PfSir2B. Together, they may allow spreading of H3K9me3/PfHP1 by removing activating acetyl marks (especially H3K9ac), but also by recruiting additional proteins that are required for heterochromatin establishment [23, 65–68]. Additional proteins suggested to be involved in heterochromatin establishment are PfOrc1 and PfAlba. PfOrc1 binds to TAREs in a PfSir2A-dependent manner and PfAlba-containing molecular complexes associate with TARE6 and colocalize with PfSir2A [23, 24, 69, 70]. Subsequent heterochromatin spreading may require, as in other organisms, binding of PfHP1 to methylated H3K9, which in turn leads to the recruitment of a H3K9 HKMT [34, 54, 59, 61]. The HKMT may methylate H3K9 on the neighboring nucleosome, which in turn would result in PfHP1 binding and heterochromatin spreading from small nucleation sites into large chromosome domains, thereby silencing the underlying genes [71, 72]. Despite identification of numerous components of *Plasmodium*'s epigenetic machinery involved in heterochromatin formation, it still remains unclear how these components work together to orchestrate heterochromatin establishment, spreading, and maintenance in *P. falciparum*.

Epigenetic Regulation of Gene Expression in *P. falciparum*

Over the past decade, the awareness that epigenetic mechanisms play an essential role for *P. falciparum*'s biology increased. They control pathogenic processes such as antigenic variation [73, 74], sequestration of infected erythrocytes [75], and

selection of erythrocyte invasion pathways [75–77]. Furthermore, they are also involved in antibiotic resistance [78–80], adaptation to changing environments, for instance, increased blood lactate levels or increased body temperatures of the host (fever) [81], and nutrition availability and uptake [78, 80, 82]. Lastly, epigenetic mechanisms were shown to be crucial for gametocyte conversion and thus for parasite transmission [54, 65, 83, 84]. In this section, we discuss two of these processes in detail to illustrate the complexity of epigenetic regulation of gene expression.

Epigenetic Regulation of Antigenic Variation

Our current knowledge on epigenetic mechanisms in *P. falciparum* mainly derives from studying the process of antigenic variation. Antigenic variation is based on multigene families (e.g., *var*, *rifin*, *stevor*, and *pfmc-2tm*) located in heterochromatic subtelomeres and intrachromosomal islands. Most of these families encode proteins that are exported and anchored to the erythrocyte membrane [85–92]. Interestingly, within these families, only a small subset of genes is expressed while other family members remain transcriptionally silenced. Remarkably, the active state of one gene and the silent state of all other family members can be maintained over generations of blood-stage parasites, being inherited from one parasite to its descendants by an epigenetic memory [46, 93]. However, a small minority of parasites begins to express a different member within the gene family giving the infected red blood cell a "fresh makeup" [94]. Through this phenomenon, some parasites can escape the host's immune system, thus ensuring parasite survival within its host and sustained infection.

The most extensively studied multigene family, the *var* gene family, encompasses approximately 60 members. Interestingly, *var* gene expression is mutually exclusive manner, meaning that a parasite expresses only a single *var* gene at a time while all other family members remain transcriptionally silent [95]. Every *var* gene consists of two exons separated by a highly conserved intron. Exon1 encodes an extremely diverse extracellular binding region, while exon2 encodes the more conserved intracellular domain. Together, they assemble the variant adhesion surface molecule "*P. falciparum* erythrocyte membrane protein 1" (PfEMP1) whose extracellular binding region is exposed at the surface of the infected erythrocyte during trophozoite and schizont stages. Thereby, PfEMP1 exposes parasite antigens to the host immune system, which can be the target for an immune response. Nonetheless, PfEMP1 is an essential virulence factor that mediates adherence of trophozoite- and schizont-infected erythrocytes to the endothelium of capillaries and venules of the host, thus enabling the parasites to circumvent spleen-mediated clearance. Adverse effects associated with the infection are adhesion-based complications such as cerebral malaria and pregnancy-associated malaria [96].

Transcription of *var* genes occurs in 8–20-h-old ring stages of *P. falciparum* [51, 95, 97–100]. During the trophozoite and schizont stage (25–48 h after invasion),

the active *var* gene is in a "poised" state, meaning that it is not actively transcribed but still retains an epigenetic makeup that prevents silencing and enables reactivation in the next generation [101]. With low frequency, a previously silenced *var* gene becomes active while the previously active gene becomes silent. It is currently unknown to which extent this infrequent switch is hardwired into the parasite's genome or linked to external factors. However, there are few indications that external factors, for instance, starvation stress, high body temperature, and lactate levels contribute to *var* gene switching; potentially through modulation of NAD^+-dependent Sir2 activity [81, 102–104].

Over the past decade, tremendous progress has been made in unraveling the complex mechanisms of *var* gene regulation encompassing interaction of genetic elements, non-coding RNAs, chromatin modifications and modifiers, and specific subnuclear localization [13, 74, 100, 105, 106]. Crucial genetic elements involved in *var* gene regulation are the 5′ upstream *var* gene promoter that gives rise to PfEMP1-encoding transcripts and the *var* gene intron that contains bidirectional promoter activity [107–110]. Based on transgenic parasites expressing episomal or episomally integrated *var* promoters, the following observations were made: (i) 5′ upstream *var* promoter and intron pairing is required for *var* gene silencing and (ii) loss of one-to-one *var* promoter and intron pairing as well as integration of an unpaired promoter leads to *var* gene activation [108, 109, 111, 112]. Important *cis*-acting elements within the *var* locus have also been unraveled, which is further discussed elsewhere [15, 100, 113].

Particular localization within the nucleus also influences *var* gene expression as telomeres, subtelomeric, and intrachromosomal *var* genes are tethered to the nuclear periphery and activation of a single *var* gene results in its "repositioning" into a special subnuclear expression site [15, 100]. Apart from genetic elements and subnuclear localization, lncRNAs were shown to play an important role in *var* gene regulation. In particular, lncRNAs transcribed in sense and antisense orientation from the bidirectional promoter within the *var* intron have gained a lot of attention [48, 74, 107, 114–116]. Sense lncRNAs are transcribed from the highly conserved exon2 and have a size of around 2–2.5 kb. They are expressed from all *var* genes at the time when the *var* upstream promoter is silent, meaning from the onset of DNA replication (~24 h post invasion) through schizogony and merozoite formation. They were shown to associate with chromatin [114–116] and were suggested to play a role in epigenetic memory of *var* expression [115]. Antisense lncRNAs are transcribed from the variable exon1 region of *var* genes at the same time as sense lncRNAs and have a size of around 1.7 kb [114, 115]. Recently, it was shown that they associate specifically with the single active *var* gene during the ring stage and are incorporated into the chromatin. Moreover, expression of these lnRNAs *in trans* induces *var* gene activation in a sequence- and dose-dependent manner. Interference with antisense lncRNAs leads to silencing of the respective *var* gene, erases its epigenetic memory, and induces *var* gene switching [74]. Additionally, another family of lncRNAs named lncRNA-TARE-4Ls was detected [48]. These are transcribed from subtelomeric regions that contain a cluster of SIP2-binding motifs in the 5′ upstream promoter

of a subset of *var* genes and are thought to play an important role in silencing adjacent *var* genes, probably by contributing to the regulation of epigenetic *var* gene memory [48, 50]. Additionally, active, "poised," and silenced *var* genes are specially marked by histone modifications and variants implying an essential role for histone chaperones and chromatin "readers," "writers," and "erasers" in regulating *var* gene expression (Figure 17.1). Both active 5′ upstream and intronic *var* gene promoters are marked with H2A.Z/H2B.Z double-variant nucleosomes and the histone PTMs H3K9ac, H3K4me2/me3, as well as H4ac in the case of the 5′ upstream promoter [26, 28, 30, 34]. The "writers" of H4ac and H3K4me2/me3 are histone acetyltransferase PfMYST and methyltransferase PfSet10, respectively, and were shown to be enriched at the active 5′ upstream *var* promoter [34, 101]. In the "poised" state, only H3K4me2 and PfSET10 associations were observed, while 5′ upstream promoter H2A.Z/H2B.Z incorporation was lost [28, 101, 117]. This implies a role for H3K4me2 in transmitting epigenetic memory, possibly by preventing H3K9me3 to spread into the *var* promoter region while the 5′ upstream promoter is transcriptionally inactive during trophozoite and schizont stages [101, 118]. In contrast, silenced *var* genes are constantly marked by PfHP1, H3K9me3, and H3K36me3, not only in their promoter regions but also over their coding sequences [30, 44, 45, 56]. Intriguingly, *var* introns are constitutively covered by PfH2A.Z/PfH2B.Z [26, 28]. Depletion of PfHP1 or PfSET2 results in simultaneous activation of all *var* genes, even outside of the perinuclear *var* gene expression site [37, 54, 56, 58]. Similarly, depletion of deacetylating enzymes also leads to upregulation of all (PfHDA2) or subsets of *var* genes (PfSir2A and PfSir2B) [65, 66, 68] (Table 17.1).

Although *var* genes represent the most investigated example of epigenetic regulation in the malaria parasite, we are far from understanding the complex interplay of various regulatory events that orchestrate mutually exclusive expression and switching. However, it seems evident that *var* gene expression depends on complex disassembly of heterochromatin at a single locus rather than on simple transcription factor activation.

Epigenetic Mechanisms Involved in Gametocyte Conversion

Asexual replication during the intraerythrocytic cycle establishes and maintains infection of the host. To ensure transmission to other hosts, some parasites develop into gametocytes, through a process called *gametocytogenesis*. Unlike asexual parasites, gametocytes are unable to replicate within the mammalian host, but are infectious to the mosquito vector that subsequently transmits the parasites to new mammalian hosts [119]. To guarantee transmission and maintenance of infection at the same time, the switch from asexual replication to gametocyte conversion needs to be tightly controlled.

Recent studies uncovered AP2-G, a member of the Api AP2 transcription factor family, as a key regulator of gametocyte conversion in both *P. falciparum* and *P. berghei* [83, 84, 120]. *P. berghei* strains that lost their ability to generate

gametocytes showed mutations within their *pbap2-g* locus and targeted disruption of the *pbap2-g* locus caused the same phenotype [84]. Similarly, levels of gametocyte formation in *P. falciparum* strongly correlate with *pfap2-g* expression levels and disruption of PfAP2-G function resulted in loss of gametocyte formation [83]. Both in *P. falciparum* and *P. berghei*, potential AP2-G-binding motifs were identified to be enriched in the upstream region of early gametocyte genes as well as *pfap2-g* itself [83, 84]. Additionally, a potential downstream target of PbAP2-G, also a member of the AP2 transcription factor family, was identified. This AP2 modulates gametocyte conversion rates, but disruption of its gene locus does not erase gametocyte formation completely [84]. Collectively, these observations strongly indicate a cascade-like regulation of gametocyte conversion with AP2-G as master regulator and, perhaps even with an autoregulatory feedback loop.

Intriguingly, *pfap2-g* is the only AP2 family member marked by H3K9me3 and PfHP1, and its expression was recently revealed to be regulated on an epigenetic level [30, 31, 50, 54, 65]. Key regulators of *pfap2-g* expression are components of the epigenetic machinery involved in heterochromatin biology, PfHP1 and HDAC PfHDA2 [54, 65]. Conditional depletion of PfHP1 resulted in increased gametocyte conversion rates and transcriptional changes of genes associated with gametocyte development [54]. PfHP1 depletion also inhibited mitotic proliferation and caused upregulated heterochromatic multigene families (see 17.4.1). Accordingly, Brancucci and coworkers suggest that epigenetic silencing of *pfap2-g* allows continuous mitotic proliferation and counteracts gametocyte conversion. Local dissociation of PfHP1 from the *pfap2-g* locus, however, activates *pfap2-g* and induces gametocyte conversion. In addition, conditional depletion of PfHDA2 resulted in a similar phenotype with upregulation of *pfap2-g* and genes associated with gametocyte development. In contrast, knockout of NAD^+-dependent deacetylases Sir2A and Sir2B does not appear to influence gametocyte conversion rates. In conclusion, PfHP1 and PfHDA2 may cooperate to strictly control gametocyte conversion rates, potentially as part of a specific silencing complex as found in other eukaryotes [54, 121, 122].

Despite these current and exiting findings, much still remains to be uncovered on the precise regulation of *pfap2-g* expression and additional factors involved in this process. For instance, different *Plasmodium* isolates and clones exhibit highly variable gametocyte conversion rates and host signals such as anemia and cytokines influence gametocyte conversion rates [46, 54, 65, 83, 84, 123–125].

Concluding Remarks and Perspective

Plasmodium parasites exhibit an exquisitely complex life cycle alternating between intracellular, differentiating stages and extracellular, invasive stages, not only in different cell types but also within two different hosts. Prerequisite for such successful stage transition and environmental adaption is a perfectly and tightly controlled gene expression. In the previous sections, two processes with

major impact on *Plasmodium*'s survival illustrated the importance of epigenetic mechanisms in controlling *P. falciparum*'s gene expression. However, the spectrum of epigenetically regulated processes during blood-stage development is much broader, ranging from erythrocytes invasion [75–77] and nutrition uptake [78, 80, 82] to growth adaptation due to environmental changes [81]. Moreover, other life-cycle stages are poorly investigated and may reveal even more epigenetically regulated processes, for instance, variable expression of sporozoite surface proteins to evade the host's immune system or variable expression of transport channels that might allow adjustments of nutrition uptake during liver-stage development similarly to *clag 3.1* and *clag 3.2* in blood-stage development of the parasites [46]. Additionally, epigenetic mechanisms and components in other *Plasmodium* species are barely uncovered. Considering the key roles of epigenetic mechanisms in processes vital to parasite survival and transmission, identification of *Plasmodium* unique components of the epigenetic machinery provides great promise for drug development. Importantly, epigenetic drugs are successfully used in cancer treatment, for instance, Vorinostat (Zolinza) and Romidepsin (Istodax) in cutaneous T-cell lymphoma treatment [126, 127] and HDAC inhibitors were shown to inhibit malaria parasite growth efficiently [67, 128]. HAT inhibition by curcumin and anacardic acid resulted in more moderate *P. falciparum* blood-stage growth inhibition [37, 42]. However, when used in combination therapy, they might still be advantageous in counteracting rapidly increasing drug resistance [129]. Recently, two potential HKMT inhibitors, BIX01294 and its structurally related analog TM2-115, were shown to inhibit parasite growth at all stages of the intraerythrocytic cycle and exhibit a rapid killing effect [130]. Despite these promising initial results, the major challenge will be to find drug targets and drugs unique for *Plasmodium* and to reduce off-target effects, which are known to occur for all current compounds as effectively as possible. Dissecting the intricate mechanisms of epigenetic regulation in detail will significantly contribute to this task.

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18
Mechanisms Regulating Transcription in *Plasmodium falciparum* as Targets for Novel Antimalarial Drugs

*Evelien M. Bunnik and Karine G. Le Roch**

Abstract

As any organism, the human malaria parasite, *Plasmodium falciparum*, is dependent on a fine-tuned regulation of gene expression for its development and successful propagation. Recent insights into this process suggest that the parasite relies heavily on epigenetic mechanisms of gene regulation, more so than its human host. As we gain a better understanding of how gene expression is orchestrated in the parasite, novel avenues for the development of antimalarial therapies are opening up. In this chapter, various mechanisms that play a role in regulating transcription in the parasite are discussed, including nucleosome landscape, histone variants, histone modifications, and nuclear architecture. In addition, molecular components that are involved in these mechanisms and that may be promising targets of novel antimalarial drugs are reviewed.

Transcription in *Plasmodium falciparum*

Regulation of transcription is an important mechanism by which *Plasmodium falciparum* controls its development through the different stages of its life cycle. Sequencing the genome of *P. falciparum* [1] has greatly increased our understanding of the basal transcription process in these parasites, by facilitating the identification of many of the specialized proteins that are part of the transcription machinery. In eukaryotes, transcription is initiated by the assembly of RNA polymerase II and the general transcription factors into the preinitiation complex (PIC) located at the promoter (Figure 18.1a). Mining the parasite genome revealed that *P. falciparum* encodes most of the components of the basal transcription machinery that are found in other eukaryotes. In particular, all 12 RNA polymerase II subunits and a total of 23 TFII components have been identified [2, 3]. A notable observation is the apparent lack of TFIID subunits with a histone fold domain. In yeast, the TFIID complex consists of TATA-binding

*Corresponding author.

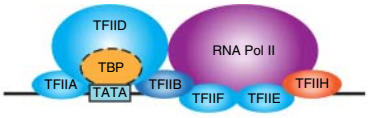
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protein (TBP) and 14 TBP-associated factors (TAFs), more than half of which contain a histone fold motif that allows the TAFs to assemble into heterodimers [4]. In *P. falciparum*, only four TAFs have been identified in addition to TBP, and none of these proteins contain a histone fold domain [3], suggesting that the structure of the parasite TFIID is highly divergent from other eukaryotes.

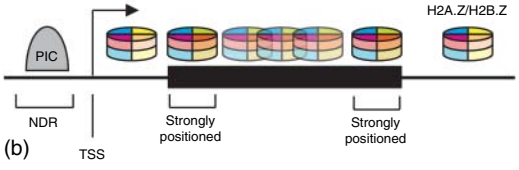
Recruitment and activation of the PIC is regulated by specific transcription factors (TFs) and large mediator complexes that bind to regulatory elements in promoters and enhancers to suppress or activate transcription initiation. In *P. falciparum*, only 3 of the 26 mediator subunits present in yeast have been uncovered (Ref. [5] and M. Featherstone, unpublished data). In addition, only approximately 30 specific TFs have been identified, 27 of which belong to a family of apicomplexan-specific TFs that contain a modified version of the AP2 domain found in plant TFs (ApiAP2) [6]. Members of this TF family are present in all *Plasmodium* species, as well as in other Apicomplexa, including *Cryptosporidium*, *Theileria* [6], and *Toxoplasma* [7]. Many of these TFs are expressed during the intraerythrocytic development cycle (IDC) of *P. falciparum*, but some are thought to specifically control the transitions between major developmental stages, such as AP2-G for gametocyte development [8, 9], AP2-O for ookinete development [10], AP2-Sp for sporozoite formation [11], and AP2-L for liver-stage development [12]. Another approximately 40 proteins with zinc finger or helix-turn-helix domains have been implicated as specific TFs [5], but experimental evidence for their function is largely missing.

Compared to the number of TFs expected to be found in an organism containing a genome of the size of *P. falciparum*, approximately two-thirds of specific TFs remain unaccounted for in this parasite. Although the relative paucity of

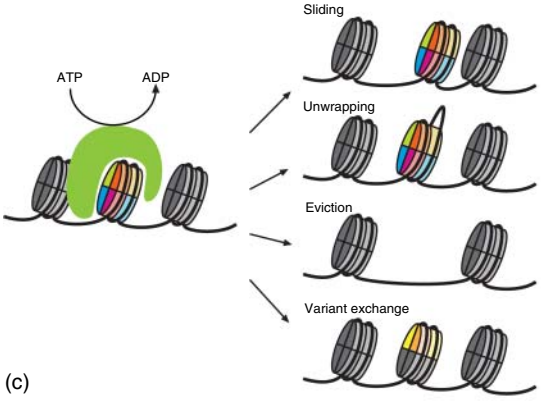
Figure 18.1 Schematic overview of the mechanisms that regulate transcription. (a) The preinitiation complex consisting of RNA polymerase II and the general transcription factors. (b) The nucleosome landscape around *P. falciparum* genes, with strongly positioned nucleosomes at the start and end of the gene, a fuzzy array of nucleosomes inside the gene, and nucleosomes containing histone variants H2A.Z and H2B.Z in the intergenic regions. The preinitiation complex (PIC) binds to the nucleosome-depleted region (NDR) upstream of the transcription start site (TSS). (c) ATP-dependent nucleosome remodelers change the nucleosome landscape by sliding nucleosomes along the DNA, partially unwrap DNA from the nucleosome, evict nucleosomes, or exchange histone variants. (d) Posttranslational modifications of histones by histone acetyltransferases (HATs), histone deacetylases (HDACs), histone lysine methyltransferases (HKMTs), and histone lysine demethylases (HKDMs). (e) Architecture of the *P. falciparum* nucleus, with colocalization of centromeres (purple balls) and telomeres. The silent *var* genes (white) cluster in repressive heterochromatin, while the single active *var* gene (green) is located in transcriptionally permissive euchromatin. Note the additional looping of the blue chromosome that brings internal and subtelomeric *var* genes in close spatial proximity, while this is not observed in the red chromosome that does not harbor any internal *var* genes. (f) Overview of the complex network of regulatory mechanisms involved in telomere biology and control of *var* gene expression, including histone posttranslational modifications, proteins interacting with DNA motifs or histone-PTMs, and lncRNAs. See the main text for more details.


TFIID
RNA Pol II
TBP
TFIIA
TATA
TFIIB
TFIIF
TFIIE
TFIIH

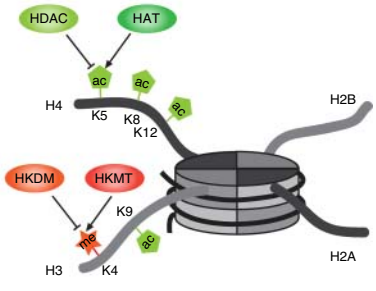
(a)


H2A.Z/H2B.Z
PIC
NDR
TSS
Strongly positioned
Strongly positioned

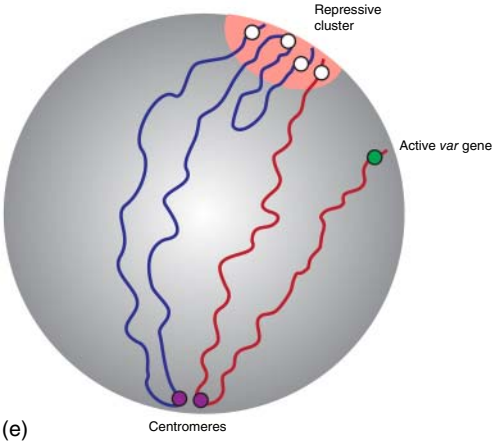
(b)


ATP
ADP
Sliding
Unwrapping
Eviction
Variant exchange

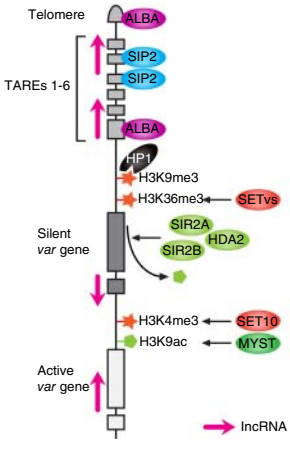
(c)


HDAC
HAT
ac
ac
ac
H4
K5
K8
K12
H2B
HKDM
HKMT
K9
me
ac
H3
K4
H2A

(d)


Repressive cluster
Active *var* gene
Centromeres

(e)


Telomere
ALBA
SIP2
SIP2
TAREs 1-6
ALBA
HP1
H3K9me3
H3K36me3
SETvs
Silent *var* gene
SIR2A
HDA2
SIR2B
H3K4me3
SET10
H3K9ac
MYST
Active *var* gene
lncRNA

(f)

identified and specific TFs in *P. falciparum* could be a result of low homology of these proteins with TFs in other eukaryotes, accumulating evidence points toward a unique dependence of the parasite on epigenetic mechanisms of transcriptional control.

Nucleosome Landscape

The genetic material of eukaryotic organisms does not float freely within the nucleus, but is tightly bound by histone proteins to form a condensed structure called *chromatin*. The basic DNA packaging unit, a nucleosome, consists of a stretch of 147 bp of DNA wrapped around a core of eight histones, two of each of histones H2A, H2B, H3, and H4. The packaging of DNA into these nucleosomal units does not only compress the genetic material to fit into the restricted size of the nucleus, but also plays a very important role in regulating access of DNA to other DNA-binding proteins, such as transcription factors. As a result, the positioning of nucleosomes around genes and other functional DNA elements shows a number of distinct features general to most eukaryotes. First, nucleosome occupancy is higher in coding regions as compared to intergenic regions [13–17]. Second, the transcription start site (TSS) is marked by an upstream nucleosome-depleted region that harbors the promoter and contains TF-binding sites [15, 16, 18]. The density of nucleosome occupancy in this region determines whether TFs can bind and recruit other components of the transcription machinery and thus has a strong influence on transcriptional activity. Third, the nucleosome-depleted region is flanked by strongly positioned nucleosomes, called the *−1 and +1 nucleosomes* that are found at nearly the same location in all cells within a population [15, 16, 18]. These nucleosomes often contain the histone variant H2A.Z instead of the canonical histone H2A, which destabilizes the nucleosome and promotes gene activation [19, 20]. Nucleosomes downstream of the +1 nucleosome are positioned in an array with increasing variation in their positioning, called *fuzziness*. Finally, the nucleosome landscape at the end of the gene mirrors the landscape at the gene start. The transcription stop site is marked by another strongly positioned nucleosome, followed by a nucleosome-depleted region [21, 22].

Nucleosome positioning in *P. falciparum* follows the general rules as observed in other eukaryotes (Figure 18.1b). The parasite genome shows a depletion of nucleosome coverage upstream of the TSS, and the degree of nucleosome occupancy in this region is inversely correlated with gene expression [23, 24]. However, its genome lacks the strongly positioned +1 nucleosome that marks the TSS itself. Instead, the most strongly positioned nucleosomes are found at the start and the end of coding regions [23, 24]. In addition, the difference between nucleosome occupancy in coding regions and intergenic regions seems to be more pronounced than in other organisms [24–26]. Finally, the variant histones H2A.Z and H2B.Z are found throughout intergenic regions, instead of being almost exclusively present in the +1 nucleosome [27, 28]. This parasite-specific

nucleosome landscape is most likely the result of the high AT content of the *P. falciparum* genome, which averages 80.7% AT genome-wide and reaches 90–95% AT in intergenic regions. AT-rich DNA is relatively rigid and does not easily wrap around a nucleosome core. The parasite may have partially solved this issue by evolving its histone variants H2A.Z and H2B.Z to become more efficient at binding AT-rich DNA.

In addition to this distinct nucleosome landscape around genes, *P. falciparum* also shows global changes in nucleosome levels during its IDC that have not been observed on the same scale in other organisms. In other eukaryotes, the process of transcription involves the local displacement of nucleosomes to allow the transcription machinery to progress along the gene (reviewed in [29, 30]). In *P. falciparum*, nucleosomes are evicted from the entire genome during the transcriptionally most active trophozoite stage [24, 26], as observed by a range of methodologies, including western blotting [31], mass spectrometry [24, 32], and ChIP-Seq [24]. This global nucleosome eviction may contribute to a general transcriptionally permissive state of the genome, allowing the efficient transcription of 70–80% of all *P. falciparum* genes at this stage of its life cycle. As the parasite prepares for evasion of the infected red blood cell, nucleosomes are reassembled and global histone levels return to those observed before the peak in transcriptional activity.

Chromatin Remodeling Enzymes

The molecular components involved in these parasite-specific nucleosome dynamics possibly make ideal targets for novel antimalarial drugs. Nucleosome positioning is organized by ATP-dependent chromatin remodeling complexes that act through different mechanisms, including nucleosome sliding, unwrapping, rearrangement, and eviction, as well as replacement of canonical histones with their variants (Figure 18.1c). Thus far, little is known about which chromatin remodeling enzymes may specifically be responsible for establishing and maintaining parasite-specific features of the nucleosome landscape in *P. falciparum* and for regulating the genome-wide nucleosome dynamics observed during the asexual replication cycle. The parasite encodes the four major classes of chromatin remodeling enzymes, SWI/SNF, INO80, ISWI, and Mi2/NURD, including 11 different variants of SWI2/SNF2 ATPases (Table 18.1) [33]. Furthermore, computational searches have identified three RuvB homologs [5, 34], ATPases that are part of INO80 chromatin remodeling complexes, as well as three high-mobility-group box proteins that are involved in DNA bending and nucleosome mobility [5, 35]. Finally, a homolog of Snf2-related CBP activator protein (SRCAP) has been identified [5], which in humans and in yeast replaces canonical H2A with the variant histone H2A.Z [36–38] (Table 18.1). Several chromatin proteins have domain architectures that are unique to apicomplexan organisms [33, 39], such as PfISWI (PF3D7_0624600), the *P. falciparum* homolog of the Snf2/Swi2 ATPase ISWI, which contains an additional five PHD domains around its core

Table 18.1 Proteins known to be involved in mechanisms of transcriptional regulation and potential targets of novel antimalarial drugs.

