Milestones in Drug Therapy
Series Editors: Michael J. Parnham · Jacques Bruinvels

Henry M. Staines
Sanjeev Krishna *Editors*

Treatment and Prevention of Malaria

Antimalarial Drug Chemistry, Action and Use

Springer

Milestones in Drug Therapy

For further volumes:
http://www.springer.com/series/4991

Henry M. Staines • Sanjeev Krishna
Editors

Treatment and Prevention of Malaria

Antimalarial Drug Chemistry, Action and Use

Springer

Volume Editors
Dr Henry M. Staines
Centre for Infection and Immunology
Division of Clinical Sciences
St George's, University of London
Cranmer Terrace
London SW17 0RE
United Kingdom
hstaines@sgul.ac.uk

Prof. Sanjeev Krishna
Centre for Infection and Immunology
Division of Clinical Sciences
St George's, University of London
Cranmer Terrace
London SW17 0RE
United Kingdom
skrishna@sgul.ac.uk

Series Editors
Prof. Michael J. Parnham, Ph.D.
Visiting Scientist
Research & Clinical Immunology Unit
University Hospital for Infectious Diseases "Dr. Fran Mihaljević"
Mirogojska 8
HR-10000 Zagreb
Croatia

Prof. Dr. Jacques Bruinvels
Sweelincklaan 75
NL-3723 JC Bilthoven
The Netherlands

ISBN 978-3-0346-0479-6 e-ISBN 978-3-0346-0480-2
DOI 10.1007/978-3-0346-0480-2

HMS dedicates this book to his wife, Zoë, and children,
Talia, Luca and Oren and SK to Yasmin's memory
and Karim, and to the rest of his exceptional family.

Preface

Malaria is a devastating disease that extracts huge health and economic costs from the poorest countries in endemic regions. Malaria is caused by single celled parasites, belonging to the genus *Plasmodium* that have infected humans (and related primates) for thousands of years. In its different specific and clinical guises, malaria is one of the strongest selective forces to have shaped our recent evolution. These parasites have already evaded one attempt at eradication in the mid twentieth century. Now, there are renewed attempts to control and eventually eradicate what remains one of the world's biggest killers.

With ambitious new targets set to reduce the global burden of malaria, we must urgently develop new tools for disease control, as well as optimising and re-evaluating our current tools. An indispensable part of controlling malaria is the capability of treating the disease effectively, despite the ability of this highly mutable parasite to develop resistance sooner or later to all classes of antimalarials. Understanding of how antimalarial drugs might work, how best to use them and how to assess for resistance to them has expanded considerably in the past few years. This book aims to capture these recent advances in our understanding of all antimalarial classes, and discuss how this information is pertinent for treating patients.

The introductory chapter details the disease, its current political, financial and technical context, alongside the policies and tools required to make eradication a possibility. Subsequent chapters cover the history, chemistry, mechanisms of action and resistance, preclinical and clinical use, pharmacokinetics and safety and tolerability of our current antimalarial drug armamentarium. Each chapter reflects the unique perspectives of its expert authors, and often describes new ideas and directions for study. There is particular emphasis on artemisinins (and related next generation peroxides) that have become the frontline treatment for malaria, as part of artemisinin-based combination therapies (ACTs). The artemisinins may have become established in ACTs in the past decade, but they are now being challenged by the potential for resistance that has recently been described and is only just being defined.

Other chapters authoritatively discuss our antimalarial drug development pipeline and how this is being shaped by public/private partnerships; molecular markers

of antimalarial drug resistance, their use in monitoring treatment failures and the insights they provide into the action of these drugs; malaria prevention strategies, including chemoprophylaxis, where the risk of catching malaria is balanced against the risk of side effects of drugs and the critical use of diagnostics to improve the identification of malaria and to refine treatment strategies.

The treatment and prevention of malaria is a fascinating and complex subject – made all the more interesting now that malaria eradication is back on the global agenda. We hope that readers will be stimulated by this volume and that they may find its contents useful in dealing with malaria.

London, United Kingdom

Henry M. Staines
Sanjeev Krishna

Contents

Antimalarial Drugs and the Control and Elimination of Malaria 1
Karen I. Barnes

4-Aminoquinolines: Chloroquine, Amodiaquine and Next-Generation Analogues 19
Paul M. O'Neill, Victoria E. Barton, Stephen A. Ward, and James Chadwick

Cinchona Alkaloids: Quinine and Quinidine 45
David J. Sullivan

8-Aminoquinolines: Primaquine and Tafenoquine 69
Norman C. Waters and Michael D. Edstein

Other 4-Methanolquinolines, Amyl Alcohols and Phentathrenes: Mefloquine, Lumefantrine and Halofantrine 95
Francois Nosten, Penelope A. Phillips-Howard, and Feiko O. ter Kuile