Function	Gene	Name	Description
Chromatin remodeling			
	PF3D7_0624600	PfISWI	SNF2 helicase
	PF3D7_1023900	PfCHD1	Chromodomain-helicase-DNA-binding protein 1 homolog
	PF3D7_0216000	SWI/SNF	DEAD/DEAH box helicase
	PF3D7_1357500	SWI/SNF	DNA helicase
	PF3D7_0604600		DNA helicase
	PF3D7_0818700		DNA helicase
	PF3D7_1104200	PfSNF2L	Chromatin remodeling protein
	PF3D7_0912100	n.a.	zf-HIT protein
	PF3D7_1433300	n.a.	Chromatin assembly factor 1 P55 subunit
	PF3D7_0110700	n.a.	Chromatin assembly factor 1 protein WD40 domain
	Pf3D7_0501800	PfCAF1	Chromosome assembly factor 1
	PF3D7_1441400	PfFACT-S	FACT complex subunit SSRP1
	PF3D7_0517400	PfFACT-L	FACT complex subunit SPT16
	PF3D7_0809700	PfRUVB1	RuvB-like helicase 1
	Pf3D7_1106000	PfRUVB2	RuvB-like helicase 2
	PF3D7_1362200	PfRUVB3	RuvB-like helicase 3
	PF3D7_0628600	n.a.	SWR1/NuA4:SWC4 homolog
	PF3D7_0919000	PfNAPS	Nucleosome assembly protein
	PF3D7_1203700	PfNAPL	Nucleosome assembly protein
	PF3D7_1202900	PfHMGB1	High mobility group protein B1
	PF3D7_0817900	PfHMGB2	High mobility group protein B2
	PF3D7_1359200	PfHMGB4	High mobility group protein B4
	PF3D7_0820000	PfSRCAP	Snf2-related CBP activator
	PF3D7_1212900	n.a.	Bromodomain protein

Table 18.1 (*continued*)

Function	Gene	Name	Description
	PF3D7_0110500	n.a.	Bromodomain protein
	PF3D7_1475600	n.a.	Bromodomain protein
	PF3D7_1033700	n.a.	Bromodomain protein
Histone modification			
	PF3D7_0823300	PfGCN5	HAT
	PF3D7_1118600	PfMYST	HAT
	PF3D7_0416400	PfHAT1	HAT
	PF3D7_0925700	PfHDAC1	Class I HDAC
	PF3D7_1472200	PfHDA1	Class II HDAC
	PF3D7_1008000	PfHDA2	Class II HDAC
	PF3D7_1328800	PfSIR2A	Class III sirtuin HDAC
	PF3D7_1451400	PfSIR2B	Class III sirtuin HDAC
	PF3D7_0629700	PfSET1	HKMT
	PF3D7_1322100	PfSETvs	HKMT
	PF3D7_0827800	PfSET3	HKMT
	PF3D7_0910000	PfSET4	HKMT
	PF3D7_1214200	PfSET5	HKMT
	PF3D7_1355300	PfSET6	HKMT
	PF3D7_1115200	PfSET7	HKMT
	PF3D7_0403900	PfSET8	HKMT
	PF3D7_0508100	PfSET9	HKMT
	PF3D7_1221000	PfSET10	HKMT
	PF3D7_1211600	PfLSD1	HKDM
	PF3D7_0801900	PfLSD2	HKDM
	PF3D7_0809900	PfJmjC1	HKDM
	PF3D7_0602800	PfJmjC2	HKDM
	PF3D7_1122200	PfJmj2	HKDM
Nuclear architecture			
	PF3D7_0317500	PfEG5	Kinesin-5
	PF3D7_1211000	n.a.	Kinesin-7
	PF3D7_0111000	n.a.	Kinesin-8
	PF3D7_0319400	n.a.	Kinesin-8
	PF3D7_1245100	n.a.	Kinesin-13
	PF3D7_0724900	n.a.	Kinesin-19
	PF3D7_1245600	n.a.	Kinesin
	PF3D7_0806600	n.a.	Kinesin-like protein
	PF3D7_1146700	n.a.	Kinesin-like protein
	PF3D7_1333700	PfCENH3	Histone H3-like centromeric protein CSE4

(*continued overleaf*)

Table 18.1 (*continued*)

Function	Gene	Name	Description
	PF3D7_1021800	PfCENP-C	Centromere protein C
	PF3D7_0414000	n.a.	Cohesin subunit (smc3 homolog)
	PF3D7_1130700	n.a.	Cohesin subunit
	PF3D7_1220900	PfHP1	Heterochromatin protein 1
	PF3D7_0604100	PfSIP2	SPE2-interacting protein
	PF3D7_0814200	PfAlba1	DNA/RNA-binding protein Alba1
	PF3D7_1006200	PfAlba2	DNA/RNA-binding protein Alba2
	PF3D7_1346300	PfAlba3	DNA/RNA-binding protein Alba3
	PF3D7_1347500	PfAlba4	DNA/RNA-binding protein Alba4

helicase domain. Further characterization of such parasite-specific proteins could be highly informative for drug development.

Histone Posttranslational Modifications

Histone proteins are subject to posttranslational modification (PTM) by the covalent attachment of small molecules to amino acid side chains. Over 100 different histone PTMs in both histone cores and N-terminal tails have been described in eukaryotes. Of these, acetylations and methylations of the N-terminal tails of histones H3 and H4 have been best characterized because of their important functions in transcriptional regulation (reviewed in [40]). Acetylation events of lysine residues are generally associated with transcriptional activation. The acetyl group neutralizes the positive charge of the lysine residue, thereby destabilizing the interaction between DNA and histone proteins. This results in a more open chromatin organization that is more permissive to transcription. Acetylated histones also recruit chromatin remodeling enzymes and other factors that influence gene expression. Methylation modifications do not directly interfere with the interaction between DNA and histone, but typically function by attracting other proteins and enzymes to the chromatin, which can have both activating and repressive effects. For example, trimethylation of the lysine at position 9 of histone H3 (H3K9me3) promotes the formation of transcriptionally silent heterochromatin by the recruitment of heterochromatin protein 1 (HP1), while trimethylation of the lysine at position 4 of histone H3 (H3K4me3) is associated with transcriptionally active genes.

More than 50 different histone PTMs have been identified in *P. falciparum*, including acetylations, methylations, phosphorylations, ubiquitylations, and sumoylations [41–44]. The majority of the *P. falciparum* genome is constituted of transcriptionally permissive euchromatin. As a consequence, the genome carries more activating than silencing histone marks, as also observed in other unicellular eukaryotes [45]. The activating histone marks H3K9ac and H3K4me3 are present at high levels across the genome [41, 46, 47], and H3K9ac is enriched upstream of active genes [46, 48]. In addition, enrichment for H3K36me3 is observed at the 3′ ends of actively transcribed genes [49], in agreement with its role in transcriptional elongation in other eukaryotes.

Repressive histone marks are mostly limited to regions of the genome harboring the clonally expressed variant surface antigen gene families (*var*, *rifin*, *stevor*, and *pfmc-2tm*) and invasion gene families (*eba* and *clag*), as well as some additional loci, such as the gametocyte-specific transcription factor *pfap2-g*. These regions are organized into transcriptionally silent heterochromatin marked by H3K9me3 [47, 50–53]. In addition, silent *var* genes and to a lesser extent *rifin* and *stevor* genes carry H3K36me3 [49]. The single active *var* gene is physically separated from this repressive heterochromatin and is enriched in H3K4me3 and H3K9ac, in particular around its TSS [54].

Histone-Modifying Enzymes

The acetylation of histones is catalyzed by histone acetyl transferases (HATs), of which at least eight variants are found in the *P. falciparum* genome [5] (Table 18.1, Figure 18.1d). Histone deacetylases (HDACs) are responsible for the removal of acetylation marks (Figure 18.1d). Five HDAC enzymes have been identified in *P. falciparum* genome [5] (Table 18.1), compared to 18 HDACs encoded by the human genome [55]. The importance of these enzymes for the control of gene expression is apparent from the observation that disruption of these genes typically results in aberrant acetylation patterns and decreased parasite survival. For example, the distribution of H3K9ac is associated with the presence of PfGCN5, a HAT with a preference for acetylation of H3K9 and H3K14 residues [48, 56]. Inhibition of PfGCN5 by curcumin results in hypoacetylation of H3K9ac and H3K14ac and induces parasite death [48]. Similarly, inhibition of HDACs by apicidin induces hyperacetylation of H3K9, H4K8, and tetra-acetyl H4 (H4ac4), as well as demethylation of H3K4me3, with large changes in transcription patterns [57]. Several compounds with selective inhibitory activity against *P. falciparum* HDACs in multiple stages of the parasite life cycle have been discovered, yielding promising leads for drug development [58, 59]. However, next-generation compounds with increased antiplasmodial activity, decreased host cell toxicity, and improved pharmacokinetic properties will need to be developed before such HDAC inhibitors can be considered as novel therapeutic candidates.

Analogous to acetylations, methylation events are controlled by histone lysine methyltransferases (HKMTs) and histone lysine demethylases (HKDMs)

(Figure 18.1d). The *P. falciparum* genome encodes 10 PfHKMTs belonging to SET domain superfamily, 2 PfHKDMs of the LSD1 family, and 3 PfHKDMs of the Jumonji-related family (Table 18.1) [60–62]. Six of the PfHKMTs are essential for parasite survival [49, 61], making these enzymes potential targets for antimalarial drugs. Thus far, two related small-molecule PfHKMT inhibitors with strong activity against *P. falciparum* and *Plasmodium vivax* have been described [63, 64]. These compounds are able to rapidly kill asexual-stage parasites at nanomolar concentrations, while displaying low toxicity in mammalian cell lines.

Histone PTMs are an important regulatory mechanism controlling the allelic expression of the *var* genes (Figure 18.1f). The NAD^+-dependent class III HDAC silent information regulator 2 proteins PfSIR2A and PfSIR2B are involved in maintaining *var* gene repression, and disruption of these genes results in loss of monoallelic expression of this gene family [53, 65, 66]. A similar function was recently attributed to the class II HDAC PfHDA2, which in addition controls repression of the locus encoding the gametocyte-specific transcription factor PfAP2-G [67]. The HAT PfMYST is recruited to the active *var* gene and acetylates H4 at multiple residues [68]. The HKMT PfSET10 also colocalizes with the active *var* gene and controls H3K4 methylation in this gene [61]. On the other hand, PfSETvs (variant-silencing) deposits trimethylation marks on H3K36 to maintain transcriptionally repressive chromatin environment at the silent *var* genes [49].

These results highlight the importance of the histone code for regulation of gene expression in *P. falciparum* and suggest that histone-modifying enzymes may be attractive targets for novel antimalarial drugs. For the development of this class of drugs, it will be very important to carefully characterize the drug mechanism of action, the potential toxicity in mammalian cells as the catalytic site of these enzymes are relatively conserved across eukaryotic species, and potential off-target effects, since inhibition of histone-modifying enzymes may also affect PTMs of nonhistone proteins.

Nuclear Architecture

Chromosomes are packaged into the nucleus in an ordered, nonrandom fashion, resulting in an organized three-dimensional genome structure that plays important roles in regulating gene expression (reviewed in [69, 70]). Chromosomes are long-chain molecules that, because of their size, are unable to freely mingle and therefore arrange into chromosome territories [71]. Looping of chromosome gives rise to long-range interactions, both within and between chromosomes. Furthermore, the nucleus is divided into compartments that separate chromatin into different transcriptional environments. Finally, large and complex metazoan genomes are organized into smaller building blocks, called topologically associating domains (TADs). TADs are separated from each other by housekeeping genes and insulator proteins and show more chromosome interactions within one domain than between different domains [69, 70].

The relatively small genome of *P. falciparum* is primarily divided into a large compartment of euchromatin that contains the majority of the genome and a small compartment of transcriptionally repressive heterochromatin that harbors the variant surface antigen gene families, including the *var* genes (Figure 18.1e). Silent *var* genes are organized into one or multiple clusters at the nuclear periphery [53, 72, 73], while the single active *var* gene is located distant from this heterochromatin environment [53, 65]. Other hallmarks of genome organization in *P. falciparum* include a folded chromosome architecture anchored at the centromere [73], with clustering of the centromeres [74, 75] and clustering of the telomeres on opposite sites of the nucleus, a similar genome architecture as observed in budding and fission yeast [76, 77]. However, the *var* genes provide additional complexity to genome organization in *P. falciparum* [73]. Most of the *var* genes are located on subtelomeric regions, but several chromosomes harbor *var* genes on internal loci. These internal *var* gene clusters colocalize with subtelomeric *var* genes at the nuclear periphery, creating loops in these chromosomes that are not observed in chromosomes that lack internal *var* gene clusters [73].

During the IDC, the parasite nucleus changes in size, reaching its maximum volume at the transcriptionally most active trophozoite stage [78, 79]. Simultaneously with nuclear expansion, the number of nuclear pores increases from 3–7 pores in the ring stage to 12–58 pores in the trophozoite stage, which are uniformly distributed around the nucleus and are located adjacent to regions of euchromatin [79]. These changes revert as the nucleus undergoes schizogony and the nuclear content and its pores are distributed between all daughter nuclei. Finally, chromosomes show more intermingling at the trophozoite stage, while chromosomes are more strictly contained within chromosomal territories in the ring and schizont stage [73]. These dynamic rearrangements of the nucleus and the three-dimensional genome during the IDC may contribute to control of gene expression. Indeed, genes that are located more closely together have more similar expression profiles than genes that are more distantly located [73]. In addition, certain genes that are known to be specifically expressed in other stages of the parasite's life cycle and that are silent during the IDC also tend to be colocalized [73], indicating that their location within the nucleus is important for their repression. Finally, the repressive heterochromatin center seems to influence gene expression over a larger distance, as the average gene expression increases with increasing distance from the repressive center [73].

Proteins Involved in Nuclear Organization

The structure of the nucleus is organized by the nucleoskeleton, a network of proteins that anchor the nuclear content to the nuclear envelope and mediate the movements of chromosomes, thus conferring shape and functionality to this organelle (reviewed in [80]). Some of these nucleoskeleton proteins directly interact with chromatin by binding to DNA, histones, and chromatin-remodeling

complexes. Not all of the proteins that form the nucleoskeleton in Metazoa are present in *P. falciparum* or other unicellular eukaryotes. For example, lamin, making up the nuclear lamina on the inside of the nuclear envelope, has not been found in *P. falciparum* [81]. Similarly, CCCTC-binding factor (CTCF), an insulator protein that forms the boundaries of TADs and tethers chromatin to the nuclear lamina, is absent in the parasite [82]. However, motor components of the nucleoskeleton, such as actin, myosin, and kinesin, are present and have been recognized as possible drug targets. A recent small-molecule screen identified a potent inhibitor against *P. falciparum* and *P. vivax* Kinesin-5 that showed no cross-reactivity in human cell lines [83].

As in other eukaryotes, the centromeres in *P. falciparum* are marked by a specialized isoform of histone H3, called PfCENH3 [74, 75]. This protein was recently shown to interact with PfCENP-C to form functional centromeric chromatin that mediates assembly of the kinetochore [75, 84]. Disruption of the dimerization domain of PfCENP-C resulted in loss of mitotic spindle integrity, demonstrating that this protein is evolutionarily conserved and essential for chromosome segregation and cell cycle progression [84]. The multiprotein complex cohesin is responsible for chromosome segregation during cell division and has additional roles in DNA repair and transcription regulation (reviewed in [85]). Cohesin is made up of four subunits, of which two have been annotated in *P. falciparum.*

The maintenance of the repressive center and regulation of *var* gene expression have been subject of extensive research and involve many different proteins (Figure 18.1f). The most important structural component of the repressive center that has thus far been identified is *P. falciparum* heterochromatin protein 1 (PfHP1), which specifically binds to the repressive histone mark H3K9me3 and is structurally and functionally similar to its homologs in other eukaryotes [86, 87]. Knockdown of this protein in *P. falciparum* results in disruption of monoallelic *var* gene expression and an arrest in parasite growth [88]. In addition, PfHP1 controls repression of the gametocyte-specific transcription factor locus *pfag2-g*, resulting in an increased rate of gametocyte differentiation in the absence of PfHP1 [88]. Repositioning of the active *var* gene from this repressive environment to a transcriptionally permissive nuclear compartment is an active process involving the recruitment of an actin protein complex to an 18-bp nuclear protein-binding element in the *var* gene intron [89].

One of the members of the family of ApiAP2 transcription factors, *P. falciparum* SPE2-interacting protein (PfSIP2), is also involved in organization of subtelomeric heterochromatin [90]. This protein interacts with arrays of SPE2 DNA motifs present in the subtelomeric regions upstream of the *var* genes, as well as in the telomere-associated repeat elements (TARE). Another family of proteins that is implicated in heterochromatin maintenance and *var* gene repression are the DNA/RNA-binding proteins PfAlba1–4 (Acetylation lowers binding affinity). PfAlba1, 2, and 4 bind to TARE6 [91], while PfAlba3 binds to telomeric and subtelomeric regions and is additionally found in *var* gene promoters [92]. The HDAC PfSIR2A, also present in the repressive center, is thought to interact with PfAlba3

and to increase its DNA-binding affinity by deacetylating lysine residues in its N-terminus [92].

Given the complexity of nuclear organization and chromosome dynamics, many other proteins are likely to be involved in these processes. Identification and characterization of these molecular components are a work in progress and advances in methodologies aimed at analyzing proteins and protein complexes will likely contribute to a more detailed map of chromatin-associated proteins in the near future. In addition, it will be very interesting to characterize the pathways involved in biogenesis of the nuclear membrane and the assembly of nuclear pores, as disruption of these processes is likely to greatly affect parasite cell cycle progression.

Long Noncoding RNAs

A variety of long noncoding RNAs (lncRNAs) are transcribed from the TAREs [93–95]. These TARE-lncRNAs have low abundance during DNA replication in the trophozoite stage and reach their highest expression levels in the schizont stage. Some evidence suggests that TARE-lncRNAs are transcribed as long precursor molecules that are processed into smaller fragments [93, 94]. The exact nature and function of these TARE-lncRNAs currently remain unknown, but it is likely that these transcripts are part of a regulatory network controlling heterochromatin and gene silencing at the chromosome ends. As such, TARE-lncRNAs show functional homology to a eukaryotic family of noncoding RNA called telomeric repeat-containing RNA (TERRA) that is involved in telomere maintenance and heterochromatin assembly (reviewed in [96]).

LncRNAs also play an important role in the regulation of monoallelic expression of the *var* gene family. Two lncRNAs are transcribed from a bidirectional promoter in the intron of the *var* genes, an antisense transcript extending into exon 1 and a sense transcript spanning exon 2 [97]. These transcripts are capped, but not polyadenylated, and are incorporated into chromatin [97]. Based on current knowledge, the sense lncRNA seems to function as a silencer of *var* gene expression, while the antisense lncRNA is transcribed from and associated with the single active *var* gene, maintaining epigenetic memory [98]. These lncRNAs thus add another layer to the complex network of epigenetic mechanisms controlling *var* gene expression (Figure 18.1f).

Posttranscriptional Control of Gene Expression

The picture emerging from combining all current knowledge on regulatory mechanisms controlling transcription during the IDC in *P. falciparum* is that only a small part of the parasite's genome is organized into strictly repressive heterochromatin. As detailed earlier, this includes the surface variant antigen gene families that are under the control of a range of, mostly epigenetic, regulatory mechanisms,

such as compartmentalization, nuclear localization, repressive histone PTMs, and lncRNAs. The majority of the genome is present in a transcriptionally permissive state and undergoes dynamic changes in chromatin structure to cycle through binary stages of transcriptional activity: low levels of transcription in ring and schizont stages versus high levels of transcription in the trophozoite stage. This single main phase of transcriptional activity does not agree with the cascade of steady-state mRNA transcripts that is observed during the IDC, suggesting that posttranscriptional control mechanisms also play an important role in regulating gene expression.

Mechanisms involved in mRNA maturation, transport, modification, stability, storage, translation, and degradation together determine the abundance of transcripts and the timing and levels of protein synthesis. During the IDC of *P. falciparum,* steady-state mRNA levels correlate with protein levels for 50–70% of genes, while a delay between the moment of transcription and the moment of translation was observed for approximately 30% of genes [31, 99], suggesting that mechanisms regulating mRNA stability and translation play a major role in controlling gene expression. Temporary storage of transcripts in P-bodies during gametocyte development [100] and a temporary inhibition in translation during sporozoite latency [101] are two other well-described examples of the importance of posttranscriptional regulatory mechanisms in malaria parasites. These processes are largely coordinated by RNA-binding proteins [102]. Identification and characterization of these RNA-binding proteins may reveal novel drug targets that can be exploited for the development of novel therapeutic approaches.

Conclusions

P. falciparum employs a multitude of epigenetic mechanisms to control the expression of its genes. Many of the proteins involved in these processes are structurally divergent from those in other eukaryotic species, making them interesting targets for novel antimalarial drugs. A handful of compounds that inhibit proteins involved in processes regulating transcription have been identified, but the search for drugs with more selective antiplasmodial activity and enhanced pharmacokinetic properties is ongoing. In addition, many regulatory components are yet unidentified and further research into these processes has the potential to reveal novel drug targets.

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19
Aminoacyl t-RNA Synthetases as Antimalarial Drug Targets

Anmol Chandele and Amit Sharma*

Abstract

The transfer of information from mRNA to proteins is partly driven by an essential set of enzymes called aminoacyl-tRNA synthetases (aaRSs). These enzymes play a pivotal role in protein synthesis and thereby maintain cell viability. Mechanistically, their primary role is to recognize and amino-acylate their cognate tRNAs with the correct amino acid prior to the delivery of the charged tRNA to the protein synthesis machinery. These enzymes have long been pursued for antibacterial and antifungal drug targeting. Over the past decade, aaRSs have emerged as multifaceted enzymes that in addition to their role in protein synthesis seem to be involved in other noncanonical biological functions. Only recently have aaRSs been explored as targets for antiparasitic drugs, and now many compounds are under development as anti-infectives. Here, we discuss the recent developments in drug discovery focusing on *Plasmodium falciparum* aaRSs.

Introduction

Malaria remains the cause of additional human misery with greater than 3 billion people at risk globally [1]. There are approximately 500 million malaria cases occur per year with approximately 0.5 million deaths mostly of young children and pregnant women [2–4]. Additionally, there are 70–80 million cases of relapsing *Plasmodium vivax* malaria each year that adds to cumulative disease burden [5]. Although continued attempts to develop a vaccine against malaria are ongoing, as for now drugs continue to be the only treatment option.

Discovery of chloroquine in the 1930s was considered to be an avenue for radical cure of malaria. In fact, chloroquine was widely used until the late 1990s. However, over time, mutations within malaria parasites resulted in the emergence of drug resistant strains – originating in South Asia and South America and then spreading to endemic areas [6]. Monotherapy with chloroquine was then replaced

*Corresponding author.

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with sulfadoxine–pyrimethamine drug combination [7–9]. However, resistance to this combination of drugs also emerged at high frequencies resulting in millions of deaths. Fortunately, a Chinese herbal medication derived from a weed *Artemisia annua* was discovered to be a potent antimalarial [10]. Artemisinin-based drugs were first used in monotherapies, but they were succeeded by artemisinin-based combination therapys (ACTs) [11]. Compliance to ACTs is good because ACT regimes are administered either once or twice a day for a period of 3 days. However, these ACT regimes are not infallible and emerging drug resistance is now being reported. Recently, it was noted that in parts of Cambodia, the proportion of patients who still had parasitemia after a 3-day treatment with dihydroartemisinin–piperaquine combination increased from 26% in 2008 to an alarming 45% in 2010 [9]. Therefore, there is an urgent need to discover both new druggable targets within malaria parasites and novel chemical classes that can be future antimalarials in drug cocktails.

Drug discovery in context of antimalarials is a very challenging process for several reasons: (i) follow-up health care in countries that require antimalarials is poor, and therefore the antimalarial of choice should be well tolerated and safe in humans; (ii) the antimalarial drug should be orally bioavailable such that it can be administered in a nonhospital setting, (iii) because drug compliance and development have historically been a concern, a 3-day therapy is more desirable if not a single-dose clearance, (iv) since malaria endemicity is typically in the developing or underdeveloped world, cost-effectiveness is a major factor, and finally, (v) combination therapies are desirable such that emergence of drug resistance can be minimized. Typically, successful drug discovery entails understanding what the final product would target, that is the "Target Product Profile" (TPP) has to provide evidence that this alternative would be superior to current treatments. A new age concept that has gained momentum is "SERCaP" – Single Exposure Radical Cure and Prophylaxis. This new generation of SERCaPs would probably be a combination of drugs that tick multiple boxes such as (i) activity against blood-stage parasites of the five human infective *Plasmodium* species, including parasites that are currently resistant to existing therapies; (ii) prevention of relapsing malaria (*P. vivax*) and safe usage in glucose 6-phosphate dehydrogenase (G6PD) deficient subjects; (iii) blocking of parasite transmission by antigametocytic activity and (iv) useful prophylactic efficacies in children and pregnant women.

Traditionally, drug discovery has revolved around identification of molecular targets in the parasite that are essential for parasite survival. Finding such targets has not been an easy task especially considering that it has to be present in both the blood and liver stages. Moreover, it is difficult to experimentally demonstrate that a target is essential for parasite viability in both the blood and the liver stages, thereby making research and development more challenging. An alternative approach that is gaining popularity is to first find compounds in a phenotypic cell-based screen and then determine their mode of action through the identification of their targets [12]. The whole genome sequence of *Plasmodium falciparum* and *P. vivax* has allowed the identification of a full range of potential targets

against which a drug can be designed, resulting in potential new classes of drugs (lead compounds) with new structural scaffolds. There are a number of drugs in the pipeline whose TPPs have been published by medicines for malaria venture (see [13]).

In the context of unexplored parasite enzymes that can serve as new foci for inhibitor development, the tRNA synthetase family members are now considered highly valuable. *Plasmodium* parasite possesses three active compartments where protein translation occurs – the cytosol, the mitochondrion, and a relic plastid termed as the *apicoplast*. The parasite cytosol contains translational machinery that resembles that of other eukaryotes, while in the mitochondrion and apicoplast, the machineries are prokaryotic-like [14]. The translational engines in the cytosol translate approximately 5385 genes, whereas the apicoplast machinery translates less than 50 genes [15]. All genes relevant for translation in the apicoplast are housed on the 35 kb circular apicoplast genome that includes rRNAs, tRNAs, ribosomal proteins, translation and elongation factors, and some other proteins that are less well characterized [16]. The mitochondrial genome is about 6 kb and only encodes three proteins of the electron transport chain along with some small fragmented *rRNA* genes [16].

Of the 20 amino acids, *Plasmodium* possesses interconversion pathways for few amino acids and demand for rest of the amino acids is met by digestion of human hemoglobin that is a rich source of all amino acids except Cys, Glu, Gln, and Met that are in limiting amounts and Ile that is completely absent [15]. The obligatory dependence on exogenous sources of amino acids denotes that parasite protein synthesis relies on amino acid transport across the erythrocyte plasma membrane, food-vacuole membrane, and the parasite plasma membrane [17].

One family of enzymes that catalyze the ligation of amino acids to their cognate tRNA during protein translation are the aminoacyl-tRNA synthetases (aaRSs) (Figure 19.1) that are present in all three compartments (Figure 19.2) [14]. Recently, these enzymes have been the subject of intense research activities

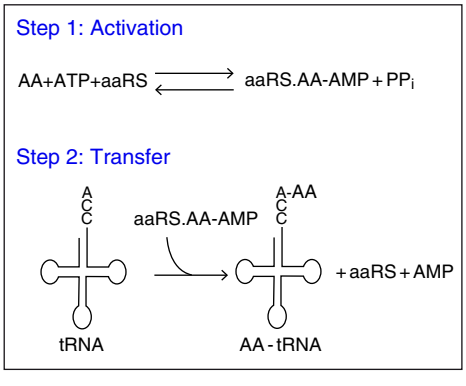


Figure 19.1 Two-step aminoacylation reaction catalyzed by aaRS family of enzymes.

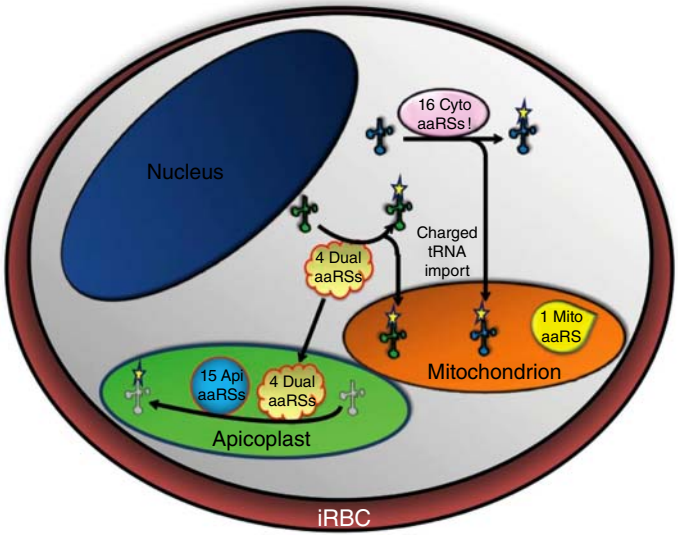


Figure 19.2 Spatial distribution of *P. falciparum* aaRSs.

with the aim to identify their suitability for antimalarial drug discovery, and very promising lead compounds have been identified.

The tRNA synthetase enzymes (aaRS) ensure faithful transmission of genetic information by bridging the transition of information from mRNA to protein. Their main function is to correctly pair particular tRNAs with their cognate amino acids to be incorporated into the growing peptide chain [18]. The aaRSs have a catalytic domain with a fold that binds to ATP, amino acid, and the 3′-terminus of the tRNA. In a two-step reaction, each aaRS first uses the energy derived from ATP to synthesize an activated aminoacyl adenylate intermediate. In a second step, either a 2′ or a 3′ hydroxyl oxygen (OH) of the 3′-adenine 76 (A76) tRNA nucleotide functions as a nucleophile in synthesis of aminoacyl tRNA in a reaction shown in Figure 19.1 [18]. Based on structural topology, aaRSs are divided into two major classes. Generally, class I aaRSs represent 10 amino acids that are Arg, Cys, Gln, Glu, Ile, Leu, Met, Val, Trp, and Tyr. Class I have a Rossmann fold in their core domain, bind ATP in an extended conformation and amino-acylate the 2′OH of their cognate tRNA [19]. Class II aaRSs represent 10 amino acids that include Ala, Asp, Asn, Gly, His, Lys, Phe, Pr, Ser, and Thr [20]. Class II enzymes have antiparallel beta strands flanked by alpha helices in their central core, bind ATP in a bent conformation, and amino-acylate the 3′OH of their cognate tRNA [19]. Interestingly, LysRS is found in either class I or class II depending on the organism. However, in apicomplexans including *Plasmodium* LysRS, is

class II type [21, 22]. Because aaRS enzymes play such a crucial role in protein biosynthesis, once they are inhibited protein synthesis is halted, which in turn results in attenuation of cellular growth. Therefore, aaRSs have been a research focus for antibacterial drug discovery and are recently coming to the forefront for antimalarial drug discovery [19, 23]. As outlined earlier, aaRSs have several functional sites that are potential targets for inhibitors: the ATP binding site, the amino-acid-binding site, the fold that recognizes the t-RNA and editing domains.

Using various computational and bioinformatics tools, 36 aaRSs in *P. falciparum* were identified and annotated, opening new avenues for discovery of novel drugs against the parasite [24]. It is now established that of these 36 *P. falciparum* aaRSs in blood-stage parasites, 16 aaRSs are cytoplasmic, 15 reside in the apicoplast, and 4 encoded by single copy genes (alanyl, threonyl-, glycyl- and cysteinyl-tRNA synthetases) that dual localize to the cytoplasm and the apicoplast [14]. Interestingly, of the 36 aaRSs, only one aaRS (phenylalanyl RS) localizes to the parasite mitochondrion [14]. The aaRS enzymes represent valuable targets for drug discovery because they meet various basic prerequisites such as (i) they are ubiquitous in cellular life while displaying considerable evolutionary divergence in sequence – allowing for selective inhibition; (ii) X-ray crystal structures of many aaRSs have been solved and thus provide a platform for rational drug design; (iii) parasites are fast proliferating and therefore rely heavily on rapid protein translation; (iv) aaRS inhibition is likely to block parasite growth; and (v) apart from protein translation, aaRSs have various noncatalytic and noncanonical roles in RNA processing, RNA trafficking, and rRNA synthesis [25]. However, in the case of *Plasmodium* aaRSs, there is currently evidence only from one aaRS (tyrosyl-RS) with an accessory function in inducing inflammation [26].

Alanyl-tRNA Synthetase (AlaRS)

AlaRS is a dual-targeted enzyme that is, found in the cytosol and the apicoplast, and is conceptually an attractive target for antimalarial compounds [27]. The enzyme also has editing activity such that misacylated amino acids do not accumulate to toxic levels in the cell. This editing activity is crucial for cell functionality, and mice with defects in AlaRS have a neurodegeneration phenotype [28]. Several putative inhibitors of *Pf* AlaRS have been screened based on docking data obtained from *in silico* screening against structural homology models. One compound, A5 {4-92-nitro-1-propenyl}1,2-benzenediol, that targets AlaRS-inhibited parasite growth at low micromolar concentration showed only limited cytotoxicity in mammalian cells [27, 29]. Further work on the suitability of similar inhibitors is warranted.

Isoleucyl-tRNA Synthetase (IleRS)

The widely used drug mupirocin (bactroban) is for topical application and inhibits IleRS. In bacterial systems, mupirocin has been shown to bind to the Ile-AMP intermediate, and this inhibits the enzyme's amino acylation activity. Recently, it was also demonstrated that mupirocin inhibits *P. falciparum* in nanomolar concentrations [30]. A "delayed-death" phenotype was observed in mupirocin-treated parasites, which is suggestive of inhibition of protein translation in the apicoplast [30]. Unfortunately, there are already reports of mupirocin-resistant parasites that have mutations in the apicoplast-derived IleRS, and therefore it is unlikely that this drug would be used an as antimalarial in the future. Another compound, thiaisoleucine, that is an analog of isoleucine was also reported to rapidly kill culture parasites *in vitro* [17]. However, this compound did not discriminate between mammalian and parasite IleRS [30]. Although neither of the two inhibitors is suitable as antimalarial, these studies validate IleRS as a drug target, more specifically the cytoplasmic IleRS in the context of malaria parasites as a target.

Threonyl-tRNA Synthetase (ThrRS)

Previous studies have demonstrated that borrelidin is an inhibitor of yeast cyclin-dependent kinase [31] and an activator of proapoptotic mediators in endothelial cells [32]. The mechanism of action of borrelidin is now becoming clear as addition of L-threonine in culture abrogates the effects of the inhibitor suggesting it targets ThrRS [33]. *Plasmodium* possesses only one ThrRS, a dual-targeted enzyme that is trafficked to the apicoplast and the cytosol [34]. Studies have demonstrated that borrelidin has an immediate effect on parasite viability and does not show delayed death as seen for other apicoplast inhibitors [34]. Novel inhibitors of *Pf* ThrRS identified by *in silico* docking analysis of small-molecule compound libraries against homology models of *Pf* ThrRS provided poorer hits that showed only moderate inhibition (IC_{50} 75–150 μM) of *P. falciparum in vitro* growth [27].

Methionyl-tRNA Synthetase (MetRS)

P. falciparum encodes two copies of methionyl-tRNA synthetases present in cytoplasm and apicoplast. Recent work from our group has shown that two known bacterial MetRS inhibitors called *REP3123* and *REP8839* are effective in killing *P. falciparum* [35]. Several related compounds that were tested for abrogation of protein synthesis are also promising hits with IC_{50} value in low micromolar range. *In silico* drug docking studies suggest distinct modes of binding for three

compounds in the catalytic site when compared with the enzyme product methionyl adenylate. This study therefore suggests that *P. falciparum* MetRSs represent new targets and that novel hit compounds can be explored in future drug discovery strategies [35].

Lysyl-tRNA Synthetase (LysRS)

Cell-based screening identified the compound cladosporin as a potent antimalarial that inhibited parasite blood stages [36, 37]. Biochemical analysis of this phenotype suggested that cytoplasmic LysRS was the target of cladosporin [36]. It was further validated that the selective difference in bioactivity of cladosporin was due to structural differences in the ATP-binding pocket of human versus parasite LysRSs [37]. In order to interrogate relative binding affinities of cladosporin, ATP, or AMPPNP (a non-hydrolyzable analog adenosine 5′-(β,γ-imido) triphosphate) for LysRS, thermal shift and microscale thermophoresis (MST) assays using highly purified recombinant enzyme were performed [29, 37]. Protein melting profiles of LysRS were slightly altered by either ATP or AMPPNP, suggesting small increments in enzyme stability upon ligand engagement. In contrast, addition of cladosporin increased LysRS melting by approximately 16 °C indicating both higher affinity and greater thermal stability of the *Pf* LysRS – cladosporin complex (Figure 19.3a) [29, 37]. Binding assays were conducted in solution to determine binding constants of cladosporin and AMPPNP with purified *Pf* LysRS enzyme [29, 37]. These experiments revealed a K_D value of approximately 4 μM for the LysRS-AMPPNP complex while the binding to cladosporin was very tight with K_D of 14 nM [36]. The superposition of catalytic domains from parasite LysRS-Lys-cladosporin and human LysRS-Lys-ATP ternary complexes revealed striking congruence in structural positioning of cladosporin and adenosine moieties respectively [29, 37]. In addition, it was discovered that in several human and animal pathogens, LysRS active sites that retain the identity of the critical cladosporin-accommodating residues Val328 and Ser344 also display high conservation in other parts of their LysRS active sites [29, 37]. These pathogens include parasites that cause trypanosomiasis (including Chagas disease), intestinal schistosomiasis, and loiasis (respective infectious agents: *Trypanosoma cruzi*, *Trypanosoma vivax*, *Trypanosoma congolense*, *Schistosoma mansoni*, and *Loa loa*). The analysis so far highlights ample space within LysRS active sites for further chemical derivatization from the THP (2,6-disubstituted tetrahydropyran) moiety of cladosporin. Such derivatives may also be useful against other human pathogens that retain high conservation in cladosporin-chelating residues within their LysRSs. Although due to its poor bioavailability cladosporin itself cannot be explored as an antimalarial. However, cladosporin provides a new scaffold whose bioavailability optimization is now required for improving its drug-like

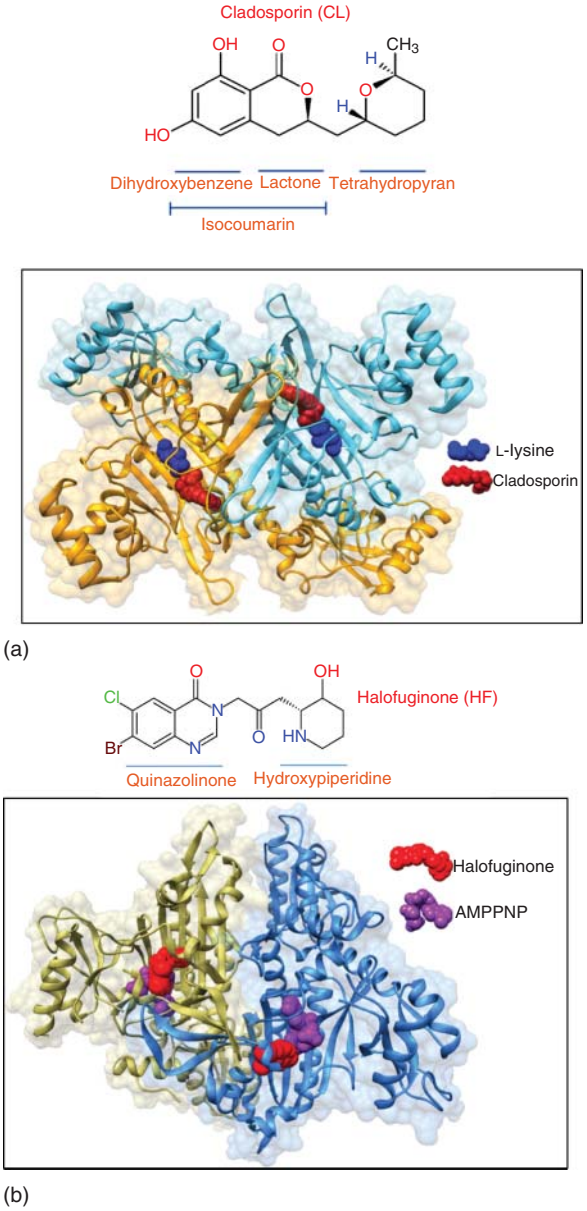


Figure 19.3 (a) Structural positioning of cladosporin in Lys-RS (PDB ID: 4PG3). View of *P. falciparum* Lys-RS bound to cladosporin (red) and to L-lysine (blue). The protein is dimeric (light blue and yellow) where each monomer binds one drug and one lysine. (b) Structural positioning of Halofuginone in Pro-RS (PDB ID: 4YDQ). View of *P. falciparum* Pro-RS bound to halofuginone (red) and to nonhydrolyzable ATP (AMPPNP – magenta). The protein is dimeric (blue and yellow) where each monomer binds one molecule of HF, proline, and AMPPNP.

properties. Hoen *et al.* recently discovered a series of new compounds that selectively inhibit *Plasmodium* apicoplastic LysRS, thus further validating the suitability of LysRS as a target for specific inhibition of *P. falciparum* [38].

Prolyl-tRNA Synthetase (ProRS)

Traditionally, the Chinese herb *Dichroa febrifuga* has been used to treat malaria. The active component febrifugine and its derivative halofuginone (HF) are now well established to be potent antimalarials [39–41]. Recently, the molecular target for HF was identified as ProRS where it was demonstrated that protein translation was restored in the presence of excess proline [41]. This ProRS inhibition was due to mimicking by HF of both L-Pro and A76 of tRNA and binding of HF to the active site in an ATP-dependent manner [42]. Several groups have now unequivocally demonstrated a direct and strong binding of HF to the *P. falciparum* ProRS and effectiveness of febrifugine derivatives as antimalarials [40]. *P. falciparum* ProRS is a dimeric enzyme that resides in the cytoplasm of the parasite [39]. It has two domains joined by a linker that separates an N-terminal editing domain from the canonical synthetase domain (Figure 19.3b). The active site for amino acylation reaction resides in the latter, where HF binds. Ternary complex structure of *Plasmodium falciparum* ProRS-HF-AMPPNP has recently been determined at 2.3 Å resolution (Figure 19.3b) [40]. Hence, we now have a structural rationale for the exquisite inhibitory properties of HF. In addition to febrifugine, derivatives such as 6-fluoro febrifugine (6F-FF) and tetrahydro quinazolinone febrifugine (Th-FF) are effective based on both cell- and enzyme-based drug interaction assays [43]. In fact, four quinazolinone-based compounds HF, FF, 6F-FF, and Th-FF have EC_{50} values in submicromolar ranges of 1–190 nM *against P. falciparum* [40]. Recombinant ProRS catalyzes amino acylation reactions that are inhibited by the four quinazolinone-based compounds with IC_{50} values of 9, 24, 13, and 352 nM, respectively [40, 43]. The addition of ATP analog AMPPNP along with Th-FF, FF, 6F-FF, and HF inhibitors results in a substantial increase in Tm of ~30–40 °C, indicating specificity, higher affinity, and greater stability of ProRS–inhibitor–AMPPNP complexes. The binding constant (K_D) of *Pf* ProRS-HF was estimated to be approximately 300 nM and interestingly increased approximately 30-fold in the presence of AMPPNP to approximately 9 nM. Atomic resolution details of active site in *Pf* ProRS-HF-AMPPNP complex delineate spaces that can be exploited for further modification or derivatization of HF [40].

Nanomolar inhibitory dose makes the enzyme target/pharmacophore relationship robust and paves the path for further modifications. Work so far has provided a high-resolution ternary complex structure of *Pf* ProRS-HF with AMPPNP, where the latter facilitates HF integration into the *Pf* ProRS active site. Structural analyses highlight spaces within the *Pf* ProRS active site architecture for derivatives of HF/FFs quinazolinone, but not the hydroxypiperidine moiety. The cell-,

enzyme-, and structure-based data on FF-based inhibitors suggest utility of their inclusion in antimalarial drug development efforts.

Conclusions

In summary, several labs worldwide have been able to characterize members of the malaria parasite aaRS enzyme family over the past 7 years [24]. Structures of tRNA synthetase–inhibitor complexes (lysyl-tRNA synthetase with cladosporin and prolyl-tRNA synthetase with HF) have been determined, and these allow for structure-based drug design strategies to be initiated. *Plasmodium* aaRSs hence provide very valuable new classes of drug targets, which can be further exploited for antimalarial drug discovery. The aaRSs are implicitly required for protein translation, and no cell can survive without aaRSs. This offers new hope and in principle as many drug targets as aaRSs in any given organism. In malaria parasites, aaRSs are excellent drug targets because their selective inhibition can potentially stall protein production in all three parasitic compartments. Therefore, of the 36 aaRSs, up to 21 can be further explored, even if one momentarily ignores the 15 apicoplast aaRSs due to potential delayed-death phenotypes. However, the caution here is that the key element of aaRS druggability remains discovery of novel and selective small-molecule inhibitors that accumulate in the parasites. Natural compounds have traditionally supplied a majority of modern drugs, and this theme is maintained for parasitic aaRSs as well – both cladosporin and febrifugine are natural metabolites of fungal and plant origin respectively. Apart from the advanced knowledge about some of the aaRS targets detailed in this review, many more remain to be examined and may potentially be exploitable. Drug discovery pipelines aimed at parasite aaRSs hence need to work overtime to further explore this promising area of *Plasmodium* biology for drug discovery.

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Part Four
Mathematical Approaches to Drug and Target Discovery

Comprehensive Analysis of Parasite Biology: From Metabolism to Drug Discovery,
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Mathematical Modeling and Omic Data Integration to Understand Dynamic Adaptation of Apicomplexan Parasites and Identify Pharmaceutical Targets

*Partho Sen, Henri J. Vial, and Ovidiu Radulescu**

Abstract

Apicomplexan parasite diseases cause severe health consequences in humans and livestock. Increasing resistance of parasites toward available drugs poses several challenges for treatment and cure. Of crucial importance for any attempt to fight the parasites is the need to understand the complex changes of regulation of gene expression and metabolism during various stages of their life cycle. This can be done by combining integrative transcriptomic, proteomic, and metabolomics studies with mathematical modeling to fill in the gaps in knowledge whenever these occur. In this chapter, we review some of the methods used for unraveling the parasite adaptation dynamics and suggest how they can be used for identification, selection, and validation of drug targets. These methods include omics based approaches, biochemical experiments, and mathematical modeling. In particular, we discuss glycerophospholipid metabolism in Plasmodium where several drug targets have been identified and validated.

Introduction

Apicomplexa are intracellular eukaryotic parasites that are responsible for diseases such as malaria, toxoplasmosis, and cryptosporidiosis. Parasitic diseases are a worldwide scourge, in terms of morbidity and mortality in humans. Increasing resistance of parasites toward available drugs poses several challenges in treatment and cure. In order to fight against the parasites, it is necessary to understand their complex biology with changes of regulation of gene expression and metabolism during the various stages of their life cycle. This can be done by integrative analyses of transcriptome, proteome, and metabolome and ask for mathematical modeling to fill in the gaps in knowledge whenever required.

Plasmodium and *Toxoplasma* both belong to the phylum Apicomplexa (or Sporozoa). *Plasmodium falciparum* and *Toxoplasma gondii* are obligatory intracellular parasites known to cause malaria and toxoplasmosis, respectively.

*Corresponding author.

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They possess distinct cellular tropism and exhibit a complex, multistage life cycle, each being reflected in stage-specific morphologies, transcriptomes, proteomes, and metabolomes [1, 2]. There are numerous significant metabolic differences between these parasites and their human host that afford distinct opportunities for drug development [3]. A common trait of Apicomplexa is the complexity of their life cycles and differentiation stages with regular alternation of sexual and asexual development in two or more hosts. *P. falciparum* has a life cycle subdivided into two parts. One part takes place in the human vertebrate host in which *Plasmodium* replicates asexually in two distinct phases: the pre-erythrocytic phase in the liver and the symptomatic phase in the erythrocyte. Once in the erythrocytes, the parasite develops through ring, trophozoite, and schizont stages, and the increasing parasite load over the time of infection causes the disease known as *malaria*. The other part of the life cycle takes place in the mosquito, where the parasite reproduces sexually and then replicates asexually with release of thousands of sporozoites that are injected to the vertebrate host.

T. gondii exists in three forms: oocysts, tachyzoites, and bradyzoites. The parasite reproduces sexually only in cats while its asexual multiplication by cell division can occur virtually in any host cell. During asexual phases, *T. gondii* exhibits two developmental forms, the actively dividing tachyzoite stage, which is found during the acute phase of toxoplasmosis, and the quiescent bradyzoite stage that forms tissue cysts and defines the chronic stage of the asexual cycle.

The transitions between the different developmental forms of Apicomplexa are accompanied by important reprogramming of the transcriptome and proteome. An important principle of this strategy is the "just-in-time" hypothesis, consisting in transcriptome reprogramming allowing rapid developmental transitions [4]. In contrast to other eukaryotes such as yeast, for which only a relatively small part of the transcriptome is involved in cell cycle progression, Apicomplexa mobilize up to 75% of their genes in their actively dividing developmental forms. The molecular mechanisms performing the switch between quiescent and active forms, of crucial importance for the proliferation of the parasite, are still largely unknown [5]. In *Plasmodium*, this picture is further complicated by epigenetic events that remodel the nuclear architecture and chromatin compaction synchronously accompanied with the changes of gene expression [6]. The cell cycle is submitted to the clock-like behavior of the transcriptome, with a large proportion of genes undergoing periodic variations in transcription during the intraerythrocytic developmental cycle (IDC) [7].

Apicomplexa mobilize important resources for rapid adaptation and biomass production during active developmental stages within a changing environment. This adaptation capacity is robust. A first aspect of this robustness results from peculiarities of transcriptional control in the parasites. Most of the studies agree on the paucity of the transcription factors in Apicomplexa. Unlike yeast, where there is one transcription factor (TF) for about 30 genes (200 TF for 6000 genes, [8]), the apicomplexan AP2 transcription factor (ApiAP2) family has 27 members for more than 5000 genes in *Plasmodium* [9]. In spite of the small number of regulators, the apicomplexan transcriptional network seems to be strongly protected

against perturbations of ApiAP2 expression; inhibitors of ApiAP2 trigger compensatory upregulation of the regulated genes [9]. Another aspect of robustness concerns the multiplicity of metabolic pathways that the parasites use for the synthesis of metabolites that are not scavenged from the host. Inhibition of one of these pathways may induce compensatory activation of parallel pathways and parasite resistance. Phospholipid (PL) metabolism is a typical example in this respect. During its intraerythrocytic development, *P. falciparum* activates several metabolic pathways that are rarely found in a single organism. The structural PLs, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), are thus produced by several distinct metabolic pathways. The human *Plasmodium* parasite exhibits additional reactions that bridge some of these biosynthetic routes, a feature otherwise restricted to different organisms, such as plants [10]. The cellular location of individual metabolic pathways, their possible cross-regulation, and possible redundancies between the pathways are not completely understood.

All these questions are of paramount importance to understand parasite biology and to design inhibitors that stop the proliferation of these parasites. As emphasized by systems biology, regulatory systems perform trade-offs between robustness and flexibility [11]. A robust system also comprises vulnerable targets that can be discovered by analyzing its behavior under perturbations via mathematical modeling. Therefore, new strategies, combining integration of various types of omics data and mathematical modeling, are needed for answering these questions. Systems biology methods were instrumental in unraveling molecular regulatory mechanisms of well-studied organisms [12]. There are now great opportunities for using these methods in the study of Apicomplexa. Genomes of *Plasmodium* [13] and *Toxoplasma* are available since the early 2000s and were used to reconstruct genome-scale metabolic networks [14, 15]. There are also good-quality transcriptomic datasets, some with unrivaled temporal resolution [7]. RNA-seq, polysome-seq, and more recently, ribosome profiling brought information about splicing and translation regulation [16, 17]. Proteomic data [18–20] is also abundant, and metabolomic datasets are becoming available [21–25].

The regulatory and compensatory mechanisms providing stability of metabolic and signaling networks are highly dynamic. Kinetic modeling, based on dynamical equations and predicting time dependence of biological variables, is crucial for understanding these processes and accurate prediction of drug targets. In this chapter, we discuss various integrative approaches for omics data analyses and mathematical modeling techniques, with a particular focus on kinetic modeling of glycerophospholipid (GPL) metabolism where various drug targets have been identified and validated in *Plasmodium*.

Omics-Based Approaches

Different omics-based approaches such as epigenomics, transcriptomics, proteomics, metabolomics, and fluxomics have been used to identify biomarkers

that indicate a specific physiological state of parasites [26]. The biomarkers can thus distinguish between the various developmental stages in different host cells and are important to characterize the growth, development, and regulation of the parasites [27, 28].

Comparative genomics of Apicomplexa [29, 30] identified, through exported proteins in *P. falciparum*, conserved motifs termed *Plasmodium* export element (PEXEL) and vacuolar transport signal (VTS). These elements are necessary for export of hundreds of proteins from the parasites that serve to remodel the host erythrocyte [30–32]. Comparative genetics has also been important in locating genetic regulatory elements, particularly the ApiAP2 transcription factors [33, 34]. Comparative genomics of *T. gondii* and *Neospora caninum* also identified an unexpected expansion of a surface antigen gene family as well as a divergence of secreted virulence factors, including rhoptry kinases. Unlike *Toxoplasma*, *Neospora* is unable to phosphorylate host-immunity-related GTPases. This defense strategy is thought to be key to virulence in *Toxoplasma* [35].

Transcriptomics

Microarray-based technology has been used to identify genes in *P. falciparum* that are expressed in various stages of the parasite life cycle [7, 36, 37]. Next-Generation Sequencing (NGS) (RNA-seq) identified new open-reading frames with untranslated flanking regions [38]. In addition to mRNA-related transcriptomics, noncoding protein RNA (ncpRNA) in the *P. falciparum* transcriptome detected 604 putative ncpRNAs [39, 40], which might play crucial roles in determining antigenic variation and virulence mechanisms [41]. Transcriptomics of *Toxoplasma* showed functional diversification of the transcription and translation machinery marked along the temporally coordinated gene expression with parasite cell cycle. Moreover, 35% of all *Toxoplasma* genes were identified to exhibit cyclic expression patterns that are coordinated with the parasite replication cycle. These dynamic expression patterns reflect a functional diversification of gene expression that allows for rapid and efficient "just-in-time" transcription of genes that are functionally relevant for the different phases of the cycle [42, 43].

Proteomics

Proteomics [18, 44] and interactomics [45] studies added functional annotations to the genes. Proteomics analyses surveyed stage-specific proteins and investigated them as potential drug targets. Parasite surface proteins (parasite proteins that are exported to the surface of the infected red blood cells) also represent new potential antigens for rational vaccine development [18, 20, 44, 46]. Comparative proteomics throughout the life cycle of *P. falciparum* identified the sporozoite proteome that appeared markedly different from other life-cycle stages [18, 47]. In *Toxoplasma,* difference gel electrophoresis (DIGE) coupled

with mass spectrometry was utilized to examine protein expression differences in tachyzoites and bradyzoites. Proteins ROP9 and GRA9 were found to have greater expression in the bradyzoite stage, although ROP9 has been previously shown as a tachyzoite-specific protein [48], and GRA9 has been associated with both stages [49].

Metabolomics

Metabolomics investigates small molecules that are the final products of cellular regulatory processes and provides a direct read out of the metabolomics state of parasite cells, which cannot be obtained from transcriptomics and proteomics.

^{31}P-NMR has been used for profiling of parasite phosphate metabolism and identified condensed phosphates in *Plasmodium*, *T. gondii*, and *Cryptosporidium parvum* [50]. ^{13}C NMR has been used to characterize abundant metabolites in *P. falciparum*-infected erythrocytes [51]. Moreover, it measured glucose flux in *P. falciparum* and *P. yoelii* infected erythrocytes [52]. Mass-spectrometry-based metabolomic analysis of the *P. falciparum* throughout its 48 h IDC showed general modulation of metabolite levels by the parasite, with numerous metabolites varying in phase with the developmental cycle. The phases of the metabolite abundances also differed from those of the host cells, irrespective of the developmental stage [24].

Metabolomics has been recently used to characterize the malarial parasites *P. berghei* and *P. falciparum* and their distinct host cells (cometabolomes), so as to gain great insight into the development of erythrocyte- or reticulocyte-specific malaria parasite, their specificity, and their exchange with host cells. The study found out key metabolic differences between the asexual and sexual stages of parasites [2]. The last point is highly interesting because the parasite has to adapt to different host types (vertebrate and mosquitoes), and how it handles these different (sometimes hostile) environments is poorly known.

Metabolite profiling and stable isotope labeling have been used to identify pathways of carbon metabolism in *T. gondii* at tachyzoite stages. The parasite stages constitutively utilize both glucose and glutamine as major carbon sources. The highly efficient and flexible energy metabolism underlies the extraordinary capacity of these parasites to proliferate within a wide range of host cells [53].

Some metabolites such as PLs constitute a major class of lipids in *Plasmodium* and *Toxoplasma*. These PLs are important for membrane biogenesis during parasite proliferation. Lipidomic analysis in *Toxoplasma* carried out by electrospray ionization–tandem mass spectrometry measured 2% of the total polar lipid, including ceramide phosphoethanolamine. *T. gondii* has higher levels of PC, but lower levels of sphingomyelin and phosphatidylserine (PS). The distinctive *T. gondii* tachyzoite lipid profile may be particularly suited to the function of parasitic membranes and the interaction of the parasite with the host cell and the host's immune system [54]. In *Plasmodium*, a comprehensive method of identifying and quantifying metabolites of this intracellular parasite using liquid

chromatography–tandem mass spectrometry (LC-MS) was proposed and implemented [21]. This method allowed for reliable measurement of water-soluble metabolites involved in PL biosynthesis, as well as several other metabolites that reflect the metabolic status of the parasite including amino acids, carboxylic acids, energy-related carbohydrates, and nucleotides.

Functional annotation of genes by metabolic profiling has been leveraging biochemical and metabolomics strategies [55]. It has already uncovered important biological insights with possible implications in terms of adaptation, evolution, and host–pathogen interactions [24, 51, 56].

Fluxomics

In the past decades, isotope labeling became a major tool for studying metabolic activity of many organisms from bacteria to human [57, 58]. Such experimental techniques have as primary aim the quantification of intracellular metabolic fluxes. Labeling techniques were used to understand PL metabolism in *Plasmodium* by incorporation of PL-radiolabeled precursors and measurement of concentration of end products and intermediate labeled metabolites [59]. These techniques were also used to understand regulation of PC biosynthesis in *Plasmodium*-infected erythrocytes [59]. Moreover, in *Plasmodium*, the fluxomic studies include the assessment of influx of glucose [52], isoleucine [60], and inorganic phosphate [61].

In *T. gondii*, fluxomic and labeling techniques identified significant accumulation of the unexpected metabolite, GABA. The enzymes responsible for the GABA shunt were subsequently identified and confirmed by genetic knockout in combination with U-^{13}C-glutamine labeling [62] (Table 20.1).

Integration of Transcriptomics, Proteomics, and Metabolomics Data

With the emergence of high-dimensional data in the postgenomic era, the role of computational and systems biology is paramount. Out of various aspects, these disciplines deal with integration of large-scale omics data obtained from high-throughput genomic, proteomic, and metabolomic experiments. Integrative approaches provide insights into the understanding of the molecular basis of parasite development, differentiation, and identification of key regulations Figure 20.1. They also lead to the identification of novel genes, proteins, metabolites, pathways, and networks of regulations within the parasite and between the parasite and the hosts. The study of quantitative and qualitative relations between genes and proteins at a large scale opened a new avenue in parasitology research. Several methods of integration between transcripts and proteins have been attempted to understand the regulation of parasite development under varying conditions. A quantitative analysis of the mRNA levels measured at nine different time points (the mosquito salivary gland sporozoite, seven erythrocytic a sexual

Table 20.1 Summary of omic techniques used in the studies of Apicomplexa.

Omics approaches	Advantages	Limitations	References
Transcriptomics			
High-density oligonucleotide microarrays	Estimates the absolute abundances, streamline handling, greater reproducibility	Limited sensitivity due to hybridization, background noise, and choice of appropriate normalization methods	[37, 63–65]
Spotted microarrays	Flexible, low background noise	Measure relative abundances, low probes per genes, comparison between arrays, or experiment is difficult	[66, 67]
qPCR	High precision and sensitivity, increasingly multiplexed	Low throughput, choice of data normalization, and reference gene selection is a subtle task	[68, 69]
High-throughput sequencing (RNA-seq)	Single base resolution, low background noise, less sample need, ability to distinguish between isoforms, allelic expression	Read mapping and uncertainty, transcript length bias	[17]
Proteomics			
2DE-MS	Large-scale separation and identification of proteins in a sample	Limited sensitivity, lack of reproducibility, poor separation of acid, basic, hydrophobic, and low abundant proteins	[70, 71]
DIGE	High sensitivity and less integral variability	Needs special equipment for visualization with expensive fluorophores. Protein without lysine cannot be labeled	[72]
Protein arrays	High sensitivity and specificity, useful for specific class of protein, low sample need	Low proteome coverage, antibodies and affinity reagents dependence, low protein expressions	[73]
ICAT	High potential, good sensitivity, detect low expression peptides	Limited to protein containing cysteine, not amenable to translationally modified and acidic proteins	[53]

(*continued overleaf*)

Table 20.1 (*continued*).

Omics approaches	Advantages	Limitations	References
SILAC	High degree of labeling Ease with quantification	Labeling of tissue sample is not possible	[74, 75]
iTRAQ	Higher confidence of identification than ICAT Good sensitivity and consistency Identify posttranslationally modified proteins, multiplex several samples	Increase MS time due to high number of peptides, peptides has to be fractionated before MS, sample complexity	[72]
LC-MS	High sensitivity, moderate to high resolutions	Matrix effects, complex data, and analysis	[44]
MUDPIT	Identify large protein complexes, high degree of separation	Not quantitative, inability to distinguish isoforms	[18, 20]
Metabolomics			
GC-MS	High separation efficiency, reproducible, robust, identify different classes of metabolites, robust, large linear range	Slow, unable to analyze thermolabile metabolites, requires derivatization of nonvolatile metabolites	[76]
LC-MS	High sensitivity, moderate to high resolutions, analyze thermolabile metabolites, no derivatization required	Matrix effects, desalting necessary, limited commercial chemical libraries	[21, 24]
CE-MS	Small sample volume, high resolutions, fast and efficient separation of charged and uncharged species	Complex methodology and quantification, poor retention time reproducibility	—
FTIR	Rapid analysis, no derivatization required, gives complete finger print of sample chemical composition	Complex and convoluted spectra, sample drying	[77]

Table 20.1 (*continued*).

Omics approaches	Advantages	Limitations	References
NMR	Rapid analysis, high resolution, no derivatization needed, nondestructive	Complex matrix, convoluted spectra, limited libraries available	[56, 78, 79]
Fluxomics			
NMR analysis	Minimal sample, high reproducibility	High cost, low throughput relative to MS, complicated data deconvolution, and statistical fitting procedures	[52]
MS analysis	Highly sensitivity, rapid, more resolvable metabolites	Complicated data deconvolution and statistical fitting procedures	[62]

stage time points and finally the sexual stage gametocytes) of *P. falciparum* using a short oligonucleotide array [37] and the semiquantitative analysis of protein levels measured at seven stages (ring, trophozoite, schizont, merozoite, gametocyte, gamete, and salivary gland sporozoite) detected by multidimensional protein identification technology (MudPIT) [18] provided abundance profiles for thousands of parasite transcripts and proteins. These abundance data correlated to specific parasite stages and time points in the parasite life cycle. Among various genes, *var* and *rif* genes that encode antigenically variant proteins exposed on the surface of *Plasmodium*-infected erythrocytes were found to be largely expressed in sporozoites. Chromosomal clusters encoding coexpressed proteins suggested a potential mechanism for controlling gene expression [18].

Foth *et al.* compared mRNA and protein abundance profiles of *P. falciparum* during the IDC at 2 h resolution using oligonucleotide microarrays and two-dimensional differential gel electrophoresis protein gels. One important finding of this study was that most proteins apparently are represented by more than one isoform, presumably because of post-transcriptional modifications [19]. Further, it was found when correlating mRNA and protein profiles that significant time shifts between transcript and protein levels in *P. falciparum* occur. Abundant protein peaks arise significantly later (median delay of 11 h) than the corresponding transcripts, which gradually decrease in the second half of the IDC along the 48 h life cycle. Computational modeling showed that varied incongruence between transcript and protein abundance may largely be caused by the dynamics of translation and protein degradation [19]. Transcripts and protein abundance levels were also compared for seven different stages of the parasite life cycle [20]. A moderately high positive correlation between mRNA and protein abundance was determined for these stages. However, a delay was found between mRNA

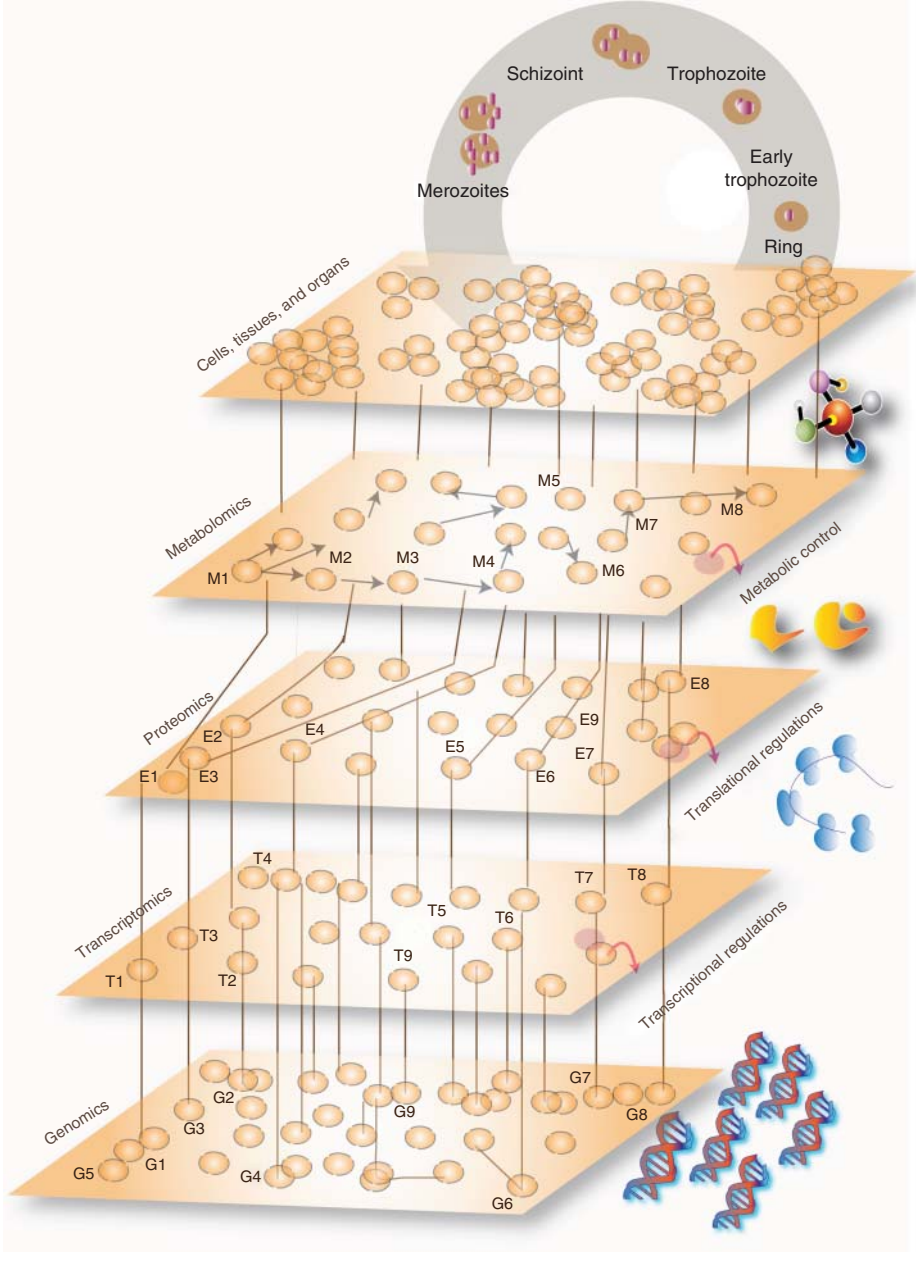


Figure 20.1 Omics data integration is needed to understand fully the regulation of biological processes during the life cycle of Apicomplexan parasites.

and protein accumulation. Potentially, post-transcriptionally regulated genes were identified, and families of functionally related genes were observed to share similar patterns of mRNA and protein accumulation.

From all these studies, it appears that there is an apparent prevailing trend of delay between the occurrence of maximal mRNA transcript concentrations and their corresponding protein products. It was therefore proposed that *Plasmodium* has an intricate system of post-transcriptional regulation that modulates the overall gene expression pattern associated with the parasite life cycle [20, 80, 81].

In *T. gondii*, integration of detected proteome with microarray results also revealed some interesting discrepancies. About 204 proteins that correspond to 25% of least abundant mRNA were detected. From the most abundant mRNAs (~1900 genes), only half of the proteins were detected by proteome analysis. It was hypothesized that many of the genes expressed with high mRNA levels do not exhibit similarly high abundances in their gene product (protein). This suggests the existence of a considerable degree of control that regulates the level of protein abundance, independent of the rate of transcription in tachyzoites [82].

While several attempts were made on integration of transcripts and proteins, very few studies were known to combine transcriptomics and proteomics with metabolomics in Apicomplexa. Integration of transcriptomics and metabolomics in *Saccharomyces cerevisiae* and *Plasmodium* showed that genes along a metabolic pathway(s) were highly coordinated [83, 84]. Clearly, this regulated coordination of gene expression along metabolic pathways is intended to affect the protein and finally the metabolite profiles. Enzymes are key players that drive the metabolic forces. Following an environmental stress, the enzymes alter the metabolic concentrations through specific reaction kinetics and redirect metabolic fluxes. Combining the omic technologies (e.g., transcript, protein, and metabolite levels) is appropriate to decipher the specific impact of drugs as shown with spermine synthase inhibitors [85]. Interestingly, global effect of inhibiting the bifunctional enzyme *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (AdoMetDC/ODC) on the *P. falciparum* IDC was measured by following global transcript, protein, and metabolite abundance.

Polyamine depletion was confirmed as the primary mode of action since, as expected, metabolite analysis revealed a significant drop in parasite polyamine levels, specifically spermidine and putrescine, with a concomitant rise in the upstream metabolite *S*-adenosylmethionine. In addition, the drug inhibition of AdoMetDC/ODC triggered a complex adaptation response involving both downstream and upstream enzymes and metabolites. Despite the general transcription arrest induced by the drug, ornithine aminotransferase (OAT) was found upregulated resulting in degradation of ornithine. On one side, this avoids the ornithine accumulation that would result from the ornithine decarboxylase (ODC) inhibition (see also Chapter 14, Creek *et al.*). On the other side, it leads to accumulation of glutamate metabolites that result from ornithine degradation [86]. This is a step forward toward parasite systems biology that illustrates how parasite metabolite levels fluctuate in response to changes in parasite

gene expression at the transcriptional and protein level and how metabolite concentrations can feedback to regulate gene expression.

Integration of transcriptomics and proteomics together with metabolomics is still a subtle task. Metabolism represents the sharp end of cellular process; changes in metabolite concentration are necessarily amplified relative to changes in the transcriptome, proteome, and enzymes activity (which could be modulated by stress, drugs, etc.). However, complex data, systematic variations, and unknown parameters are bottlenecks for such integrative studies. Various bioinformatics and mathematical tools were proposed for effective integration of omics data [87–89]. They open up great opportunities but also have limitations as discussed in the next section.

Mathematical Modeling

The research on Apicomplexan parasites offers many opportunities for computational approaches and mathematical modeling. Mathematical models provide scaffolds to overlay biological data. They are also deployed to fill in the gaps in undetermined gene and metabolic networks. Simulation of validated models leads to a better understanding of mechanisms that govern parasite development and differentiation and can be used for testing drug action *in silico*. Furthermore, models serve as guides to postulate new hypothesis that can be tested experimentally. As complementary parts of a virtuous circle, modeling and data production generate new relevant information such as regulation of gene expression, and metabolic pathways.

Data Mining and Machine Learning Studies

Data mining and machine learning designate a wide range of mathematical techniques for automatic extraction of information and knowledge production out of experimental data. By combining different types of data and mathematical models, data mining and machine learning can fill the gaps in the comprehension of biological phenomena. Recent developments in this field were spurred by the heterogeneity of biological data types and formats and by explosive data volumes [90, 91].

Establishing new biological hypotheses based on large datasets requires a new generation of scientists that combine mathematical skills with a profound understanding of biological processes. It should be nevertheless emphasized that data mining and machine learning, sometimes called *data science*, are not miracle solutions to all complex problems in biology. It is well known that these methods suffer from the curse of dimensionality, a generic phenomenon that affects the analysis of data in high-dimensional space and imposes strong limitations to the size of the models that can be learned from data. In order to solve a particular problem, data scientists are confronted with numerous trade-offs. However, implemented properly, data mining techniques can be

valuable tools, for instance, in the context of apicomplexan biology. Indeed, Apicomplexa have intricate life cycles with several stages inside the vector and the mammalian host. In spite of extensive data production in the past years, little is known about the mechanisms regulating the events in the life cycle of these organisms.

The transcriptome and proteome of malaria parasites have been extensively studied with a variety of data mining approaches. Transcriptomic studies using microarrays [66] showed that 75% of the genes are activated only once during the 48 h of IDC and time-dependent mRNA profiles of many genes are almost sinusoidal. This clock-like behavior was also found by subsequent RNA-seq studies [17]. Modeling studies can be used to infer gene–gene interactions networks from time-dependent expression profiles. For instance, probabilistic genetic networks, a special type of Markov chain model that uses nonlinear threshold functions to describe the response of a gene to its regulatory inputs, have been used as models of interacting genes in *Plasmodium* [92]. Confronted to the IDC transcription microarray dataset, this approach has successfully identified a glycolysis gene network and has predicted putative apicoplast proteins.

Statistical approaches, using both Bayesian and frequentist (correlation analysis, position weight matrices) frameworks, were used to unravel *Plasmodium* interactome by integration of functional genomics data. Such approaches contributed to functional annotation of proteins, based on their association ([93], among many others) and to *in silico* prediction of putative regulatory elements [94, 95]. Of particular interest are the mutual interactions among the genes of the *var* family, whose switched time-dependent patterns are the hallmark of mechanisms used by *Plasmodium* to deceive the immune system of the host. Markov chain models, describing the switching from one variant to another, were learned from transcription data and revealed a highly structured pattern of transcriptional changes [96].

Although complex in their phenomenology, processes such as IE transcriptional clock or *var* gene switching patterns could eventually rely on simple mechanisms. For instance, negative feedback circuits involving a small number of genes could lead to oscillatory, clock-like activity, whereas networks of mutually repressing genes could undergo complex switching patterns. The knowledge of the mechanisms and of the main molecular actors of these processes is prone to provide lethal weapons against the parasites. In spite of some attempts to elucidate these phenomena, the whole network of interactions among genes and proteins of *Plasmodium* remains largely unknown and is an important task for the future. Furthermore, no mechanistic models are available so far, for the complex regulatory phenomena discussed earlier.

One promising direction toward the elucidation of mechanisms triggering various events in *Plasmodium* life cycle is the study and mathematical modeling of multiple epigenetic control of gene expression, such as histone modifications, gene localization within the nuclear architecture, and nucleosome remodeling. In particular, clustering of *var* genes into distinct nuclear compartments could be important for the epigenetic silencing of these genes [6, 97]. In this field as

well, significant progress resulted from the combination between several types of datasets such as ChIP-seq, Hi-C, FISH, 3D mathematical models of nuclear architecture, and machine learning techniques [6, 97].

Constraint-Based Modeling of Metabolism

The possibility of genome-wide reconstruction of metabolic pathways from the DNA sequence, combined with growing amounts of fluxomic and metabolomic data, gave a noteworthy advantage to metabolic modeling with respect to the more difficult problem of expression regulation. Annotation of the genome has led to reconstruction of genome-scale metabolic networks in *P. falciparum* and *T. gondii*. Genome-scale networks can be subjected to topological analysis, or they can be simulated to predict phenotypes such as growth, flux distribution, and metabolic capabilities.

Flux balance analysis (FBA) is a constraint-based approach that predicts the phenotypes of different organisms under various conditions, given the biomass composition. FBA can predict the effect of *in silico* knockouts on the distribution of metabolic fluxes and viability of the parasites. Network analysis is a fully topological approach that computes graph theoretical indices of network vertices (degree, centrality) in order to estimate the relative importance of network components. On the other hand, network analyses studies do not rely at all on parameters and consider graph theoretical indices such as centrality, derived from network topology, to estimate the relative importance of network components.

Several works were proofs of principle that FBA and network analyses studies could guide the search for drug targets and the discovery of new type of inhibitors [98, 99]. These two mathematical approaches are suitable for situations when the uncertainty about the reaction mechanisms and about the values of the kinetic parameters is high, because they do not use this type of information and depend on only a few or no parameters at all. However, they are based on strong assumptions, such as the optimal production of biomass in the case of FBA and the equivalence of the fluxes in the case of network analysis. Furthermore, both methods are static and do not deal with dynamic changes of gene expression during the developmental cycle of the parasite.

An improved version of FBA that employs extra constraints of the metabolic fluxes by mRNA abundances has been applied to the *Plasmodium* network [100]. This method showed some improvement in drug target identification (decrease of false positives). Similarly, a constraint-based model *iCS382* consisting of 571 reactions and 492 metabolites was deployed to understand metabolic contributions in development and strain-specific infectivity differences in *T. gondii*. Further, by combining FBA with mRNA expression data, this model was used to predict strain-specific differences in drug susceptibilities, which were validated by drug-based assays [101]. ToxoNet1, another reconstructed genome-scale metabolic model of *T. gondii*, predicted a minimal set of 53 enzyme-coding genes and 76 reactions to be essential for parasite replication. Double-gene essentiality

analysis (using FBA) identified 20 pairs of genes for which simultaneous deletion is deleterious to parasite viability [14] (Table 20.2).

Kinetic Pathways Modeling

Kinetic modeling of metabolism is a promising alternative to static approaches. In kinetic models, biochemical reactions are represented mechanistically by the stoichiometry of reactants, products, and cofactors. Furthermore, the reaction rates are functions of the concentrations of the reactants, enzymes, and cofactors. The reaction rates and stoichiometry allow obtaining kinetic differential equations describing the evolution of concentrations of metabolites in time. The rate functions and therefore the kinetic differential equations contain parameters that can be measured by biochemical methods or result from fitting the model to data.

Fully parameterized kinetic models can be used to simulate the time-dependent distribution of metabolic fluxes and concentrations of metabolites under physiological conditions or under perturbations resulting from knockouts and knockdowns of specific genes or inhibitions by drugs. Kinetic models are more precise than static models in identifying critical variables and parameters. Furthermore, they offer the possibility to integrate metabolic pathways with gene regulation and protein–protein interactions, which is crucial for predicting adaptation and resistance to treatment of parasites. The main limitation in their development is the scarcity and incompleteness of kinetic data.

As a matter of fact, *in vitro* biochemical assays may provide parameter values that could differ from the *in vivo* ones [106]. Therefore, *in vivo* parameters should be learned by confronting fluxes and metabolite concentrations predicted by the model to fluxomic and metabolomics data. Several experimental protocols that facilitate incorporation of radiolabeled (hot) precursors and measurement of concentrations of their end products and intermediates in the metabolic process are useful to this end. The rates of replacement of hot by cold forms of the metabolites or *vice versa* depend on the topology of the metabolic network and on the rates of various biochemical reactions within the network. It helps to understand the synthesis and clearance rates of metabolites with appreciation of half-life time in the first case. Model parameters can be learned from the change of concentration ratios of unlabeled to labeled compounds over time. Learned parameter values correspond to minima of an objective function representing the goodness of fit. In general, finding these minima is a difficult optimization problem. For some special cases, metaheuristic methods (restricting the search in a large set of possible solutions to a specific smaller subset) can simplify the task.

Hybrid algorithms combining local optimization for continuous rate parameters and nonlocal optimization for discretized flux variables worked well in the special case of Michaelis–Menten kinetic networks [104]. It is usual that the model has insensitive parameters or that several local optima have comparable goodness of fit. For these reasons, some parameters are less constrained and

Table 20.2 Summary of main mathematical modeling approaches used to unravel mechanisms of regulation of Apicomplexan life cycle.

Class	Mathematical methods	Data handled					Comparison	
		Genome	Epigenetic	Transcriptome	Proteome	Metabolome	Strengths	Weaknesses
Data mining, machine learning	Markov chains [92, 96] Bayesian and frequentist [93] Kernel methods [97] Optimization [97]	✓	✓	✓	✓	✓	Lack of bias Multicriteria Integrative Predict networks and mechanisms	Curse of dimensionality Start from scratch
Constraint-based	FBA [98, 99] FBA + expression constrains [14, 100]	✓		✓		✓	Genome-wide Do not depend on rate functions and kinetic parameters Predict effects of drugs and knockouts	May lack precision Static
Kinetic modeling	Networks of chemical reactions [102–105]		✓	✓	✓	✓	Dynamic Integrative Predict effects of drugs and knockouts Predict adaptation and resistance	Parameter uncertainty Need mechanistic details and rate functions

therefore less precise than others. Therefore, robustness of parameter inference should be tested by sensitivity analysis (SA) [104].

Radiolabeling methods are sensitive and accurate, but may become cumbersome for large-scale networks. Numerous experiments are needed for complete parametric reconstruction. In this case, isotopomer fluxomics, with distinct labeling techniques, provides additional and prominent features about metabolite interconversions. This technique generates richer information that can be used to simultaneously probe fluxes in parallel pathways.

In spite of its limitations, kinetic models are precious tools for fundamental research, pharmacokinetics, biomarkers, and drug design. A detailed kinetic model of glycolysis in *P. falciparum* has recently been proposed [103]. The model uses information on glycolytic enzymes whose mechanisms were precisely characterized for a variety of *Plasmodium* species. It has been validated by comparing its predictions to the measured glycolytic steady-state fluxes and the concentrations of hexose phosphate intermediates in the trophozoite stage of the parasite. A kinetic model of the methylerythritol phosphate pathway of *P. falciparum* [105] has been used for *in silico* inhibition studies. The model has shown that *in silico* inhibition of two critical enzymes can cause a large decrease in the pathway flux. A kinetic model of GPL biosynthesis in *Plasmodium knowlesi* [104] is discussed in a separate section.

Kinetic models were also used to understand and characterize stage-specific host-cell invasion by *Toxoplasma*. For some strains, the time taken to penetrate the host-cell plasma membrane was predicted to be 26 s (95% CI: 22–30 s). Findings from the model suggest that the parasites that ultimately invade remain attached three times longer to the host cells than the parasites that eventually detach. About 25% (95% CI: 19–33%) of parasites invade while 75% (95% CI: 67–81%) eventually detach from their host cells without progressing to invasion. The key feature revealed by the model added to the better understanding of the parasite invasion stages that cannot be observed directly [102].

Role of Kinetic Models to Elucidate Mechanism of Effectors and Identify Putative Drug Target

Current drug discovery programs still rely on simplistic approaches screening compounds on individual targets rather than focusing on biomolecular networks and ignoring the complexity of biological systems. The mechanism of drug action depends on their activity on particular enzymes, receptors, and transporters embedded within the metabolic network [107]. Characterization of the kinetic properties of these networks would help to identify regulatory or rate-limiting steps essential for parasite's survival. Knockout or inhibition of these steps would have deleterious effects. This would also identify parallel or redundant pathways that serve as an alternate source to fuel the respective parasite's development. However, the costs of experiments, restriction on clinical trials, and patient recruitment and retention are the bottlenecks in the drug discovery process [108]. It also limits our understanding of biological system as a whole.

Computational and *in silico* approaches based on kinetic modeling are good tools in filling these gaps with limited resources and therefore should be developed to understand the behavior of the system as a whole [104, 109].

As a case study, we discuss how a quantitative kinetic model together with the biochemical experiments has been deployed to achieve these objectives. The quantitative model was used to elucidate the functioning of the multiple PL synthetic pathways and identification of critical steps in *P. knowlesi*, which is discussed in the next subsection.

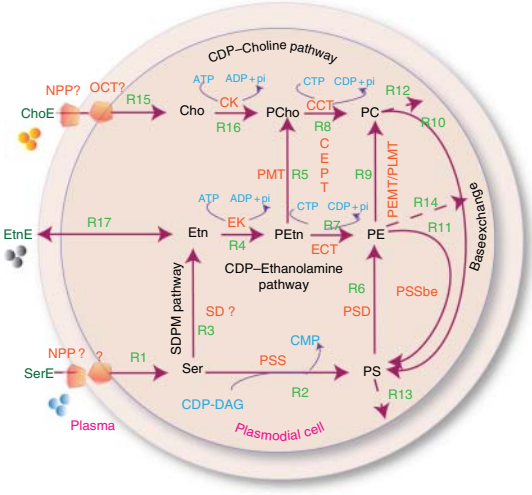
Phospholipid Synthetic Pathway Model in *Plasmodium*

GPLs are the main *Plasmodium* membrane constituents, with a preponderance of PC and PE and with an increase in phosphatidylinositol (PI) involved in signaling. Upon *P. falciparum* or *P. knowlesi* infection, the PL content in the erythrocytes increases to sixfold. In purified parasites, the main PLs are PC (40–50%), PE (35–45%), PI (4–11%), and SM and PS (<5%). At the blood stage, *Plasmodium* species thus display an unexpected puzzling number of metabolic pathways that are rarely found together in a single organism [110]: (i) the ancestral prokaryotic-type CDP–diacylglycerol dependent pathway; (ii) the eukaryotic-type *de novo* CDP–choline and CDP–ethanolamine (Kennedy) pathways; and (iii) *P. falciparum* and *P. knowlesi* exhibit additional reactions that connect some of these routes. A plant-like pathway named the serine decarboxylase–phosphoethanolamine methyltransferase (SDPM) pathway relies on serine to provide additional PC and PE. This route is of great interest as it involves serine decarboxylase (SD) that has been characterized in plants and is distributed sporadically throughout animal genomes [110, 111]. Kinetic modeling [104] together with biochemical experiments showed that the main source of PC is the CDP–choline Kennedy pathway and that SDPM and PE transmethylase pathways provide a smaller proportion of PC.

Model Reconstruction and Simplification

A detailed metabolic network of GPL metabolism in *P. knowlesi* was reconstructed and manually curated with the knowledge from literature and experts' discussion. The pathway scheme with metabolites, enzymes, and the associated reactions (R) is shown in Figure 20.2.

In the CDP–choline pathway, PC is produced from phosphocholine (PCho) in two steps. First, PCho is converted into CDP choline, and then CDP choline is transformed to PC. The intermediate CDP choline is produced in extremely small quantity, which was difficult to measure experimentally [59, 113]. The yield of labeled PCho was found to be 35 times greater than that of CDP choline. Thus, the formation of PC from CDP choline is very rapid relative to the formation of CDP choline from PCho [59], and CDP choline is a quasi-steady-state species. Therefore, the fast intermediate was ignored and two reactions were combined into a single reaction (labeled R8 in Figure 20.2). This was similar for the



Reaction	Reaction rate	Kinetic law
R1 = SerE -> Ser	Vm1*SerE/(km1+SerE)	Henri–michaelis–menten
R2 = Ser -> PS	Vm2*Ser/(km2+Ser)	"
R3 = Ser -> Etn	Vm3*Ser/(km3+Ser)	"
R4 = Etn -> PEtn	Vm4*Etn/(km4+Etn)	"
R5 = PEtn -> PCho	Vm5*PEtn/(km5+PEtn)	"
R6 = PS -> PE	Vm6*PS/(km6+PS)	"
R7 = PEtn -> PE	Vm7*PEtn/(km7+PEtn)	"
R8 = PCho -> PC	Vm8*PCho/(km8+PCho)	"
R9 = PE -> PC	Vm9*PE/(km9+PE)	"
R10 = PC -> PS	Vm10*PC/(km10+PC)	"
R11 = PE -> PS	Vm11*PE/(km11+PE)	"
R12 = PC ->	Vm12*PC/(km12*PC)	"
R13 = PS ->	Vm13*PS/(km13*PS)	"
R14 = PE ->	Vm14*PE/(km14*PE)	"
R15 = ChoE -> Cho	Vm15*ChoE/(km15+ChoE)	"
R16 = Cho -> PCho	Vm16*Cho/(km16+Cho)	"
R17 = EtnE <-> Etn	kf17*EtnE - kb17*EtnE	Mass action

Figure 20.2 Schematic overview of plasmodium reactions in structural phospholipid biosynthesis as demonstrated by experimental work. R1 to R17 denote the reaction rates/fluxes. List of species: SerE = exogenous serine, Ser = intracellular serine, PS = phosphatidylserine, EtnE = exogenous ethanolamine, Etn = intracellular ethanolamine, PEtn = phosphoethanolamine, PE = phosphatidylethanolamine, ChoE = exogenous choline, Cho = intracellular choline, PCho = phosphocholine, PC = phosphatidylcholine, DAG = diacylglycerol, SD = serine decarboxylase, PSSbe = phosphatidylserine synthase I, PMT = phosphoethanolamine-*N*-methyltransferase, PEMT = phosphatidylethanolamine-*N*-methyltransferase, CCT = choline phosphate cytidylyltransferase, ECT = ethanolaminephosphate cytidylyltransferase, CEPT = choline/ethanolamine phosphotransferase, CK = choline kinase, EK = ethanolamine kinase, NPP = new permeation pathway, OCT = organic cationic transporter, and ? = putative genes found. The full mathematical model can be found (ID BIOMD0000000495) in BioModels database (www.ebi.ac.uk/biomodels, [112]).

CDP – ethanolamine pathway (labeled R7 in Figure 20.2). All steps/reactions of the model, with the exception of ethanolamine influx, were considered irreversible. After model simplification, the Michaelis – Menten kinetics was used to model the rate of each reaction (with the exception of ethanolamine influx that was modeled by mass action law) and ordinary differential equations were written, which represent the rate of change of metabolites.

Model Training and Parameter Estimation

The GPL model has kinetic parameters such as maximum rates (V_{max}) and Michaelis constant (K_m) for each reaction step. In order to estimate the parameters, the GPL model was trained with two datasets, which also served as model inputs: (i) incorporation of serine [59] and (ii) incorporation of choline [114] as precursors that lead to the production different metabolites (PS, PE, PC) involved in the GPL metabolism pathways. The experimental datasets include the

steady-state concentrations of the radiolabeled precursors (serine and choline) with respect to their exogenous concentrations. The steady-state concentration of all serine- or choline-derived metabolites were predicted and used to fit the experimental data by least-squares optimization technique. A multiobjective function was designed for this purpose [104].

Model Analysis

The fitted parameters (V_{max} and K_m) were available to derive the reaction rates or fluxes. Fluxes quantitatively determine the flow of metabolites or their interconversion in the biomolecular network. The distribution of fluxes in a particular network represents a condition-specific phenotype.

Comparison of Kinetic Model (ODE Model) with FBA

In a study, exogenous concentration of serine was given as a feed to fully parameterized kinetic (ODE) model. Distribution of flux in different PL pathways was estimated. FBA, an alternative method, was deployed to maximize the lipid production and corresponding flux distribution in the pathways was also estimated. Although the two methods (FBA and ODE) showed similar trends in flux distribution, some marked differences were highlighted and discussed [104].

Fluxes obtained from the ODE model showed high flux in a direct decarboxylation of serine (SD, labeled R3) and CDP ethanolamine (EK (R4), ECT (R7)) (EK, ethanolamine kinase and ECT, ethanolamine-phosphate cytidylyltransferase) pathways (Figure 20.2). This suggested that R3, R4, and R7 are the preferred pathways for the formation of PE derived from serine; this is in agreement with the experimental findings [59]. On the contrary, fluxes from FBA showed higher flux into PSD (labeled R6, Figure 20.2) than ECT, which means that a major part of PE derived from serine was furnished by R6; this contradicts the experimental findings [59, 104]. This contradiction may be due to the lack of relevance of the biomass optimization in the situation when only serine is incorporated. This also emphasizes the utility of kinetic modeling whose precision supersedes that of FBA.

Incorporation of several parameters such as enzyme activities and affinities in the kinetic model makes it more reliable for the prediction of phenotype. FBA methods do not incorporate such information and solely depend on the stoichiometry and connectivity of the networks. Several efforts made to improve FBA by constraining the models with kinetic data have been discussed in the previous section.

Sensitivity Analysis and Determination of Rate-Limiting Steps

Kinetic models depend on parameters that characterize biochemical properties of reaction steps in the metabolic network. Perturbations of these parameters could alter the distribution of fluxes in the network and affect the functioning of the network and the final phenotype. The degree at which the network functioning

is affected by varying the parameters linked with the system is studied by SA. SA consists in computing sensitivity coefficients (SCs) defined as the derivative of the logarithm of a flux F at a given time, with respect to the logarithm of a rate constant k:

$$C_k^F = \frac{\partial \log(F)}{\partial \log(k)'}$$

where k is V_{max} or K_m.

In a particular case, this sensitivity coefficient is equivalent to the flux control coefficient (FC) as stated in metabolic control theory (see the Chapter 22, Niekerk *et al.*), where k is V_{max} and F is the steady-state flux. In this case, F is a homogeneous function of the parameters and the corresponding flux control coefficients satisfy the usual summation theorems [115].

SA was performed to characterize rate-limiting steps in CDP–choline pathway for PC biosynthesis. It was found that carrier-mediated choline entry (R15, Figure 20.2) into the parasite and the choline phosphate cytidylyltransferase (CCT) reaction (R8, Figure 20.2) have the largest sensitivity coefficients in this pathway [104]. This finding has been partially exploited in the search for antimalarial drug targets. Choline entry into the CDP–choline or Kennedy pathway is in fact targeted by a new class of potent antimalarial drugs, which are currently in clinical trials [116–118].

Relevance or Essentiality of Multiple Pathways

In order to understand the relevance of different pathways at varying concentration of the substrate or precursor for the production of PC, PE, and PS; *in silico* knockout experiments were designed. In these experiments, PMT (phosphoethanolamine-*N*-methyltransferase) and PEMT (phosphophatidylethanolamine-*N*-methyltransferase)/PLMT enzymes were deleted simultaneously and the effect on the production of PC, PE, and PS was monitored. *In silico* knockout experiments showed comparable importance of PMT and PEMT/PLMT for PC synthesis in *P. knowlesi*. These findings confirmed earlier hypotheses about the existence of both PMT and PEMT activities in *P. falciparum* and *P. knowlesi* [119–121]. Further, *in silico* knockout experiments prove partial dependence of PC production on both PMT and PEMT, meaning that single knockout of any of these enzymes will reduce but not completely eliminate PC production from serine in *P. knowlesi*. The absence of PMT [119–121] in other mammals makes it a target for the development of selective antimalarial with a broad specificity against different *Plasmodium* species. PMT has been known to be inhibited by amodiaquine and NSC158011, two drugs known to have potent antimalarial activity [122].

Conclusion and Future Perspectives

The omics experiments, data integration, and mathematic modeling are of huge interest to grasp the knowledge of Apicomplexan parasite biology and to identify

the crucial factors that are involved in their proliferation and differentiation, depending on their host cells. Elucidating regulations and nodes of regulation based on essentiality, limiting steps, and specificity regarding the host can guide the research for novel drug targets and the design of inhibitors. Quantitative kinetic modeling has been deployed to fill the gaps in metabolic networks, which was then used to understand the regulation of essential metabolic pathways in *Plasmodium*.

Kinetic models can be modified to incorporate different types of omics data. This will extend our understanding of the multiple layers of regulation that occurs within the parasite cells along their life cycles. The association and interrelation between or among the genes, proteins, and metabolites is the key to understand the functioning of the life cycle and to design effective weapons against the parasite. The next-generation kinetic models aim to resolve such issues. This could, for instance, be done by relating model parameters to time-dependent expression data. In the particular case of the GPL kinetic model discussed in this chapter, a simple modeling choice would be to consider that $V_{\max}$ parameters are proportional to the time-dependent concentrations of enzymes that are readily available or result from models of gene expression. This modification of the model would allow for understanding stage-specific functioning of parasite metabolism that can then be used to optimize drug therapies.

Failure of drug candidates in phase 2 or 3 clinical trials (when the human subjects are exposed to the drugs), the so-called late-stage attrition, is expensive to the pharmaceutical industry [123]. The constantly evolving model of drug development now dictates that systems biology should be employed for the early detection of likelihood of failure of potential drug candidates. Systems biology, which encompasses analysis of genomics, proteomics, and metabolomics data, will find more and more applications throughout the drug development process as well as after a product enters the market [124]. Combining omics data to mathematical models along with the experiments would allow for optimizing the drug discovery process via an in-depth analysis of biological systems and novel metabolism-targeted therapeutics. With the emergence of drug resistance and the need for rapid solutions to counteract such phenomena, the role of systems biology and mathematical modeling is paramount.

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21
Understanding Protozoan Parasite Metabolism and Identifying Drug Targets through Constraint-Based Modeling

*Francis Isidore Totanes, Sanu Shameer, David R. Westhead, Fabien Jourdan, and Glenn A. McConkey**

Abstract

The 21st century has seen a monumental advancement in genome sequencing and high-throughput "omics" techniques. The availability of massive amounts of data and the need to synthesize these data into a coherent form combined with increasing systems-level approaches has led to the development of computational techniques capable of modeling, simulating, and studying the full embodiment of metabolism in whole cells. In this chapter, we provide an introduction to the approach of genome-scale metabolic network reconstruction and flux balance analysis in the study of parasitic metabolism. We also summarize upon the various tools and databases available for use in this approach. Lastly, we also discuss the application of this approach to parasitology, prospect for enhancing drug target discovery, and the current limitations.

Introduction

Genome-Scale Models

Systems biology is the study of multiple molecular components and of their interactions at the cellular and organism levels. It involves using large datasets from high-throughput measurements, reconstruction of cellular systems, mathematical modeling, and *in silico* simulations [1]. The main objective of systems biology is to provide an understanding of complex biological systems, and to attain this, development of mathematical models is required. These models attempt to closely replicate wet lab experiments with the goal of computationally generating hypotheses that can be experimentally validated at the organism scale (also called *genome scale*) [2]. Genome sequencing data, gene–protein

*Corresponding author.

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reaction knowledge, and biochemical and enzymatic data on the metabolism of an organism are combined to create a genome-scale model. This genome-scale model is formatted following a defined and standard structure compatible with various software suites. Computation based on these models then allows calculation of possible phenotypic states of the model organism [3]. Genome-scale models can also be used to predict the function of previously uncharacterized genes and rectify incorrectly annotated genes. Gene deletions and gene over or underexpression strategies are applied to genome-scale models to predict genes and pathways that may be altered for bioengineering the production of therapeutically or industrially important compounds [4]. These models can also be used to predict genes and enzymes that are essential for the survival of an organism. These predicted essential genes and enzymes may be potential drug targets and therefore important in drug discovery and development [5].

Genome sequence and gene annotation data are used to identify specific roles of individual proteins within the system. A metabolic network (i.e., a network of metabolites interconnected via reactions involving the said metabolites) is developed utilizing published data on elucidated protein function and cellular location, enzyme thermodynamics, and reaction stoichiometry. Data from closely related organisms, for example, orthologous gene data, are sometimes used in the absence of reported information on the organism of interest [6]. Reactions and corresponding metabolites are tabulated into a matrix that accounts for the number of metabolites consumed and produced within the given reactions. Additional constraints on the fluxes through the reactions (often expressed in metabolite amount per dry weight of the parasite per hour with upper and lower boundaries) are incorporated to control the flux values and represent the reversibility or irreversibility of reactions [7].

Constraint-Based Modeling

Constraint-based modeling is an important *in silico* approach as it takes into account the different biochemical processes (i.e., reactions) and the flow of metabolites (i.e., species) in order to closely represent the metabolic network of an organism without the necessity for individual enzyme kinetics. It models the possible steady states of the metabolic network (metabolite concentrations do not change with time), thus enzyme kinetic parameters (e.g., Michaelis–Menten) that would need to be derived from recombinant expression and biochemical assays for all enzymes are not required. This is an important advantage for genome-scale modeling since these are seldom known for every enzyme encoded in a genome. Moreover, these enzyme parameters are strongly dependent on environmental conditions (pH, for instance). Even with the steady-state assumption, too many fluxes will need to be computationally predicted. In order to focus on more relevant flux distributions, specific constraints, often based on experimental data, are entered into the system to represent limits of enzymatic fluxes as well

as available metabolites. The steady-state assumption allows the use of linear programming (i.e., a mathematical technique that computes the optimal output of a model whose constraints are given by a set of linear equations) to solve for the maximum or minimum flux values [8]. Finally, the growth of the organism is predicted based on the production of essential components for increasing biomass [3]. This set of constraints in terms of metabolite consumption and biomass component production is the reason why this approach is called *constraint-based modeling*.

The genome-scale metabolic network is assigned an objective, most often the production of metabolites such as proteins, lipids, nucleic acids, and carbohydrates representing the biomass components that are essential for the survival of the organisms. ATP production can also be assigned as the objective function of the network [9]. The production of these components is then correlated with the growth of the organism in the given system. The flux values for all the reactions in the network are calculated as those values that maximize the objective function. The utilization of biomass components to satisfy the objective function is represented by the drain of these metabolites out of the system. Since in flux balance analysis (FBA), biochemical reactions are assumed to occur rapidly, steady state is achieved instantaneously and hence is assumed in all reactions. Linear programming is used to incorporate the constraints in the model to identify flux values that will result in the maximized production of biomass components at steady state. This process defines FBA. Flux variability analysis (FVA), on the other hand, aims to identify the minimum and maximum flux values on reactions that correspond to similar optimal values for the objective function. This identifies alternate pathways that contribute to achieving the objective function. It also gives an idea of the flexibility of the organism metabolism. For instance, this may help in identifying essential pathways where only small changes in fluxes through reactions are allowed. One of the major drawbacks of this technique is that since it does not take into consideration the reaction kinetics and the initial concentration of each metabolite, it will be unable to predict the concentration of these metabolites over time as in kinetic modeling [3]. There has, however, been a release of algorithms such as dynamic FBA, which tries to address the issue of varying concentration of medium components [10, 11].

Constraint-based modeling has been used to predict the cellular response of an organism in different conditions. This allows a more in-depth comprehension of the complex metabolic networks in organisms [12]. As a result, functional annotations for hypothetical proteins and correction of erroneous annotations are possible [7]. By restricting the amount of specific metabolites, changes in the production of biomass components are noted to predict the growth rate of the organism [9]. Altering the entry of metabolites into the system can also simulate the presence or absence of specific components in the media, and thus, components that are essential for the growth of the organism can be identified. Gene knockout can also be simulated in the FBA model in order to identify essential genes. A gene knockout is represented in the model by forcing a zero

flux through specific enzymatic reaction/s associated with the gene. Single gene knockouts have been used to pinpoint possible drug targets against pathogenic organisms [7].

Double gene knockouts have also been simulated and outcomes predicted using FBA. There are two types of lethal double-gene knockouts that have been defined in the recent literature [7]. Trivial knockouts are lethal gene pairs where one of the two genes is lethal when deleted individually. On the other hand, nontrivial or synthetic lethal genes are gene pairs that are lethal when inhibited together, but are nonessential when taken singly [7, 13]. Cell-scale networks have been utilized in order to identify drug targets in cancer cells [14]. This technique can also be utilized in the development of bacterial strains used for the production of metabolites of nutritional or pharmaceutical interests. Gene knockouts that will redirect the consumption of precursor metabolites to allow the overproduction of metabolites of interest can be identified using FBA [13].

Existing Metabolic Models

Leishmania major

Chavali *et al.* [7] developed a reconstruction of the *Leishmania major* metabolic network utilizing published literature and gene/enzyme databases. The network takes into account a total of 560 genes, 1112 reactions, and 1101 metabolites. Stoichiometric equations of metabolic reactions were atom- and charge-balanced, and thermodynamic properties of these equations were also considered. Biomass production was assigned as the overall objective of the metabolic network. Biomass components include amino acids, fatty acids, and DNA. The estimated amount of amino acid per gram of dry weight was computed based on the open reading frames in the genome of the organism, while the DNA component was computed by taking into account the G-C content of *L. major* DNA. Fatty acid components were based on previously published literature. For the computation of fluxes, subcellular locations of the different reactions were also considered. Linear programming was used to compute the flux distribution for the entire network at maximum biomass production.

To identify essential genes, single- and double-gene deletions were simulated by forcing zero flux through reaction/s associated with particular gene/s. The effect of the deletion on the growth of the organism was then categorized as lethal (0% growth), growth-reducing (between 0% and 90% growth), and no effect (>90% growth). Lethal double-gene deletions were further classified as either trivial or nontrivial. A total of 69 lethal single-gene deletions were identified, while 19 285 and 56 trivial and nontrivial double-gene deletions were identified, respectively [7]. Furthermore, using the sequences of the enzymes involved in the predicted set of essential reactions, inhibitors (e.g., antipsychotics and antibiotics) were identified from existing drug databases and were tested experimentally for target validation [15].

Plasmodium falciparum

A metabolic network reconstruction of *Plasmodium falciparum* involving 1001 reactions and 616 metabolites in four distinct intracellular compartments was developed by Plata *et al.* [16]. The biomass objective function was based on that used in the iND750 yeast metabolic model [17] with the individual compositions modified for *Plasmodium.* FBA was done and the production of biomass components was used as the objective function of the network. A total of 55 lethal single-gene deletions and 16 nontrivial double-gene deletions were identified using this model. Published materials on metabolic gene knockouts in *P. falciparum* and *Plasmodium berghei* were used to validate the results obtained from this model. All 14 published essential and nonessential metabolic gene knockouts were consistent with knockouts identified by FBA. On the other hand, 17 previously published drug inhibition studies coincided with those obtained from the FBA of the metabolic network.

Gene expression data from published microarray studies were also incorporated into the FBA model of Plata *et al.* Gene expression data at different stages (i.e., ring, late trophozoite, and schizont) were used to calculate proportional constraints in relation to the level of gene expression. In order to validate the results, trends in metabolite production, in terms of changes in metabolite concentration (i.e., increase or decrease, as opposed to actual concentrations) from ring to trophozoite and from trophozoite to schizont stages, were compared against experimentally obtained shifts in metabolite concentrations in the media. Calculated shifts in flux values of exchange reactions (i.e., reactions involved in transporting metabolites into or out of the given network) were interpreted as change in metabolite concentration. Thus, a negative or positive shift in flux represents a decrease or increase in concentration, respectively. This comparison revealed consistent results in 46 out of 66 shifts in concentrations of 33 identified metabolites. Moreover, the magnitudes of change in concentration observed in the FBA model correlated significantly with the experimental values [16].

An extensive *in silico* model of *P. falciparum* by Huthmacher *et al.* [18] was developed through the compilation of metabolic networks from a number of databases available online. The authors also integrated the parasite metabolic networks with that of the erythrocyte model. Data were obtained from published literature and online resources to take into account the exchange of metabolites between the host and the intraerythrocytic parasite. In this merged metabolic network, limits in the uptake of metabolites were based on the existing data on the proportions of these metabolites in the plasma, while the parasite is allowed to consume glucose and hemoglobin from the red blood cells.

Stage-specific gene expression data from five published data sets on *P. falciparum* and *Plasmodium yoelii* were used to compute binary variables to represent gene expression, where 1 is assigned to a gene that is expressed, while a value of 0 is assigned to a gene that is not expressed. A reaction related to a gene that is not expressed is forced to have a null flux while a reaction that is expressed is allowed

a nonzero flux. In addition, constraints on biomass components important in specific stages were taken into consideration. Similar to the previously presented metabolic networks, Huthmacher *et al.* simulated gene knockouts by fixing zero flux into individual reactions while maintaining an objective of producing metabolites that are essential to the survival of the parasite at a specific stage. It is important to note that essential metabolites differ between parasite stages since each stage has its own survival objectives (e.g., replication, invasion, etc.). Through this technique, 307 essential reactions were identified, 35 of which were reported in published literature to be essential to parasite growth or survival (out of a list of 57 essential reactions reported in literature). By blocking reactions associated with the same gene product, they were able to identify two more reactions in the list of essential reactions reported in the literature. Finally, in order to identify potential drug targets, sequences of essential parasite proteins were used to screen for human orthologs and those found were excluded, thus resulting in a total of 30 potential drug targets. Additionally, identified drug targets that were not in the list of 57 previously reported essential reactions were scored based on (i) nonhomology of the corresponding gene to human genes, (ii) if the function of the parasite enzyme is targeted in any organism (using the SuperTarget database) [19], and (iii) essentiality of the reaction in all stages of the parasite. The top 30 targets were then presented as essential reactions [18].

Forth [20] developed a malaria metabolic network that focused on 247 reactions representing important high-flux reactions. The reconstruction was built manually and utilized a Cytoscape session for easy visualization and inspection of the network. Metabolite concentrations of 10 carbon compounds that were obtained through proton nuclear magnetic resonance assay of media samples from *in vitro P. falciparum* 3D7 culture were used as a basis for the FBA constraints. The network objectives were also based on laboratory-measured biomass components (i.e., DNA, RNA, and protein) extracted from *P. falciparu*m culture. The model predicted 117 essential reactions associated with 79 lethal and 19 growth-reducing gene deletions. Only 22 of these identified essential genes were also predicted in the Plata *et al.* model; however, among the genes predicted to be nonessential, eight were predicted to be essential by the Plata *et al.* model.

Trypanosoma brucei

The TrypanoCyc Pathway/Genome Database (PGDB) [21] is a community-annotated metabolic database on *Trypanosoma brucei* metabolism. The initial reconstruction was developed in Pathway Tools using the genome of the *T. brucei* 927 strain sequenced at the Wellcome Trust Sanger Institute. The draft has since then been curated by a team of 37 experts, each specialized in his/her area of trypanosome metabolism. The TrypanoCyc PGDB currently has 9 compartments, 227 pathways, 1008 enzymatic reactions, and 835 metabolites. The unique feature of the TrypanoCyc database is the presence of reaction annotations. These annotations were submitted though the semiautomated

TrypAnnot pipeline and includes the localization of enzyme(s) catalyzing the reaction and the life-cycle stage(s) where it is known to be active. Annotators are provided with additional space to submit comments and publications to support their statement. TrypanoCyc also employs a modified version of the confidence scoring system proposed by Thiele and Palsson [22] to represent confidence of each reaction based on the annotations. The TrypanoCyc PGDB can be converted to the more popular SBML (systems biology markup language) format using the Pathway Tools suite. The TrypanoCyc community is also growing and is expected to continue collecting annotations on the *Trypanosoma* metabolism, continuously improving the quality and accuracy of the TrypanoCyc model. Work on the model generated from this database is currently ongoing.

Trypanosoma cruzi

The iSR215 is a metabolic network reconstruction of *Trypanosoma cruzi* strain CL Brener core metabolism developed by Roberts *et al.* [23]. In this study, two models were created for *T. cruzi.* The full model was based on direct genetic and biochemical data involving *T. cruzi* as well as data on other related species obtained from published literature. It takes into account 215 genes and 162 reactions in four subcellular compartments. Another model simulated the metabolic network of the epimastigote form of the parasite. Proteomic data from epimastigote cultures was obtained to identify specific proteins that are present in this stage of the parasite. Proteins absent in the epimastigote are removed from the full model by forcing a null flux into the involved reactions. Redirections of metabolic fluxes through certain pathways in the epimastigote model were observed as a result of the absence of trypomastigote and amastigote stage-specific reactions. The model was validated by comparing the predicted metabolic by-products under aerobic and anaerobic conditions with the data presented in the published literature. By-products observed in the model were found to be mostly consistent with the data reported in the literature.

Single reaction knockouts were predicted for each reaction in the full and the epimastigote models. A greater number of lethal reaction knockouts (40 reactions) were observed in the epimastigote model compared with that of the full model (26 reactions). This has been associated with the limitations imposed by the redirection of pathways in the absence of a number of reactions in the epimastigote model. Double reaction knockouts were also simulated in both models. Similarly, the epimastigote model yielded greater number of trivial and nontrivial deletions, 2880 and 183, respectively, as compared with the full model (1872 and 96, respectively). The predicted essential reactions were then compared with published experimental data on the species related to *T. cruzi* to further validate the results of the metabolic model. In total, 46 out of the 58 published gene targets available were consistent with the results of the metabolic network. All nonlethal reactions in the published literature were consistent with the results of the network [23].

Genome-Scale Reconstruction

Automated Genome-Scale Metabolic Network Reconstruction

A genome-scale metabolic reconstruction is a representation of a cell as a network of all the metabolic reactions that have been identified to occur within the given cell. As it is time-consuming to manually add every single reaction one after the other, many automated tools [24–26] and algorithms have been developed to help generate a draft, which can then be manually curated to better describe the cellular network.

Automated genome-scale draft reconstruction tools use an annotated genome of the organism of interest to mine biochemical databases (or reactions pools) in order to identify a set of reactions associated with the enzymes encoded in the genome. Some of these tools also predict the cellular localization of the enzymes in order to develop multicompartment models. This subset of chemical reactions along with their gene–protein reaction relationship forms the draft of the metabolic reconstruction [8]. Some automated genome-scale metabolic reconstruction tools such as the SEED server is even capable of annotating the genome of interest and have proved to be quite efficient with prokaryotic reconstructions [24]. All automated draft reconstruction tools have their own reaction pools from which reactions are selected for the draft. PathoLogic, the automated draft reconstruction tool employed in Pathway Tools, for instance, uses the MetaCyc database as its pool [25]. The AUTOGRAPH pipeline is well known for implementing the use of a user-defined manually curated metabolic model as the reaction pool for generating the initial draft [26]. More details on these tools are discussed further along in this chapter.

Most draft reconstruction tools and servers use nonorganism specific reaction pools to enable their application on diverse species. These drafts are hence prone to false-positive and false-negative hits that result in the draft containing reactions known to be absent in the organism of interest and not containing reactions that are known to be present in the organism of interest, respectively. Organism-specific reactions, which are absent in the reaction pool, are also missed in these automated reconstructions. Many automated genome-scale reconstructions also fail to identify and incorporate transport reactions into the model that are necessary for successful simulation of these models. Manual curation is hence necessary to fix these issues and create a more realistic representation of the genome-scale metabolism of interest.

Manual Refinement of Genome-Scale Model

The manual curation step in genome-scale reconstruction involves modifying the model draft so that it better represents the organism in real life. It involves adding reactions missing in the draft and removing reactions known not to exist

in the organism of interest. The manual curation stage involves re-evaluation and refinement based on literature and experimental observations. The manual curation stage is in essence a never-ending process. It is usually coupled with validation of the model with experimental data in order to identify how well the model is able to represent the actual metabolism. Another important part of manual curation is the addition of metadata to the model such as InChI and SMILES identifiers to the metabolites and Enzyme Commission (EC) number, and pathway and enzyme localization information to the reactions. With the recent increase in genome-scale metabolic reconstructions, many detailed protocols for manual curation have been published [22].

A simplified representation of the steps involved in genome- scale metabolic reconstruction, model validation, and model prediction is shown diagrammatically (Figure 21.1).

Standardization and Model Formats

In 2005, Le Novère *et al.* reported that "most of the published quantitative models in biology are lost for the community because they are either not made available

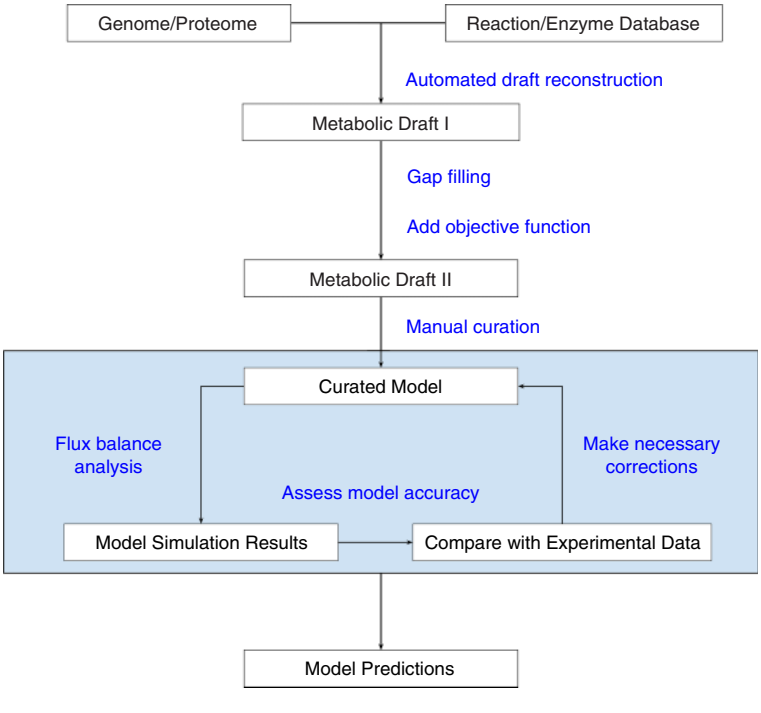


Figure 21.1 Steps in genome-scale metabolic model reconstruction and prediction.

or they are insufficiently characterized to allow them to be reused" [27]. With the increasing importance of metabolic models in research, standard formats were established to improve exchange and reusability. Currently, metabolic models are accepted in the scientific community in the form of standardized machine readable formats such as SBML [28], CellML [29], and BioPax [30]. Additionally, specific standards were developed to encode systems biology graphics notations (SBGNs [31]) and simulation descriptions (SedML [32]).

One of the major issues that affect the understanding and reusability of existing metabolic models is the lack of a single identifier system for metabolites and reactions. This leads modelers to generate their own identifiers or borrow identifiers from popular biochemical databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) [32], Biochemical Genetic and Genomic (BiGG) [33], and BioCyc [34]. Since genome-scale reconstructions tend to have more than a thousand reactions and metabolites, the use of different systems makes comparison of models or mapping experimental data difficult and time-consuming. As a result, the scientific community stresses on the inclusion of metadata along with the model elements in the form of annotations. Some modelers also provide additional information on model components in the form of InChI, SMILES, EC numbers, and so on. The Minimum Information Required In the Annotation of Models (MIRIAM) guidelines published in 2005 [27] describe an efficient solution to this problem. According to the MIRIAM guidelines, the model should clearly provide a description of all model elements, relate to a publication, and list its authors and contact information along with the simulation conditions. In the case of model elements' annotations, the MIRIAM guidelines advice authors to link those to external databases using an annotation triplet: "data type," "identifier," and "qualifier." Here, the "data type" refers to the general part of the link to a database resource and the "identifier" refers to the specific ID in the particular database. The *qualifier* is a term (selected from a predefined namespace) used to represent the relationship to the resource. According to the Biomodels database [35], there are two types of qualifiers: (i) model qualifier that represents the relationship between a modeling object and its annotation and (ii) biological qualifier that represents the relationship between a biological object represented by a model element and its annotation [36]. The implementation of the MIRIAM guidelines can thus improve the reusability of metabolic models. However, since different modelers can use references to different database resources, comparison of different models implementing different identifier systems is still not straightforward. The InChI system [37] developed by International Union of Pure and Applied Chemistry (IUPAC) provides a unique identifier for a chemical entity (i.e., metabolite), and hence, this can be used to determine identical metabolites and reactions between two different models. With the implementation of these standardizations, metabolic models developed can be easily read, understood, integrated with experimental data, and even combined to generate larger metabolic models.

Tools and Databases

Automated Draft Reconstruction Tools and Servers

Pathway Tools is a genome-scale reconstruction software suite developed by SRI International and was first introduced to the scientific community in 1996 [38]. Pathway Tools is capable of generating automated genome-scale reconstruction based on an annotated genome using PathoLogic [25]. PathoLogic not only recognizes reactions associated with a given enzyme, but also intuitively identifies potential pathways to which the reaction belongs, with the assumption that the predicted pathway plausibly exists in the organism of interest. The model generated by Pathway Tools is stored in a database called *PGDB*. A Pathway/Genome Editor helps users in curating the PGDB generated by PathoLogic. Besides reactions, metabolites, and pathways, users can add/modify experimental evidence, publications, summary/description, information on curators and affiliations, and so on, using the PGDB editor. Another important component of Pathway Tools is the Pathway/Genome navigator. This component lets the user browse through the PGDB, query, and visualize the different components of the model. The navigator also allows the user to enable an independent web server mode, which generates browsable web pages of the PGDB. The TrypanoCyc and LeishCyc [21, 39] are examples of web resources generated by the Pathway/Genome navigator. With the introduction of MetaFlux [9], Pathway Tools is also now capable of developing flux balance models.

The SEED server [24] is another important resource in genome-scale reconstruction. The ModelSEED component of this server implements an efficient pipeline, which can be used to generate good-quality metabolic model drafts. The genome of interest is first annotated using the RAST server [40] to generate a preliminary model draft. The pipeline then generates a biomass equation for the draft and performs an autocompletion step so that the model is capable of producing the biomass components. This model can then be "fitted" to experimental data through FBA. The ModelSEED pipeline also contains a modified version of the GrowMatch algorithm, which helps in model optimization by identifying missing transport reactions, conflicts between GPRs and gene essentiality data, gaps in the metabolic network, and extra reactions in the model [41]. ModelSEED is also a repository of genome-scale models with about 236 public models and 247 pathways, as of February 2015.

Most automated draft reconstruction tools are based on organism-nonspecific databases to generate the primary metabolic draft. In 2006, Notebaart *et al.* published the AUTOGRAPH pipeline in which existing, manually curated genome-scale model of a taxonomically related organism was used to generate metabolic draft [26]. In this pipeline, orthologous genes between the template (for which there exists a manually curated model) and the target genomes were identified using bioinformatics tools such as INPARANOID [42].

The list of orthologs was then used to select components of the manually curated model to build the target organism metabolic draft. Selecting the right template organism and model is an important step in this pipeline and determines the quality of the resulting metabolic draft. This method has been proved to generate more accurate metabolic drafts.

Databases

Manually curated metabolic models take a considerable amount of effort and time to build. They are rich in information such as gene–protein reaction relationships and metabolic capabilities of the organism. BioModels is one of the most popular model databases for both metabolic and nonmetabolic models [35]. At the time of writing, it has 562 curated models and 696 noncurated models published in peer-reviewed literature. BioModels also hosts automatically generated 112 898 metabolic, 27 531 nonmetabolic, and 2641 genome-scale metabolic models [43]. An important feature of BioModels is that it allows users to query not just the model components but even the annotation and metadata provided in the model, making it a very important resource. The BioCyc database collection (www.biocyc.org) is a huge set of PGDBs generated by Pathway Tools. As of 7 November 2014, BioCyc contains 5500 databases in three categories: Tier 1, Tier 2, and Tier 3. Tier 1 databases are literature-based manually curated databases while Tier 2 and Tier 3 databases were computationally predicted using the PathwayTools software and lack intense manual curation [34]. BioCyc is hence a very rich biochemical resource providing easy-to-browse metabolic models. The BiGG database [33] is another database of 10 extensively curated published genome-scale metabolic models. These models provide a good source of biochemical information for specific organisms and also provide cross-links to other databases. Another example of a metabolic model repository is the JWS online [44], the browser-based simulation tool, which maintains kinetic models.

Biochemical information in databases provides an excellent resource on the metabolic capabilities of an organism (Table 21.1). One of the most popular databases among them is the KEGG [32]. The KEGG database collects information on genes, enzymes, metabolites, biochemical reactions, and biological pathways among many other types of information. KEGG also has a popular web service, allowing other tools and databases to quickly mine them for information. As of April 2014, the KEGG web service was reported to have 400,000–500,000 unique users per month. KEGG pathway maps are also frequently used by many to map omics datasets. These maps are generated by merging all known biological pathways and are well maintained. MetaCyc [34], one of the most important PGDBs in the BioCyc collection, is another important biochemical database. It is a collection of all pathways, reactions, and metabolites generated from all the BioCyc PGDBs that provides a good biochemical reaction pool during gap filling. PathoLogic, the draft reconstruction tool in Pathway Tools, completely relies on MetaCyc to generate the draft PGDBs. Enzyme databases such as BRaunschweig ENzyme DAtabase (BRENDA) [45], ExplorEnz [46], and

Table 21.1 Summary of databases useful in model reconstruction.

Database	Biochemical data	Pathways	Proteins	Gene	Experimental data	Metabolic models	Metabolite	Organism specific	References
BiGG						✓		✓	[33]
BioModels						✓		✓	[35]
BRENDA	✓				✓		✓	✓	[45]
ExPASy	✓								[47]
ExploreEnz	✓								[46]
ChEBI	✓						✓		[50]
ChemSpider							✓		[51]
EuPathDB	✓	✓	✓	✓	✓			✓	[53]
GeneDB			✓	✓				✓	[51]
KEGG	✓	✓	✓	✓			✓	✓	[32]
LAMP[a)]	✓	✓	✓	✓			✓	✓	[61]
MaGnET[a)]			✓	✓	✓			✓	[56]
MetaCyc	✓	✓	✓	✓			✓		[34]
MetaTIGER		✓	✓					✓	[59]
MPMP[a)]		✓	✓	✓	✓			✓	[57, 58]
TrypanoCyc[b)]	✓	✓	✓	✓			✓	✓	[21]
UniProt			✓	✓				✓	[48]

a) *Plasmodium* only.
b) *Trypanosoma* only.
This table summarizes information that can be retrieved from some of the commonly utilized databases that are useful in genome-scale reconstruction of *Plasmodium falciparum* and *Trypanosoma brucei*.

(Expert Protein Analysis System) ExPASy [47] enzyme databases are quite useful resources for thermodynamic information of reactions in systems biology. In addition, BRENDA allows users to submit biochemical reactions identified in various species, providing other users with substrates specific to the reaction in a particular species. Gene and protein databases such as NCBI and UniProt [48] (formerly, EMBL and SwissProt) carry many useful annotations on gene/protein functions. GeneDB [49] is another such database that stands out for providing up-to-date annotated sequence information on protozoan parasites. Chemical Databases such as ChEBI [50] and ChemSpider [51] provide a good resource on the metabolites for model reconstructions. These databases report chemical formula, InChIs, alternative names, and chemical properties for chemical entities.

Transport reactions are very important in generating functional metabolic models. Transport Databases such as TransportDB [52] provide a good resource on these reactions. TransportDB also allows users to BLAST search protein sequences to identify transporters.

The EuPathDB [53] collection of databases provides sequence information along with related protein information and experimental data on Eukaryotic pathogens. Users are also allowed to submit comments on genes/proteins, improving the value of these data. EuPathDB maintains well-curated databases for Trypanosomatidae and *Plasmodium* through TritrypDB [54] and PlasmoDB [55], respectively. PlasmoDB and TritrypDB contain genomic data (sequence and/or annotation) of 8 *Plasmodium* species (i.e., *P. falciparum*, *Plasmodium vivax*, *P. yoelii*, *P. berghei*, *Plasmodium chabaudi*, *Plasmodium knowlesi*, *Plasmodium reichenowi*, and *Plasmodium gallinaceum*) and 14 Trypanosomatidae species (*Crithidia fasciculata*, *Leishmania braziliensis*, *Leishmania donovani*, *Leishmania infantum*, *L. major*, *Leishmania mexicana*, *Leishmania tarentolae*, *T. brucei*, *Trypanosoma congolense*, *T. cruzi*, *Trypanosoma evansi*, *Trypanosoma grayi*, *Trypanosoma rangeli*, and *Trypanosoma vivax*), respectively. Apart from genomic data, these databases also include functional data on RNA and protein expression, putative functions, protein interactions, localization and features, as well as gene polymorphisms, orthology, and homology. Users Queries can be done by performing a keyword search using gene names or gene IDs. Initial search results can be combined with succeeding search parameters using Boolean operations (i.e., "and," "or," "not") and are presented in a workflow showing the total number of hits at each step. Users are required to register in order to store search strategies, save and download data, and add comments on gene sequences [53].

The Malaria Genome Exploration Tool or MaGnET utilizes a MySQL database storage and Java for the user interface. Queries can be done to display the location of a given open reading frame and to visualize protein–protein interaction and 3D motifs. Gene ontology annotation is also included in the results page when available [56].

The Malaria Parasite Metabolic Pathway (MPMP) is a web-based resource that aims to collate and synthesize biological, physiological, and biochemical data on *P. falciparum* genes to provide a more profound understanding of these genes [57].

MPMP focuses on metabolic pathways that are involved in the erythrocytic stage of the parasite. It shows pertinent metabolic pathways from KEGG, with some pathways developed as a result of the consolidation of different metabolic maps. A pathway is considered to exist if at least three to four enzymes in sequence for the pathway are encoded in the *P. falciparum* genome; however, if there is biochemical data that supports the functionality of a given pathway, then it is also considered relevant. In addition to the pathways, "Transcription Clocks" are included beside each enzyme providing transcription data corresponding to the specific enzyme. EC numbers in the pathways have external links to other resources for further information, including description, cellular localization, and references [57, 58].

MetaTIGER is a tool that provides phylogenetic information on the metabolic profiles of over 500 organisms. Apart from the use of existing genomic sequence data, expressed tag sequences, PSI-BLAST searches through SHARKhunt, and hidden Markov models were utilized to further enrich the MetaTIGER database. Comparison of different organisms can be done using this tool and results can be presented as phylogenetic trees, KEGG metabolic maps, or table formats along with their corresponding E-values [59]. Information from this tool can be used to fill gaps in pathways in a metabolic network by comparing the pathway with that of closely related organisms in conjunction with the likelihood of the involved enzyme/s existing in the organism in question.

With more than 250 public databases, MetExplore [60] is a web server developed to study experimental data in the context of metabolic networks. MetExplore supports mapping of metabolites using standard identifiers such as InChI and molecular mass. Users registered on MetExplore can import their own metabolic networks into the server, visualize, curate, and share them. Analysis of metabolic pathways using experimental data through MetExplore, which does not rely on predrawn pathways, contributes to better understanding the changes in the metabolism of an organism of interest.

Metabolic Model Simulation

Flux Balance Analysis

The initial step to FBA is the creation of a mathematical representation of the metabolic reactions in a given metabolic network. Reactions and corresponding metabolites involved in specific reactions are tabulated into a matrix (i.e., Stoichiometric matrix or S-matrix) that accounts for the number of metabolites consumed and produced within the given reactions. Columns of the matrix represent the reactions while rows represent the metabolites. The number of metabolites produced or consumed in a given reaction is represented in the matrix as a positive or negative number, respectively. The stoichiometry of each reaction provides a constraint onto the resulting network. In addition, each reaction is assigned a flux boundary (i.e., upper and lower bounds), which represents the permissible

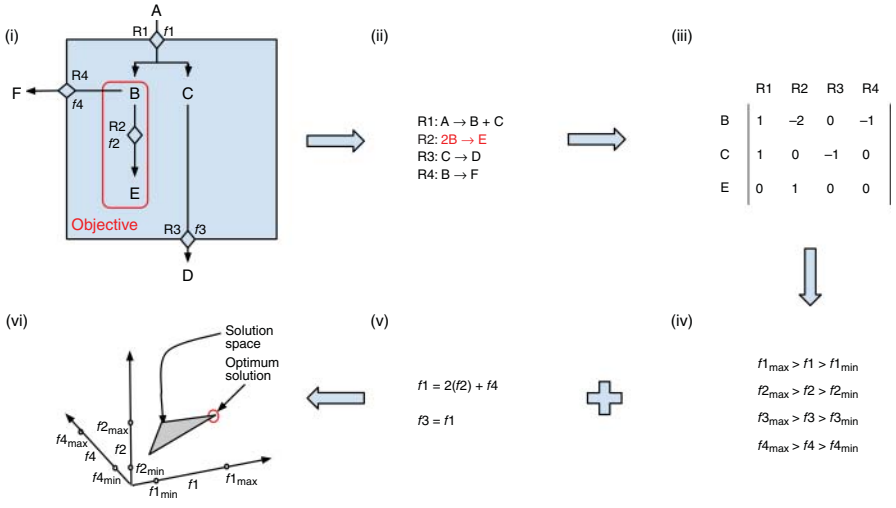


Figure 21.2 Constraint-based modeling.

fluxes for the said reaction. These constraints therefore define the allowable rates at which metabolites are produced or consumed within the system [3].

Following the creation of the S-matrix and the assignment of flux boundaries for the reactions, an objective function is assigned. A reaction representing the said function (e.g., production of biomass components from precursors) is included into the matrix. Biomass reactions, for example, are based either on experimentally obtained data [3, 20] or on the data obtained from closely related organisms [6, 16, 18]. Simulation tools that utilize linear programming software are then used to calculate the permissible solution space and can identify the optimal solution for the given objective reaction through FBA. FVA [62] can be performed to identify alternative solutions that will still satisfy a given condition. An example of such condition is the production of at least 90% of the biomass. The fluxes of individual reactions (within the assigned limits) are maximized and minimized in order to find alternative solutions [3] (Figure 21.2).

This figure describes the summary of FBA process. Here, we have considered a simple metabolic model (i) with six metabolites (A, B, C, D, E, and F) and four reactions (R1, R2, R3, and R4) (ii), summarized in the stoichiometric matrix (S-matrix) (iii). Flux values ($f1, f2, f3, f4$) for the reactions have maximum and minimum constraints, $[f1_{max}, f1_{min}]$, $[f2_{max}, f2_{min}]$, $[f3_{max}, f3_{min}]$, and $[f4_{max}, f4_{min}]$, respectively (iv). As for the objective function, we have considered the maximization of the production of metabolite E. Using this S-matrix, linear equations of the fluxes are determined (v). Linear equations and constraints are used to determine the solution space. The optimum solution (vi), in this case, is the solution with maximum permissible flux through the objective function $f2$.

Simulation Tools

The constraint-based reconstruction and analysis (COBRA) Toolbox is a collection of methods for the development, analysis, and simulation of metabolic networks. These methods are based on an approach that utilizes physicochemical, biological, and data-driven constraints to identify phenotypic states of a given metabolic network under a given condition [63]. Version 1.0 of the toolbox was initially released in 2007 and was then updated to version 2.0 in 2011. Apart from the COBRA archive, installation of this toolbox requires MATLAB [64], libSBML and the SBML Toolbox (www.sbml.org), and linear programming software (e.g., GLPK, Gurobi, CPLEX). Currently, it offers a wide range of functionalities as shown in Table 21.2 [65]. COBRAPy is a version of the COBRA Toolbox that runs using Python instead of MATLAB, which is a proprietary software. Furthermore, COBRAPy is based on Python programming language, increasing its flexibility [66]. In terms of user interface, the COBRA Toolbox requires the users to provide a flat file, which contains all the species (i.e., molecules), reactions, and the reaction equations via MATLAB or Python interface. The MATLAB COBRA Toolbox accepts models in MATLAB, SBML, and Microsoft excel formats, and its Python implementation accepts SBML, JSON, and COBRA model object. Although the toolbox has functions to evaluate model correctness, error detection may not be as good compared with other applications. One example of such an issue is that if a reaction in the input file contains an undeclared metabolite, the function, instead of returning an error message, proceeds to add the reaction to the cobra model object without the undeclared metabolite leading to an incorrect and unbalanced reaction. However, the COBRA Toolbox provides a number of advanced features that are not present in most FBA applications/software as shown in Table 21.2. Finally, it is important to note that the COBRA Toolbox is

Table 21.2 Features of COBRA toolbox (version 2.0) [65].

Feature	Details
Flux balance analysis	Flux variability, gene deletion studies, geometric FBA, growth-rate optimization, loop law, MOMA, robustness analysis
Fluxomics	C13 data fitting and flux estimation, experimental design
Gap filling	DetectDeadEnds, gapFind, growthExpMatch
Input/output	Read/write SBML (level 2, version 4)
Metabolic engineering	GDLS, OptGene, OptKnock
Reconstruction	Create submodels using omics data, model curation tools
Sampling	Artificial centering hit and run (ACHR) sampling, updated ACHR sampling (parallel/multipoint)
Test suite	Examples are provided for testing, verify installations
Visualization	Display maps, overlay data (flux distributions, flux variability)

well maintained, and it is constantly being improved using methods and programs contributed by active community members [67].

SurreyFBA is a free stand-alone command line tool that is written in the C++ programming language. It uses the GLPK software for linear programming. Alongside the main application is the JyMet, which is a graphical user interface that is implemented in the Python programming language written in Java (Jython). JyMet has a menu-based interface in order to access the different simulation commands easily and displays the model in a spreadsheet format. The features of SurreyFBA include FBA, FVA, robustness analysis, phenotypical phase planes, elementary mode analysis, essentiality scans and prediction of reaction, and gene/enzyme knockout results, as well as model validation that includes the detection of live reactions, orphan metabolites, nonconserved metabolites, and connected components. After applying the optional matrix reduction algorithm, FVA on the iND750 model (*Saccharomyces cerevisiae*) using SurreyFBA was 41% faster compared to conducting the same analysis using the COBRA Toolbox [68].

OptFlux is a free software application that uses GLPK software for linear programming and LibSBML to read and handle metabolic models in SBML format, although other formats can also be loaded (e.g., Metatool or flat files). FBA simulations can be performed alongside adjustments in environmental conditions. The software also uses the BioVisualizer plug-in to visualize pathways and networks in the form of nodes and edges. OptFlux has a user-friendly graphical user interface with a menu bar that is easy to navigate. Similarly to the COBRA Toolbox, OptFlux can also perform FVA and Minimization of Metabolic Adjustment (MOMA) [69].

Acorn is a web-based application with a desktop-based graphical editor that has been designed to handle large genome-scale metabolic model. Users are required to install Acorn and set up an account in order to utilize the application. Models in SBML format can be imported into the application for analysis. FBA as well as FVA can be conducted with objective parameters and nutritional conditions set by the user. Essential genes and reactions can also be identified by the software. Pathways can be visualized using the desktop editor showing the corresponding flux values calculated for each reaction. Selected models and results of perturbations can also be shared using the application [70]. Table 21.3 summarizes the different FBA application software .

Applications of Flux Balance Analysis in Identifying Potential Drug Targets

Potential drug targets in pathogens should meet several important criteria. Primarily, the protein or enzyme must be necessary for the survival of the pathogenic organism. In relation to the metabolic network, the protein is often involved in pathways ultimately leading to the production of biomass components. Enzymes involved in carbon, lipid, nucleotide, or protein metabolism are potential targets [7]. Moreover, the druggability of a protein target, which is defined as the likelihood of altering the function of the target upon exposure

Table 21.3 Tools for metabolic model simulations.

Project name	Operating system	Programming language	License	Project home page	References
Acorn	Platform independent	Java, C++	GNU GPL v2	http://code.google.com/p/a-c-o-rn/	[70]
COBRA toolbox v2.0	Platform independent	MATLAB	GNU GPL version 3	http://opencobra.sourceforge.net/openCOBRA/Welcome.html	[65]
COBRApy v0.2.1	Platform independent, including Java	Python (≥2.6)/Jython (≥2.5)	GNU GPL version 3 or later	http://opencobra.sourceforge.net	[66]
OptFlux	Platform independent	Java	GNU GPL version 3	http://www.optflux.org	[69]
SurreyFBA	Platform independent	C++	GNU-GPL	http://sysbio3.fhms.surrey.ac.uk/	[68]
Sybil	Platform independent	R statistical environment	GPL-3	http://www.cs.hhu.de/en/research-groups/bioinformatics/software/sybil.html	[71]

This table summarizes some of the popular flux balance analysis tools available.

to a given compound, is assessed through a number of ways. An important characteristic of a druggable target is the presence of potential binding sites in the three-dimensional structure of the protein. These binding sites can further be assessed based on their shape, surface lipophilicity, and predicted affinity for drug compounds [72]. Similarities in protein structures with known drug targets, also referred to as *guilt by association*, have also been utilized in determining the druggability of a target [73]. Comparison with homologous targets in other pathogens has also been used to identify potential targets with similar binding sites and possible ligand interactions, particularly with antibiotics [74]. Lastly, high homology to the binding pockets of important human enzymes makes the protein in the pathogen an unlikely drug target, owing to an increased likelihood of side effects [13].

Enzymes that are essential for the survival of a pathogen can be identified using FBA by simulating individual gene or reaction knockouts and observing its effect on the objective function. In this case, it is important to accurately define the specific gene–protein reaction association [75]. It is crucial that targets identified are involved in primary metabolic pathways, rather than in secondary metabolism that is often nonessential to the growth of organism. Additionally, the objective function can be adjusted to represent the production of the minimum component requirements of an organism. This may result in an accurate prediction of the essentiality of certain reactions or genes [76]. Genes that encode isozymes may be considered nonessential since the organism is capable of producing another enzyme with a similar function. On the other hand, a gene that contributes to a protein complex that is essential to an organism is considered essential [13].

Potentially, double-gene knockouts can be targeted by a drug combination that can slow down, if not prevent, the development of drug resistance. In addition, the use of drug combinations may have synergistic effects. *In vitro* testing has demonstrated that some drug combinations exhibited greater inhibitory effect when compared to the sum of the effect of individual drugs when given separately. Yet little is known of the flux restrictions that produce the synergy [15].

Apart from focusing on enzymes in a metabolic network, a metabolite-centered approach can be applied toward identifying potential drug targets. Metabolic choke points, or reactions that solely produce or consume one or a few metabolites, are presumed to be essential if an inhibition at this point will result in the deprivation of an essential by-product or the accumulation of a possibly toxic metabolite [18]. Molecules similar to the metabolites involved in choke point reactions (i.e., single reaction connected to a number of important reactions or pathways) may be used as a competitive inhibitor for the given reaction and may be able to target more than one enzyme [77].

Drug repositioning or repurposing involves the use of a currently available drug in addressing a different disease pathology. Repurposing of drugs that have been approved for another purpose benefits from the fact that these drugs have already been tested on humans and thus toxicity and adverse effect information are already available [6, 14]. This shortens the time frame for drug development and

reduces the developmental costs [78]. Available resources, such as the DrugBank, provide an extensive database of drugs and their corresponding drug targets [79]. A number of studies have utilized the DrugBank to identify potential drugs that can target essential proteins to inhibit the growth of pathogenic organisms and cancer cells [6, 14, 80].

In a study by Chavali *et al.* [15], data on identified essential genes obtained from a previously developed *L. major* model were used to screen FDA-approved drugs that can potentially be used as antileishmanials. The protein sequences of *L. major* genes included in the metabolic network reconstruction were aligned against the sequences of drug targets in the DrugBank and STITCH databases. Inclusion and exclusion criteria such as FDA approval status, druggability, and drug toxicity were also incorporated in the drug screening. As a result, 15 genes representing potential drug targets for a total of 240 FDA-approved drugs were identified. Protein sequences of eight genes involved in nontrivial deletions were also identified as potential drug targets for a total of 37 FDA-approved drugs. Among the combined list of potential drugs targeting essential genes (single genes or gene pairs), 9 have been reported in literature to exhibit antileishmanial activity, while 71 have been shown to exhibit activity against *L. major* in previous high-throughput *in vitro* drug screenings. It is important to note that through this screening, halofantrine, an antimalarial drug, has been identified and tested *in vitro* to exhibit antileishmanial activity at concentrations of 3 μM and higher. Furthermore, drug combinations with disulfiram, a drug used to treat chronic alcoholism, and antibiotics/antipsychotics have also been demonstrated to show antileishmanial activity *in vitro.* These drug combinations have also produced significantly greater inhibition when compared with the sum of the inhibitory effects of the individual drugs.

Conclusion

Recently, the number of genome-scale metabolic reconstructions has increased greatly. With the rapid increase in the number of sequenced genomes, simplicity of tools, better predictive algorithms, and intuition of automated metabolic reconstruction tools, genome-scale metabolic draft reconstruction has been made much easier. Although manual curation is still time-consuming and requires the dedication of one or more individuals, the improvement in the quality of automated drafts makes the process faster. Model simulation tools are also constantly being improved with better, faster algorithms, and new features are added regularly. Discussion forums such as the COBRApy and sbml.org google groups provide a perfect opportunity for systems biologists and computer programmers to discuss issues and drawbacks of existing tools and in turn facilitate the development process [81–84].

Genome-scale reconstruction and simulations aim to combine all known information on a particular organism to further understand their biology and predict

their metabolic behavior under different environmental conditions. With the discovery of new information on their metabolism, metabolic models need to be updated in order to take into account these details. Recon 2 (*Homo sapiens*) [85] and iJO1366 (*Escherichia coli*) [86] are the best examples where existing models have been improved to provide better representation of known metabolism.

Finally, it is important to remember that the accuracy of the model highly depends on the accuracy of existing knowledge on the metabolic pathways in the organism. In addition, constraints in the model are mainly limited to available information on reaction stoichiometry and flux boundaries and may not take into consideration other enzyme reaction parameters. Thus, it is important to take this into consideration when interpreting and evaluating simulation results. The objective of performing simulations of genome-scale metabolic models is never to replace experimental work but to generate hypotheses and help direct experiments saving both time and resources in the process.

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22
Attacking Blood-Borne Parasites with Mathematics

*David D. van Niekerk, Gerald Penkler, François du Toit, Jacky L. Snoep, Barbara M. Bakker, and Jurgen R. Haanstra**

Abstract

Central carbon metabolism is important to cells as it supplies free energy in the form of ATP and the building blocks for new cells. Parasites harvest many of the components they require from their hosts, but they still have to generate ATP themselves, making the metabolic pathways that generate ATP essential to the parasites' survival and thereby potential target pathways for antiparasitic drugs. Metabolic networks often consist of many components that interact with each other via nonlinear kinetics. The behavior of the network arises from the interaction of the components within and outside the network. To understand network behavior, experimental measurements on the components should be integrated through computational approaches. In this chapter, we present an overview of how experiment-driven mathematical models have provided insights on important aspects of parasite metabolism and have aided in elucidating potent antiparasitic drug targets within metabolism.

The Importance of Flexible Metabolism for the Parasites *Trypanosoma brucei* and *Plasmodium falciparum*

Parasites can have multiple hosts, which result in different environments during their life cycle. Both *Trypanosoma brucei* and *Plasmodium falciparum* have an insect and a human host, and in these environments, they experience host-specific immune responses and different carbon source regimes. Within their insect vectors, parasites often move from the gut to the salivary glands, while in their mammalian hosts, many parasites move through different parts of the body and can have extracellular and/or intracellular stages. In this chapter, we mainly focus on two parasites: one lives extracellularly in its human host (*T. brucei*), while the other is intracellular (*P. falciparum*).

*Corresponding author.

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Trypanosomiasis and Metabolism through the Life-Cycle Stages of *T. brucei*

T. brucei causes African sleeping sickness in humans and a similar disease called *nagana* in cattle [1]. It is transmitted between mammals by flies of the *Glossina* genus, also known as tsetse flies. After the tsetse fly has taken a blood meal containing trypanosomes, they initially reside in the insect's midgut as procyclic-form (PCF) trypanosomes. Subsequently, they migrate to the salivary glands from where they can be injected to a new mammalian host. In mammals, *T. brucei* lives freely in the lymphatic fluid and the bloodstream. There it proliferates as the so-called bloodstream-form (BSF) trypanosomes. Infection is sustained through evasion of the immune system by changing the expression of variant surface glycoproteins [2]. Eventually, the parasites will cross the blood–brain barrier and migrate to the central nervous system. To survive in these different, often hostile, environments, they must be able to use the available carbon sources. For example, the availability of glucose strongly varies during the different life-cycle stages of the parasite. In the human serum, blood glucose levels are maintained at 5 mM, but in the tsetse gut, there is only glucose available immediately after a blood meal.

In BSF *T. brucei*, glucose is the dominant carbon source that is readily available from the blood. BSF trypanosomes rely entirely on glycolysis to generate ATP. Most of the glucose that is consumed by BSF *T. brucei* is excreted as pyruvate [3] and is not further metabolized. NMR and mass spectrometry studies have shown that some of the carbons from glucose also end up as alanine, acetate, and some other extraglycolytic metabolites [4, 5]. Fluxes to these alternative end products are small compared to the flux to pyruvate, but knockdowns of key enzymes in the pathways to these alternative end-products revealed that they are important for parasite survival.

In PCF *T. brucei*, glucose can still be used if present, but the capacity of glycolysis is downregulated in PCF cells compared to their BSF counterparts (Ref. [6] and references therein). Pyruvate is now not the end product of glucose metabolism, but the glucose is further metabolized to acetate and succinate [7]. In addition to glucose, PCF *T. brucei* can also metabolize the amino acids proline and threonine. Threonine is broken down to acetate, and proline is degraded in parts of the tricarboxylic acid (TCA) cycle in the mitochondrion, mainly to succinate or CO_2 [8].

Malaria and Metabolism in the Intraerythrocyte Stage of *Plasmodium* Species

Parasites of the *Plasmodium* genus cause malaria [9] and are transmitted to a host by the bite of an infected female *Anopheles* mosquito. When a *Plasmodium*-infected mosquito takes a blood meal, sporozoites are transmitted through its saliva. The sporozoites in the bloodstream travel to the liver and rapidly invade the hepatocytes. In the liver, the parasite undergoes replication (exoerythrocytic schizogeny), leading to the formation of a number of merozoites, which are

released into the bloodstream. Free merozoites invade erythrocytes where they grow and differentiate into rings, trophozoites, and finally schizonts, which release merozoites upon rupture of the erythrocytes. These merozoites infect new erythrocytes or undergo differentiation into gametocytes that can be ingested by mosquitoes and undergo sexual reproduction in the midgut.

Of the different phases, the in-host phase in the mammalian blood is clinically of most interest since it is in this phase that the typical malaria symptoms manifest. In this intraerythrocytic phase, the parasite has access to the host's red blood cell cytosol, containing the nutrient-rich hemoglobin, as well as to the host's blood serum. As the parasite does not have any carbohydrate reserves, it relies completely on glycolysis for ATP production [10]. Here, it employs the classic Embden–Meyerhof pathway and a glycerol-producing branch. The primary end product of its glucose metabolism is lactate, but also glycerol and pyruvate are excreted. In this stage, there is only minimal conversion of glucose-derived pyruvate to acetyl-CoA and subsequent TCA cycle intermediates [11], possibly due to the localization of the pyruvate dehydrogenase complex in the apicoplast. In addition, a small flux through the pentose phosphate pathway allows for the production of NADPH and nucleotides and assists in the detoxification of oxidative radicals resulting from the degradation of hemoglobin [12].

Metabolism as a Drug Target

Both parasitic diseases need novel treatment options [13–15]. To serve as a target pathway for drugs, a cellular pathway should be essential for the survival of the target cell. Metabolic processes provide the cell with the energy and the building blocks to proliferate, for maintenance, and, for example, in trypanosomes, also for cell motility. The central role of metabolism in the cell makes metabolic pathways prominent drug targets. An advantage of inhibiting metabolism is that it generally leads to rapid cell death. Since the same metabolic pathways may also be important in the cells of the host, classic studies focus on parasite-specific (variations of) pathways. We show in the later sections that quantitative differences between metabolic pathways can also convey selectivity to drug targets in essential pathways that are active in both parasite and host.

Several drugs against *T. brucei* and *Plasmodium* have metabolic targets; this includes both older and currently used drugs. One of the more recently registered drugs against *T. brucei* – eflornithine or DL-alpha-difluoromethylornithine – is an irreversible inhibitor of eukaryotic ornithine decarboxylase [16], an enzyme that is part of the polyamine synthesis pathway. Other antitrypanosomal drugs, such as suramin and melarsoprol, have been reported to affect glycolytic enzymes in *T. brucei* in conjunction with other cellular targets [17–20].

In the case of the antimalarial drugs, pyrimethamine, which, together with sulfadoxine, is part of a previous-generation combination treatment against malaria, inhibits the enzyme dihydrofolate reductase in the folate biosynthetic

pathway [21]. Currently, artemisinin-combination therapy is generally used against malaria. Although the mode of action of the group of artemisinins is still unknown, it was recently reported that one of them, artesunate, targets a membrane glutathione S-transferase involved in glutathione metabolism [22].

Because BSF *T. brucei* can only generate ATP in glycolysis, this pathway is considered a potent target pathway for novel drugs [23]. A compendium of results from several independent studies, using inhibitors or RNAi against glycolytic enzymes, revealed that 40–60% inhibition of the glycolytic flux is sufficient to kill BSF *T. brucei* [24]. For many enzymes of trypanosome glycolysis, inhibitors have been developed (as reviewed, e.g., in [23, 25]).

Similarly to *T. brucei*, blood-stage *Plasmodium* species rely primarily upon glucose metabolism for their energy needs [10]. When the erythrocyte is infected by the fast-growing malaria parasite, the metabolic activity of the erythrocyte is increased to more than 60 times of that of the uninfected erythrocyte [26, 27]. This increased glycolytic activity is thought to contribute to the clinical problems associated with malaria such as lactic acidosis and hypoglycemia. For these reasons, the glycolytic pathway is an attractive drug target against *Plasmodium* species [10, 28].

The finding that a metabolic pathway, such as glycolysis, is a good target pathway does not necessarily imply that every metabolic enzyme within that pathway is equally well suited as a target to kill the parasite. Not all enzymes may be essential for the output of the pathway (e.g., because they may be located in a side branch of minor importance or because redundant enzymes or pathways exist), and enzymes may be in such excess in the cell that sufficient inhibition may be difficult. Access to the target can also influence drug target potential, but in this chapter, we mainly focus on network-based drug target potential. As enzymes, similarly to other proteins, do not operate in isolation, but in the context of a network, the setup of this network will have an influence on the drug target potential of each individual enzyme. To understand enzymes in the context of their cellular network, computational approaches are becoming increasingly important.

Computer Models Can Aid Our Understanding of Metabolism

Metabolism comprises of a large and complex network of reactions that are strongly interconnected. Metabolic enzymes catalyze the interconversion of different intermediary metabolites, and as such, the enzymes communicate via their shared metabolites. This not only connects subsequent enzymatic steps but can also connect different parts of pathways since, for example, multiple metabolic enzymes use cofactors such as ADP/ATP or NAD^+/NADH. Through this, a change in the activity of one enzyme will affect the activity of potentially many more enzymes within and beyond the pathway it is part of. If the network of reactions contains alternative routes to the same metabolites, a change in the activity of the constituent enzymes can also influence the extent to which

alternative routes are used. The changes elsewhere will in turn also affect the enzyme that was changed in the first place, even at the short metabolic timescales. Hence, the global quantitative effects of changes in enzyme levels or metabolite levels are not necessarily easy to predict.

To understand metabolism both at a qualitative and at a quantitative level, computational approaches are increasingly employed in addition to experimental measurements.

Different Strategies to Model Metabolism – From "Top-Down" to "Bottom-Up"

There are different ways to model metabolic pathways. Each of these approaches has its own advantages and can help in answering different types of questions. In the next sections, we discuss the top-down and bottom-up approaches in more detail. In addition, there are hybrids of the two (middle-out; see, e.g., [29]), but this is beyond the scope of this chapter.

Top-Down: Genome-Scale Models

With the advance of high-throughput (next-generation) sequencing techniques, the genomes of many organisms have been sequenced, including those of many parasites. After the genomic sequence has been annotated, the genetic information can be used to create a genome-wide metabolic map showing all the metabolic reactions that are possible from the genome. The quality and usefulness of such a metabolic map strongly depend on the quality of the annotation and often need extensive manual curation. Metabolomics approaches can identify many intracellular metabolites, and this information can be used to fill in the gaps in the map. Metabolomics with isotope-labeled carbon sources are especially powerful: if metabolites at each side of a gap in the pathway have a label at similar positions on the molecule, this can be a strong hint that an enzyme catalyzing their conversion should also be there [5, 30, 31]. With this information, the genome sequence can be further scrutinized to find putative genes that could be coding for enzymes involved in the conversion.

When only genomic information is used in the construction of these models, they represent the most extended stoichiometric network possible from the organism's genome. The organism, however, not always employs this maximal capacity of the network as not all of the genes are necessarily expressed at any given moment. To restrict the genome-wide models to specific cell types or life-cycle stages, transcriptomic and proteomic datasets can be used [32, 33]. Genes that are not expressed in a cell type or life-cycle stage, or only at low levels, are then omitted from the network.

The advantage of the genome-scale metabolic models is that they reflect the entire metabolic capacity and with the advances of sequencing, transcriptomics, proteomics, and metabolomics, the datasets underlying them are becoming increasingly detailed.

A powerful method that uses these large-scale metabolic reconstructions for further understanding of the biochemical network is flux balance analysis (FBA)

(for reviews see, e.g., [34, 35]). In FBA, a stoichiometric matrix $\boldsymbol{S}$ of the reaction network is combined with a flux vector $\boldsymbol{v}$. The stoichiometric matrix $\boldsymbol{S}$ has columns for each reaction and rows for each metabolite and its values show how many molecules of each metabolite are consumed (negative values) or produced (positive values). The flux vector $\boldsymbol{v}$ lists the reaction rates. In a network at steady state the concentrations of metabolites remain constant and the reactions producing each metabolite need to be balanced by reactions consuming it. Mathematically, this implies that the product of the stoichiometric matrix and the flux vector equals zero:

$$\boldsymbol{S}\cdot\boldsymbol{v} = 0 \tag{22.1}$$

This equals a set of linear equations that describe the entire network, which has multiple solutions. The system can be further constrained by adding upper and lower bounds to the steady-state fluxes on the basis of, for example, experimentally determined consumption and production fluxes J:

$$J_{\min} < J < J_{\max} \tag{22.2}$$

To interrogate how fluxes could be distributed over the network, an objective function is utilized. For example, the objective could be to maximize biomass production from metabolites in the network. Ideally, the biomass equation is tailored to the biomass composition of the organism of study. Alternatively, the objective function can describe the production of a compound of interest (e.g., a compound with production value or a toxic side product of which production should be avoided). Linear programming is used to find flux distributions that maximize (e.g., for biomass or product of interest) or minimize (e.g., for toxic byproducts) the objective function.

Genome-scale metabolic models and FBA are often used to investigate the effects of a complete removal of a step (a knockout) on a desired outcome [36], such as the production of a specific compound or ATP. A particularly strong analysis comes from double knockouts or specifically synthetic lethality studies. Here, two reactions are removed simultaneously and the effects are compared to deletion of only one of the steps. A combination of two steps is synthetic lethal when, if they are depleted at the same time, they block the flux to an essential metabolite [37, 38]. Such an analysis identifies bypasses that can circumvent blockage of a main pathway and are otherwise difficult to find in large networks.

Following the sequencing of the genomes of the Trypanosomatid species [39–41], which includes *T. brucei*, as well as the genome of *Plasmodium* species [42, 43], genome-wide metabolic maps have been constructed for both *T. brucei* [44] and *P. falciparum* [45]. Based on gene expression datasets and other available experimental data, the genome-scale metabolic model of *T. brucei* has been constrained to reflect particular life-cycle stages of the parasite. In addition, for glucose metabolism in PCF *T. brucei*, a constraint-based model was made by taking into account the metabolic fluxes that have been measured *in vitro* for this life stage and was used to study flux distributions [46].

Bottom-Up: Detailed Kinetic Models

The Added Value of Detailed Kinetic Information Many metabolic enzymes are essential as there are no bypass reactions to enable a specific output. However, not all of these enzymes are equally interesting as drug targets. Even at high concentrations, drugs usually do not fully block enzyme activity. The best drug targets among the essential enzymes are those that need the smallest activity reduction to inhibit the pathway flux sufficiently to compromise parasite survival. Some essential enzymes are expressed far in excess of metabolic flux requirements, and near-complete inhibition would be needed to strongly affect a pathway flux. A prominent example is hexokinase (HXK) in *T. brucei*. HXK is the glycolytic enzyme that phosphorylates glucose. A full ablation of HXK activity truncates both glycolysis and the pentose phosphate pathway that branches off at the level of glucose-6-phosphate. HXK is thus essential for ATP generation and survival of *T. brucei*. However, experimental studies in which HXK expression was downregulated in *T. brucei* by RNAi revealed that 80% reduction in enzyme activity affected the glycolytic flux only moderately [47, 48]. Similar results were obtained for *T. brucei* glutathione synthetase: the protein is essential, but activity has to drop below 3% to affect growth [49]. This exemplifies that being essential alone is not the entire story – quantitative aspects of the entire network are also important in making a protein a good drug target.

Similarly, *P. falciparum* glycolytic enzymes have been indicated as potential drug targets, often on the basis of structural differences in the enzymes of the parasite and the host [28]. Although specific drugs can be developed for glycolytic enzymes in *Plasmodium*, these are usually only tested against either the enzyme activity (e.g., for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [50]) or on specific growth rate (e.g., for lactate dehydrogenase (LDH) inhibition [51]). Thus, it is hard to analyze the extent to which the specific enzyme activity must be inhibited to decrease the glycolytic flux (or the specific growth rate) sufficiently.

Kinetic models of metabolism can help to answer such questions. Drug action can be simulated in kinetic models by titrating down the activity of a target enzyme. Because kinetic models contain a detailed rate equation for each enzyme, even specific mechanisms of action of inhibitors can be tested.

The Construction of Kinetic Models In the construction of kinetic models of metabolism, for each reaction step in the model, a Michaelis–Menten-type rate equation is included that generally contains the maximal velocity of that step ($V_{\max}$) and dissociation constants of the substrates (K_S) and products (K_P) (an indication of affinity). A general form of the reversible Michaelis–Menten equation for an enzymatic reaction with one substrate (S) and one product (P) is (reviewed in [52]):

$$v = \frac{V_{\max,f} \cdot \frac{[S]}{K_S} - V_{\max,r} \cdot \frac{[P]}{K_P}}{1 + \frac{[S]}{K_S} + \frac{[P]}{K_P}} \tag{22.3}$$

$V_{\max,f}$ and $V_{\max,r}$ are the forward and reverse maximal velocities, respectively. When the reaction is in equilibrium, the rate equals 0:

$$V_{\max,f} \cdot \frac{[S]_{eq}}{K_S} - V_{\max,r} \cdot \frac{[P]_{eq}}{K_P} = 0 \tag{22.4}$$

The ratio of the concentrations of P and S at equilibrium is known as the *equilibrium constant* (K_{eq}), and therefore, the kinetic constants are constrained by the Haldane relation [53]:

$$K_{eq} = \frac{[P]_{eq}}{[S]_{eq}} = \frac{V_{\max,f} \cdot K_P}{V_{\max,r} \cdot K_S} \tag{22.5}$$

Eq. (22.5) can be used to rewrite Eq. (22.3) to:

$$v = \frac{\frac{V_{\max,f}}{K_S} \cdot \left([S] - \frac{[P]}{K_{eq}} \right)}{1 + \frac{[S]}{K_S} + \frac{[P]}{K_P}} \tag{22.6}$$

The use of this equation ensures that a change to K_S, K_P, or $V_{\max,f}$ cannot violate the thermodynamic equilibrium.

For reactions with more substrates and/or products, similar equations can be derived. The order of parts of the catalytic cycle (e.g., one of the substrates binds first) can also lead to variations on this equation. For many types of enzymatic reactions, rate equations have been derived [54], but not for complex and unusual catalytic cycles such as the reaction catalyzed by multistep enzyme trypanothione synthetase of *T. brucei*. For this enzyme, instead of a rate equation, a detailed computer model was made based on mass action kinetics in the catalytic cycle [55]. The parameters in the model of this enzyme were fitted to a large matrix of steady-state data obtained for different substrate/product combinations. The resulting model describes the full kinetic profile of this enzyme and was validated to correctly predict time profiles of (intermediate) product formation. Such a model of a single enzyme can be used as an alternative for a rate equation.

Enzymes communicate through their substrates and products. The dynamics of metabolite concentrations are described by ordinary differential equations (ODEs), which depend on the concentrations themselves. For a small two-enzyme pathway, in which enzyme 1 converts a substrate S into intermediate X and enzyme 2 converts X further into product P:

$$S \leftrightarrow X \leftrightarrow P \tag{22.7}$$

the change in X over time is described by the ODE:

$$\frac{d[X]}{dt} = v_1(S, X) - v_2(X, P) \tag{22.8}$$

For the rate equations (e.g., Eq. (22.6)), values for parameters and initial concentrations are required. The enzyme parameters and metabolite concentrations can be

based on literature data or on novel measurements. Especially for the enzymes of metabolism, these parameters have often been measured, but mostly in different laboratories and under different conditions. If possible, it is best to (re)measure the parameters in one laboratory and under *in vivo*–like conditions [56, 57]. The larger a model becomes, the more parameters need to be measured and therefore detailed kinetic models usually contain less reactions than their genome-scale counterparts due to the willful lumping of reactions/metabolites or less important (for the specific study) reactions/branches being left out (see Section 22).

Over the years, many detailed kinetic computer models have been made for different pathways in different organisms, cell types, and parasites. Examples for parasites are the kinetic models of glycolysis in *T. brucei* [58] and *P. falciparum* [59] (more details below), and *Entamoeba histolytica* [60] as well as nonglycolytic pathways in *T. brucei*, for example, polyamine metabolism [61]. In the next section, we focus on the glycolysis models of *T. brucei* and *P. falciparum.*

Construction and Validation of Detailed Kinetic Models of Glycolysis of BSF *T. brucei* and of the Trophozoite Stage of *P. falciparum*

The Glycolysis Model of *T. brucei*

Construction and Updates

In 1997, a first version of a computer model of glycolysis of BSF *T. brucei* was constructed [58]. The construction was aided by the availability of a vast amount of biochemical data for individual *T. brucei* enzymes, mostly from the lab of Fred Opperdoes and Paul Michels. Most of these kinetic data had been obtained under standardized conditions (see Ref. [58]). The original model consisted of 20 reactions in two compartments: the cytosol and the glycosome. Glycosomes are specialized peroxisomes that have a high content of glycolytic enzymes. In *T. brucei*, a large part of glycolysis is sequestered in glycosomes [62, 63].

Throughout the years, the *T. brucei* model has been updated based on new data. Some updates were small [64], other were bigger: in 2005, all enzyme V_{max}'s were remeasured in lysates of *in vitro*–cultivated BSF trypanosomes and some hitherto missing enzymes were included as their kinetic constants had been measured in the meantime [47]. This brought the model closer to representing the *in vitro* parasite cultures that were more and more used for experimental studies by that time. In 2013, the model was extended with the pentose phosphate pathway [65]. This pathway, important for nucleotide synthesis and the generation of NADPH, branches off from glycolysis at the level of glucose-6-phosphate and has a dual localization in the glycosome and the cytosol (Figure 22.1).

Validation and Use of the *T. brucei* Model

The original version of the model quantitatively described the glycolytic flux under both aerobic and anaerobic conditions [58]. The update with new V_{max} values

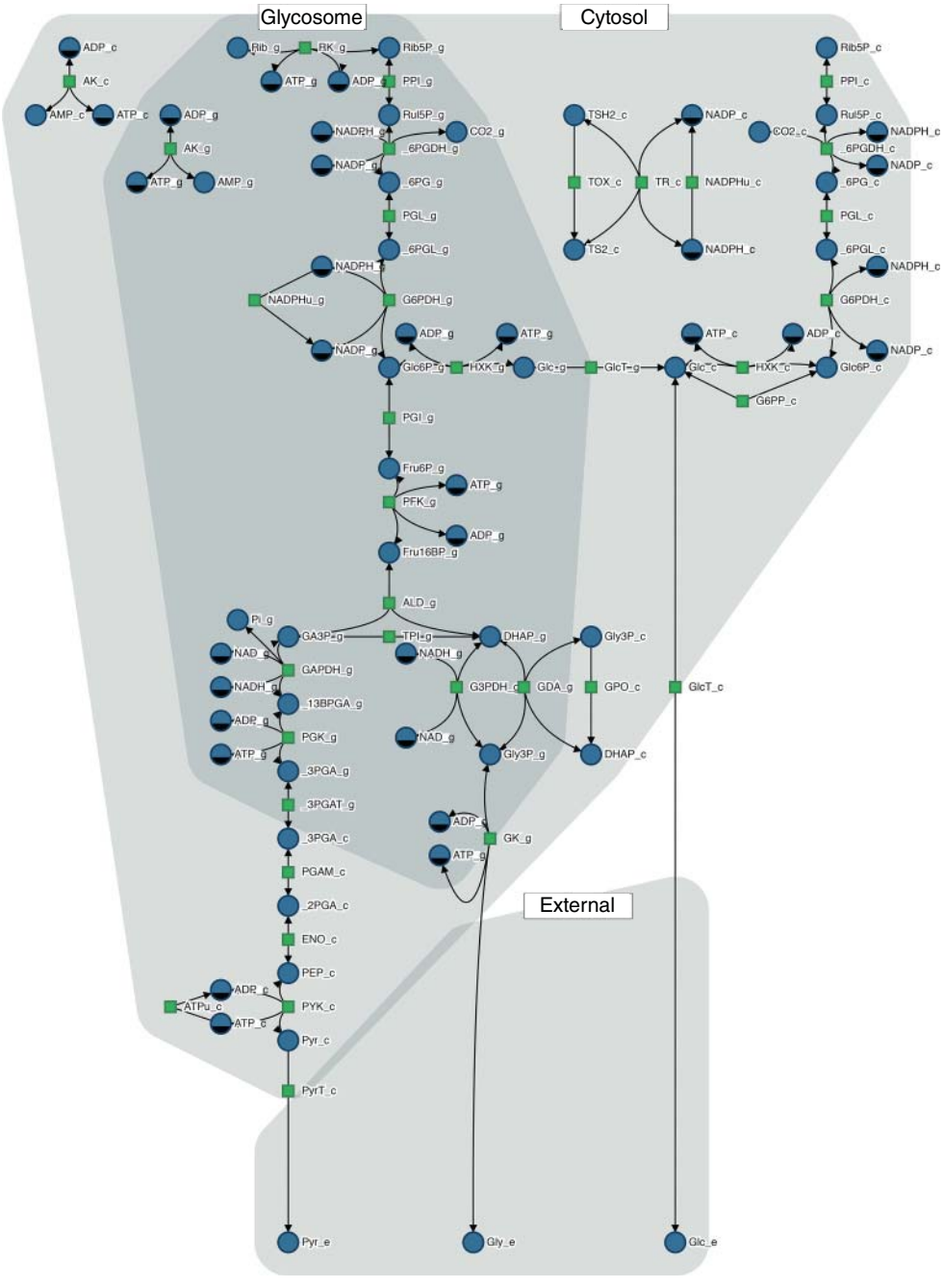

Glycosome
Cytosol
External
ADP_c
AK_c
AMP_c
ATP_c
Rib_g
RK_g
Rib5P_g
ATP_g
ADP_g
PPI_g
Rul5P_g
NADPH_g
CO2_g
6PGDH_g
NADP_g
6PG_g
PGL_g
6PGL_g
NADPH_g
NADPHu_g
G6PDH_g
NADP_g
ADP_g
ATP_g
Glc6P_g
HXK_g
Glc_g
GlcT_g
PGI_g
Fru6P_g
ATP_g
PFK_g
ADP_g
Fru16BP_g
ALD_g
Pi_g
GA3P_g
TPI_g
DHAP_g
Gly3P_c
NAD_g
GAPDH_g
NADH_g
G3PDH_g
GDA_g
GPO_c
GlcT_c
13BPGA_g
ADP_g
PGK_g
ATP_g
NAD_g
Gly3P_g
DHAP_c
3PGA_g
3PGAT_g
3PGA_c
ADP_g
GK_g
ATP_g
PGAM_c
2PGA_c
ENO_c
PEP_c
ADP_c
ATPu_c
PYK_c
ATP_c
Pyr_c
PyrT_c
Pyr_e
Gly_e
Glc_e
TSH2_c
NADP_c
TOX_c
TR_c
NADPHu_c
TS2_c
NADPH_c
CO2_c
Rib5P_c
PPI_c
Rul5P_c
NADPH_c
6PGDH_c
NADP_c
6PG_c
PGL_c
6PGL_c
NADPH_c
G6PDH_c
NADP_c
ATP_c
ADP_c
Glc_c
HXK_c
Glc6P_c
G6PP_c

Figure 22.1 Schema of the kinetic model of *Trypanosoma brucei* glycolysis with the pentose phosphate pathway. Schema of model version C from Kerkhoven *et al.* [65]. It is shown here as it is available on JWS Online (http://jjj.bio.vu.nl/models/kerkhovenC/simulate/). At JWS Online, the SBML file can also be downloaded. Green squares indicate reactions, blue circles indicate metabolites. Circles that are blue and black denote metabolites involved in more than one reaction (e.g., members of a moiety conserved cycle). Nomenclature is the same as in [65] and can be found there.

improved agreement with experimental data upon inhibition of the anaerobic flux by its product glycerol [47]. When the effect of a knockdown of enzyme activity on the glycolytic flux was determined, both in the model (via lowering the V_{max}) and in experiments (via RNAi against the enzyme) [47, 48], there was a good agreement between the model and the experiments for four of the five enzymes tested, namely for HXK, phosphoglycerate mutase (PGAM), enolase (ENO), and pyruvate kinase (PYK). For HXK, 80% reduction had only a marginal effect on the flux through glycolysis, which was in agreement with the model prediction [47]. However, when phosphofructokinase (PFK) activity was lowered, the experiment showed a stronger effect on the glycolytic flux than the model predicted [47]. The reason for this discrepancy is still unknown, but may hint at yet unidentified regulatory mechanisms.

Models can also be used to simulate experiments that are difficult *in vitro* or *in vivo*. One example concerns the present-day metabolic function of glycosomes. Glycosomes are essential organelles for *T. brucei*, and this precludes the generation of trypanosomes that lack glycosomes. Computationally, however, this compartment was easily removed and the enzymes were redistributed over the cytosol [66]. When the glycolysis model without a glycosome was given a glucose pulse, several phosphorylated intermediates of glycolysis increased to unphysiologically high levels. The explanation was that the ATP generated in the final reaction of glycolysis boosted the activity of the upstream kinases and caused an imbalance between upper and lower glycolysis, leading to the accumulation of phosphorylated sugars. Accumulation of intermediates did not occur when there was a glycosome, leading to the hypothesis that the glycosome protects *T. brucei* from this unbalanced situation. An experimental test for this model prediction was long unavailable, but here, the other life stage of the parasite (PCF) provided a workaround: PCF *T. brucei* have glycosomes but are less reliant on glycolysis as they can use proline as an alternative carbon source. Glycosomal-import-deficient PCF trypanosomes have the glycolytic enzymes in the cytosol but can be cultivated on proline before they are subjected to a glucose pulse. For these mutants, glucose is toxic [67, 68] and phosphorylated sugars accumulated over time, as predicted by the model [69]. Further validation came from simulations and experiments with glycerol as a carbon source, since the same mechanism was predicted to work for glycerol phosphorylation and subsequent metabolism. Indeed, the intermediate glycerol-3-phosphate (G3P) strongly accumulated in

both the model and experiment if glycosomal metabolism was moved into the cytosol. Depletion of glycerol kinase (GK) could attenuate the G3P accumulation in the model and could rescue the cells from glycerol-induced cell death *in vitro* [69]. This shows that glycosomal compartmentation is important to prevent accumulation of glycolytic intermediates. Therefore, the biogenesis of glycosomes and the integrity of the glycosomal membrane are promising antitrypanosomal drug targets [25, 70, 71].

Another example is the permeability of glycosomes. Recently, pore-forming activities were found in the glycosomal membrane [72]. Based on their measured properties, they are probably similar to size-selective pores in the peroxisomes of other organisms. When pores of different channel sizes were included into the *T. brucei* glycolysis model, this again hinted at the necessity of separate ATP/ADP pools in glycosome and cytosol. If ADP and ATP were allowed to traverse the glycosomal membrane via a specific transporter, but smaller intermediates were not, this led to accumulation of hexose phosphates similar to the measurements for import-deficient trypanosomes with glycosomal enzymes in the cytosol [65]. Inversely, when glycolytic intermediates up to the size of ADP and ATP (but *not* including them) were allowed to diffuse through the pores, no intermediate accumulation was observed, while including ADP and ATP did elicit the unbalanced metabolic state [73].

The model has also been used to understand the trypanocidal effects of a novel drug, 2-phenoxy-1,4-naphthoquinone, a naphthoquinone derivative [74]. An interaction screen revealed binding of this compound to two glycosomal enzymes, GK and GAPDH, and inhibition of these enzymes was confirmed. Model simulations indicated, however, that inhibition of GK by this compound only affects the flux under anaerobic conditions. Under aerobic conditions, GK inhibition does not provide additional inhibition of the glycolytic flux. Therefore, only the inhibition of GADPH seems relevant under the conditions that are prevalent in mammalian blood.

P. falciparum Trophozoite Model

Model Construction

Until recently, many of the glycolytic enzymes of different *Plasmodium* species had been characterized in isolation, but datasets were generated under different conditions (e.g., using purified enzymes or nonphysiological conditions) and were incomplete regarding allosteric regulation of the enzyme activities. In a 2015 paper by Penkler *et al*. [59], the first integrated analysis of all glycolytic enzymes was done under a single physiologically relevant condition and a detailed enzyme kinetic model of glycolysis in the trophozoite stage was constructed. Unlike *T. brucei*, *P. falciparum* does not comprise a glycosome, and the glycolytic enzymes are all located in the cytosol (Figure 22.2). The current model therefore consists of 17 reactions (including 3 transport reactions for glucose, lactate, and pyruvate) in one compartment with all but 5 reactions characterized experimentally in the same

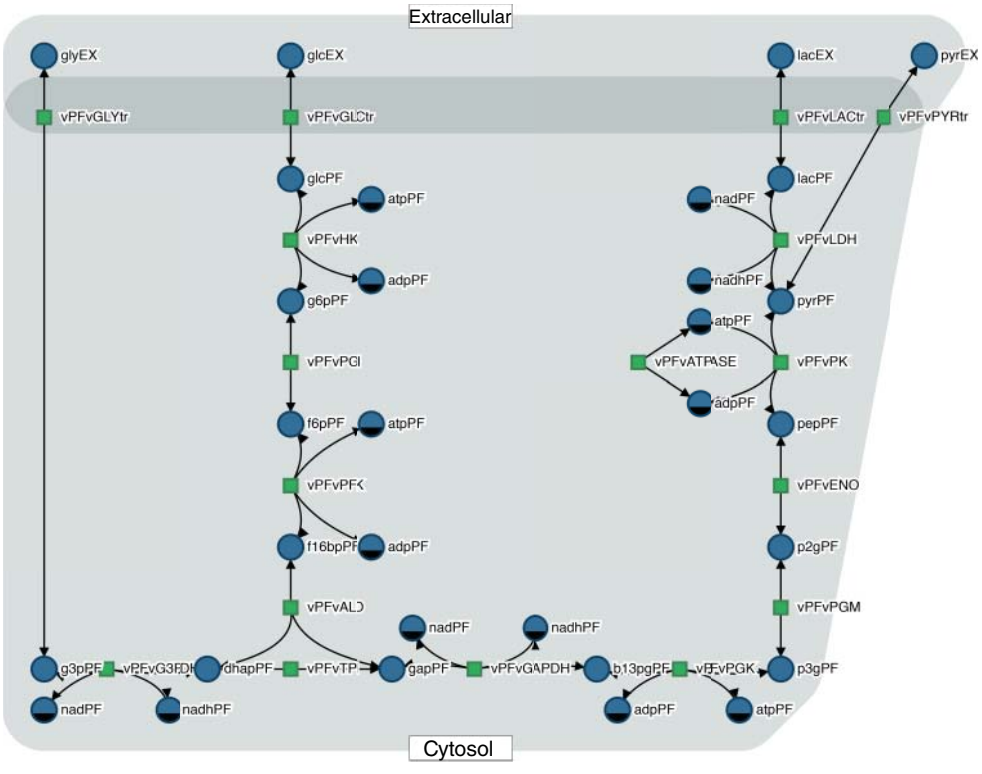


Figure 22.2 Schema of the kinetic model of glycolysis of *Plasmodium falciparum* trophozoites. Schema of model by Penkler *et al.* [59]. It is shown here as it is available on JWS Online (http://jjj.bio.vu.nl/models/penkler1/simulate/). At JWS online, the SBML file can also be downloaded. Green squares indicate reactions, blue circles indicate metabolites. Circles that are blue and black denote metabolites involved in more than one reaction (e.g., members of a moiety conserved cycle). Nomenclature is the same as in [59] and can be found there.

study. The noncharacterized processes were the conversion of G3P to glycerol and transport of glycerol across the membrane, pyruvate and lactate transport across the membrane, and ATP utilization. The reaction that produces glycerol is currently unknown for *P. falciparum*, whereas for its transport, an aquaporin has been suggested [75]. Pyruvate and lactate transport had been characterized before [76] and were included in this model. ATP-consuming reactions were grouped into a single reaction. The model consists of 91 parameters, of which 11 were taken from the literature and 2 (for the ATPase) were fitted to the physiological ATP/ADP ratio. All other parameters were experimentally measured in the same study.

Validation and Use of the *P. falciparum* Trophozoite Model

The model quantitatively predicts the glycolytic flux under the conditions prevalent in the human erythrocyte. Specifically, steady-state values of the

glucose consumption rate, lactate, pyruvate, and glycerol production rates, as well as hexose phosphate intermediate concentrations, were validated. The lactate production flux upon titration with the glucose transport inhibitor cytochalasin B was correctly predicted by the model. Further validation experiments are in progress.

Metabolic Control Analysis Ranks Enzymes for Drug Target Potential

Metabolic Control Analysis – A Framework to Decipher a Network Quantitatively

Metabolic control analysis (MCA) provides a quantitative framework to decipher which enzyme, when changed in activity, has the strongest effect on a pathway flux. MCA has extensively been reviewed in [77], but the concepts relevant for this chapter are described here (Adapted from [48].). In MCA, the relative importance of an enzyme i on a steady-state flux J is described quantitatively by the flux control coefficient. A slightly simplified definition is

$$C_i^J \approx \frac{\text{fractional change } J}{\text{fractional change } v_i} \tag{22.9}$$

where v_i is the rate of enzyme i and its fractional change should be small and the sole cause of the change in flux. Thus, control is a measure of how much of the strength of the local perturbation (fractional change in v_i) is propagated to the system level (the output seen as the fractional change in J). Because the control coefficient is based on fractional changes rather than on absolute changes, the units in which the flux and the enzyme rate are measured do not matter and the control coefficient is dimensionless. When we consider a change in a parameter p_i (e.g., the amount of the enzyme) that specifically affects the enzyme rate by a small percentage, we rewrite Eq. (22.9) as:

$$C_i^J = \frac{\left[\dfrac{d \ln J}{dp_i}\right]_{\text{steady state}}}{\left[\dfrac{\partial \ln v_i}{\partial p_i}\right]_{\text{instantaneous}}} \tag{22.10}$$

where the subscript "steady state" indicates that the change of flux is considered after relaxation to a new steady state. The subscript "instantaneous" reflects that, for the corresponding partial derivative, only the direct change in the local rate of enzyme i is considered due to the change of parameter p, while all other pathway components (notably, concentrations of metabolites and of other enzymes) are unaltered. The use of a partial derivative (∂) stresses this local analysis. For $p_{i,}$ often $V_{\max}$ of the enzyme i is used as $V_{\max}$ is proportional to the enzyme concentration. In unbranched pathways with simple kinetics, flux control coefficients are usually between 0 and 1. In more complex networks, and depending on the network structure and the kinetic properties of the enzymes, flux control coefficients can also be negative or have absolute values that are larger than 1 [78]. The higher the

flux control coefficient is, the stronger a perturbation of that step affects the pathway flux. For ideal pathways without metabolite channeling (i.e., when subsequent enzymes do not directly transfer intermediates) and coenzyme sequestration [79, 80], the sum of the flux control coefficients of all the enzymes in the pathway equals unity:

$$\sum_i C_i^j = 1 \tag{22.11}$$

Eq. (22.11) is a summation theorem and reflects that if the activity of all the enzymes of the pathway together is changed by 1%, the flux will also change by 1%. In principle, this theorem includes all reactions of the cell, although in practice, many reactions in the cell reside outside the pathway of study and are assumed to have only minor control over the pathway flux. If one of the enzymes has a control coefficient of 1, it is a true rate-limiting enzyme: a change in the activity of this enzyme will change the flux by the same proportion. In this case, all the other enzymes must be without any control on the pathway flux or balance their positive and negative control among themselves (cf. Eq. (22.11)). The higher the flux control coefficient, the better an enzyme qualifies as a drug target; of all the enzymes in the pathway, the enzyme with the highest control will require the least amount of inhibition (i.e., the lowest inhibitor concentration) to decrease the flux in the pathogen to a desired extent.

Application of MCA to the Models of *T. brucei* and *P. falciparum*

When applied to the *T. brucei* glycolysis model, MCA indicated that the glucose transporter over the plasma membrane has the highest control over the glycolytic flux in BSF *T. brucei* [81]. The high control coefficient was confirmed experimentally with the transport inhibitor phloretin [82]. In 2012, the uncertainty with respect to the exact value of the parameters of the *T. brucei* model was explicitly taken into account [83]. In this analysis, all parameters of the model were sampled from their probability distributions, leading to a collection of models that differ only in their parameter values. In 40% of those models, the glucose transporter had the highest control over glycolytic flux. In a substantial fraction of models, some of the control over the glycolytic flux had shifted to other enzymes (see Eq. (22.11)), and either PGAM or GAPDH had the highest control coefficient. For the latter enzyme, this is in agreement with an earlier coarse-grained sensitivity analysis that showed that GAPDH was one of the enzymes that acquired control if control of the glucose transporter decreased [81] (at that time, PGAM was not part of the model). In line with the high flux control coefficient of glucose transport on glycolysis that was predicted, as well as the overall importance of glycolysis for survival, it was shown experimentally that the glucose transport inhibitor phloretin kills BSF *T. brucei in vitro* [24].

MCA applied to the *P. falciparum* model indicated that the majority of the control over the lactate flux resides in the top part of glycolysis [84]. Control is distributed over glucose transport and the HXK and PFK reactions. The GAPDH

has a small positive control and the ATPase a small negative flux control. The same reactions that have a high flux control on the lactate production also have a high control on the glycerol production rate, but in addition, now the dehydrogenases (glycerol 3-phosphate dehydrogenase (G3PDH), GAPDH, and LDH) also have a strong control, illustrating the important role of the glycerol branch for the redox homeostasis of the system.

While these two parasites have the majority of the control residing in the initial steps of glycolysis, this is *not* a general feature of glycolysis itself. Quantitative differences in enzymes expression levels (affecting $V_{\max}$), and differences in other kinetic properties of the enzymes between cells or cell types can affect control distribution in a given pathway.

Work in Progress: Future Extensions and Use of the Kinetic Models of *T. brucei* and *P. falciparum*

One straightforward next step for the kinetic models of glycolysis will be to extend them with other metabolic pathways. But extensions may also deal with (regulatory) layers outside of metabolism. Furthermore, to assess drug selectivity, the parasitic models should be compared to similar models made for human cells. We highlight three of these upcoming approaches in this section.

Adding Gene Expression to Metabolic Models

Currently, the kinetic models of metabolism described earlier are valid for the short timescales at which metabolic reactions operate. After perturbations (most notably, the transfer to another host), gene expression is often altered. In particular, the maximal velocity ($V_{\max}$) of an enzyme can change through gene expression, as this parameter is proportional to the enzyme concentration. Gene expression for metabolic enzymes changes during the life cycle of the parasite [6], but also upon changing nutrient availability. An example is the inhibition of glucose transport in *T. brucei*, which leads to remodeling of the metabolic network [24]. When one knows the changes in activity of the enzymes, one can easily generate a model for the new condition and compare the networks under two conditions. The dynamics of the transition can also be modeled by adding a gene expression module to these metabolic models. Based on measured kinetics of the processes from DNA to protein (including transcription, splicing, mRNA degradation, protein synthesis, and degradation), a first gene expression model was made for the three-isoform *T. brucei* phosphoglycerate kinase locus [85]. Using genome-wide data on mRNA dynamics, the gene expression model was used to simulate steady-state levels of each *T. brucei* mRNA [86]. Comparison of simulated and measured mRNA abundances and subsequent additional modeling suggested a role for cotranscriptional degradation of pre-mRNA species in determining mRNA abundance. The gene expression model [85] can be used as a scaffold for including gene

expression into the metabolic models. Changes in gene expression influence the enzyme concentration. By replacing V_{max} values in the model by the product of the catalytic capacity per enzyme molecule (k_{cat}) and the enzyme concentration, altered gene expression can be modeled to directly affect the enzyme concentration. Some of the parameters needed to model gene expression for each metabolic enzyme have been measured in recent analyses of mRNA abundances and mRNA half-lives in *T. brucei* [86, 87].

From Parasite to Disease State

Apart from a fundamental understanding of parasite dynamics, a very important goal of these modeling projects is to understand and manage the disease state, which manifests at the whole-body level. A quantitative description of the disease phenotype as well as the importance of various drug targets requires a multiscale approach, linking the detailed parasite models to models describing their immediate environment and subsequently to models of relevant whole-body physiology. Investigation of drug effects, on the other hand, requires integration of pharmacological models with models of physiological processes and parasite metabolism. As the systems biology community progresses in characterizing the relevant reaction networks and develops models in a transparent way (as discussed in Section Concluding Remarks), higher levels of detail in these multiscale models become increasingly feasible. Since construction of such whole-body models generally follows a modular "slotting-in" approach where existing models are reused and extended, it becomes crucially important for all modelers to adhere to good modeling practice and community-wide modeling standards.

Comparing Parasite Models to Host Models: Differential Network-Based Drug Design

If a pathway is essential in both a parasite and a host, selective targeting of the parasite is considered difficult, as the enzymes in the pathway are well conserved in terms of function and the host enzyme may therefore also be affected by inhibition. Nevertheless, because of the strong reliance of BSF *T. brucei* on glycolysis [23], there has been a long line of research into inhibitors against glycolytic enzymes of *T. brucei* that exploited structural differences between *T. brucei* and mammalian enzymes (as also reviewed in [25]). In addition to possible structure-based drug selectivity, quantitative differences between the parasite and host networks can affect network-based selectivity of drug targets: the distribution of control over the different enzymes or other quantitative aspects that could affect drug function, such as the levels of intermediates, can be different in parasite and host. There is thus potentially a network-based differential effect of inhibitors. To identify these, overall drug effects can be compared between parasite and host models [88]. In both the *T. brucei* and the *P. falciparum* glycolysis models, the glucose transporter has a high control over the glycolytic flux and is hence a promising

drug target from the antiparasitic perspective. But human cells also need to transport glucose. The inhibitors used so far to inhibit glucose transport in *T. brucei* and *P. falciparum*, cytochalasin B (for *P. falciparum* [89–92] and *T. brucei* [93]), and phloretin (for *T. brucei* [82, 94]) also inhibit, for example, the glucose transporter of the human red blood cell [95–98]. Interestingly, analysis of a kinetic model of erythrocyte glycolysis [99] shows that the glucose transporter has very low control over the glycolytic flux, suggesting that such inhibitors may be selective. A glycolysis-wide comparison between parasite and host may reveal other targets where network-based differences can accommodate parasite specificity. Such an analysis should be extended to other relevant host tissues when metabolic models for those cell types become available.

Do It Yourself: Databases and Tools to Do Your Own Simulations with the Detailed Kinetic Models

Various software tools are available to serve the needs of model authors as well as users. These do not only function as research tools by facilitating model construction and analysis (e.g., COPASI [100], Cell Designer [101], and JWS Online (jjj.bio.vu.nl; [102])), but can also serve as repositories for model review during the publication process and postpublication as repositories for model reuse (Biomodels [103, 104] and JWS Online (Figure 22.3) [102]).

In principle, to make the model construction process transparent and enhance the reusability of experimental data, all the files that were used for model construction and validation need to be made public, ideally in annotated form (e.g., RightField spreadsheets [105] for experimental data and files in Systems Biology Markup Language (SBML) [106], annotated according to MIRIAM standards [107], for models). This allows a user to add complementary data to the dataset and fit custom equations to the data that might be more suitable for their project. This assists in reuse of models, which is essential in modular approaches, where validated models for different parts of metabolism are linked to construct kinetic models of larger systems [108].

One tool that can be used to this end is the complete model management online platform known as *SEEK* [109], which is now part of the FAIRDOM project (http://www.fair-dom.org). The *P. falciparum* model discussed in this chapter and all the experimental data used during model construction and model validation have been made available via doi:10.15490/seek.1.investigation.56. In the ISA (Investigation, Study, Assay) structure of SEEK, the Model Construction Study contains an experimental assay with the kinetic data, a SOP file with the experimental specifications, and a model file that imports the experimental data and specifies the parameterization methods for each of the reactions. The Model Validation Study in SEEK links to the validation experiments and the complete model description file. The model files are available as Mathematica

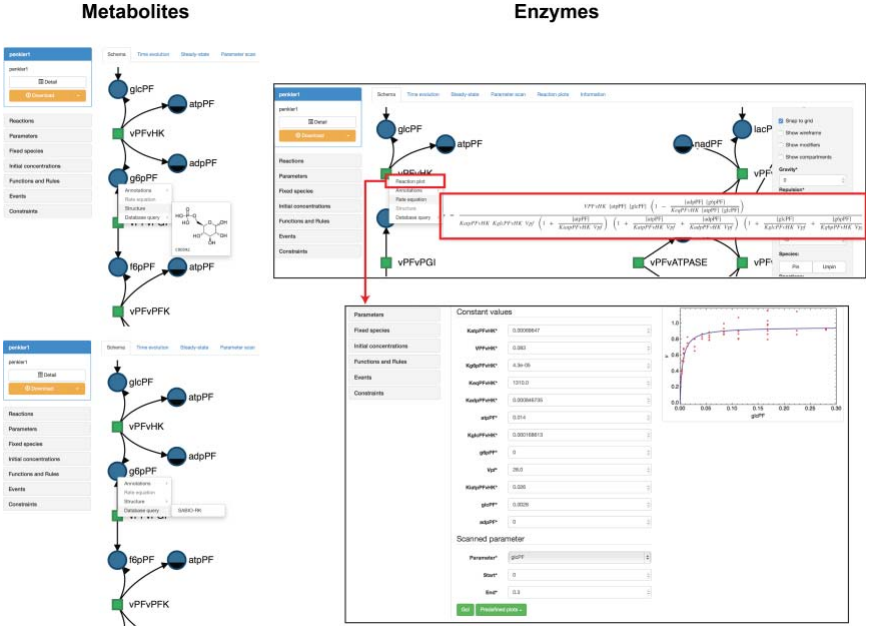


Figure 22.3 Do it yourself on JWS Online. Some features of the JWS Online website (http://jjj.bio.vu.nl) here demonstrated on the model of Penkler *et al.* [59] (full scheme is shown in Figure 22.2). Left panels show the items in the drop–down menu after a right-click on a metabolite. It includes information on the structure of the metabolite and direct links to the SABIO-RK database (http://sabiork.h-its.org/), which provides (kinetic) information on the reactions that the metabolite can be involved in. Right panels show the items in the drop–down menu after a right-click on an enzyme, such as a display of the rate equation. The lower right panel shows the result of a "Reaction plot" where the rate of the reaction can be plotted for custom parameter ranges and metabolite concentrations. If experimental data points are available, these will be shown in the plot.

(Wolfram Research, Inc, Illinois) notebooks and as MIRIAM-annotated SBML files. All the models described in this chapter can be simulated or downloaded in SBML format on JWS Online by searching on the name of the first author of the publication associated to the model [102].

Concluding Remarks

Metabolic pathways are interesting target pathways for antiparasitic drugs as they are often essential for parasite survival. However, a cell's metabolic network is very complex, even in parasites with their relatively small genome. To find the most promising drug targets in metabolism, we need to understand the function of each metabolic enzyme in the context of the network. We have seen in Section Construction and Validation of Detailed Kinetic Models of Glycolysis of BSF *T. brucei* and of the Trophozoite Stage of *P. falciparum* that different computational approaches can help in answering different types of questions: genome-scale metabolic models can identify which genes are essential to maintain a metabolic function or to sustain growth. Detailed kinetic models, although smaller than their genome-scale counterparts, excel in answering quantitative questions and can be used to simulate the effects of enzyme inhibitors – which only rarely fully block enzyme activity and are therefore different from gene knockouts. In this chapter, we mainly focused on detailed kinetic models of glycolysis in BSF *T. brucei* and *P. falciparum* trophozoites. In addition to aiding the understanding about the quantitative setup of metabolism in these parasites, MCA for these systems makes it possible to rank targets for their putative antiparasitic effects. Drug response can be compared between parasite and host metabolic models and can elucidate network-based differences in pathways that are essential in both parasite and host. The work described in this chapter shows the power of computational models as tools to understand (parasite) metabolism. After publication, models become available to the community, in ways that tailor either to experts or to novices. In the databases, model scripts are available in a common language (SBML) and, for example, by using JWS online, simulations can be done in a graphical user interface. This allows other researchers to add the power of existing metabolic models to their own research.

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Abbreviations

BSF	Bloodstream form
ENO	Enolase
FBA	Flux balance analysis
G3P	Glycerol-3-phosphate
G3PDH	Glycerol 3-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GK	Glycerol kinase
HXK	Hexokinase
LDH	Lactate dehydrogenase
MCA	Metabolic control analysis
ODE	Ordinary differential equations
PCF	Procyclic form
PFK	Phosphofructokinase
PGAM	Phosphoglycerate mutase
PYK	Pyruvate kinase
SBML	Systems Biology Markup Language
TCA	Tricarboxylic acid

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