Antifolates: Pyrimethamine, Proguanil, Sulphadoxine and Dapsone 113
Alexis Nzila

Naphthoquinones: Atovaquone, and Other Antimalarials Targeting Mitochondrial Functions 127
Akhil B. Vaidya

Non-Antifolate Antibiotics: Clindamycin, Doxycycline, Azithromycin and Fosmidomycin 141
Sanjeev Krishna and Henry M. Staines

Artemisinins: Artemisinin, Dihydroartemisinin, Artemether and Artesunate 157
Harin A. Karunajeewa

Second-Generation Peroxides: The OZs and Artemisone 191
Dejan M. Opsenica and Bogdan A. Šolaja

Combination Therapy in Light of Emerging Artemisinin Resistance 213
Harald Noedl

New Medicines to Combat Malaria: An Overview of the Global Pipeline of Therapeutics 227
Timothy N.C. Wells

Molecular Markers of *Plasmodium* Resistance to Antimalarials 249
Andrea Ecker, Adele M. Lehane, and David A. Fidock

Prevention of Malaria 281
Patricia Schlagenhauf and Eskild Petersen

Malaria Diagnostics: Lighting the Path 293
David Bell and Mark D. Perkins

Index 309

Antimalarial Drugs and the Control and Elimination of Malaria

Karen I. Barnes

Abstract Malaria remains a massive global public health problem despite being readily preventable and treatable. The past decade has seen unprecedented levels of political, technical and financial support that have facilitated the scaling-up of malaria control interventions, particularly the implementation of artemisinin-based combination therapy (ACT) policies. During this window of opportunity for reducing the burden of malaria globally and possibly eventually eliminating malaria, attention now needs to be focussed on ensuring that countries select and implement treatment policies that are not only highly effective, but will also have a prolonged useful therapeutic life, reduce malaria transmission safely and effectively and, where applicable, be active against *P. vivax*. To reduce the probability of resistance, antimalarials should be used in quality-assured fixed-dose combinations and treatment doses need to be optimised on the basis of pharmacokinetic assessments conducted within therapeutic efficacy studies in each key target population. As important is ensuring optimal targeting and adherence with these treatment policies.

1 Introduction

Malaria is a massive global public health problem. Nearly half the world's population lives at risk of malaria, which causes an estimated one million deaths and 450 million *Plasmodium falciparum* and 390 million *P. vivax* cases each year [1, 2]. Those with malaria also carry an increased burden of HIV/AIDS, measles, respiratory tract infections, diarrhoea, malnutrition and anaemia [3]. Malaria in pregnancy increases the infant risk of low birth weight, abortions and stillbirths, in addition to

K.I. Barnes (✉)
Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Anzio Road, Observatory, Cape Town 7925, South Africa
e-mail: karen.barnes@uct.ac.za

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_1,

the maternal burdens of anaemia, severe malaria and maternal mortality [4]. The indirect burden of malaria includes its adverse effects on education, worker productivity and investment. It has been estimated that malaria costs Africa $12 billion per year, with a fivefold reduction in per capita gross domestic product (GDP) after controlling for other socio-economic determinants [5].

Efforts to reduce malaria morbidity and mortality include control of the mosquito vector (using insecticide-treated bed nets and indoor residual spraying) and prompt treatment with effective antimalarials. Unprecedented levels of political, technical and financial support have facilitated the scaling-up of malaria control interventions, particularly changes in malaria treatment policy from the inexpensive yet failing monotherapies, chloroquine and sulfadoxine–pyrimethamine, to the recommended artemisinin-based combination therapies (ACTs). ACTs are generally considered as the best current treatment of uncomplicated falciparum malaria [6], as they have high cure rates, have more rapid parasite clearance times and have the potential to reduce both antimalarial resistance and malaria transmission. Over the last decade, the bar for recommending an antimalarial regimen as policy for uncomplicated falciparum malaria was raised from requiring an adequate clinical and parasitological response (ACPR) rate at 14 days of merely 75%, to at least 95% at $\geq$28 days [6]. Fortunately, there are now a number of ACTs in most settings that meet this stringent criterion. While most malaria endemic countries have adopted ACT policies, the implementation of these policies has been slower.

The extent to which malaria can be eradicated in the foreseeable future is a subject of active debate, but it is generally agreed that the tools are available to reduce the global burden of malaria substantially. How these tools are selected and, more importantly, deployed will be critical in determining the success achieved. Optimising the impact of ACTs on the control and eventual elimination of malaria depends on careful selection of the regimen implemented. In addition to the usual considerations of effectiveness, safety and cost, treatment policy selection should consider the likely useful therapeutic life (the time until ACPR rates at $\geq$28 days decrease below 90%), impact on malaria transmission and, where relevant, efficacy against non-falciparum malaria. As important are the selection of evidence-based dosage regimens that are appropriate for each key target population, especially young children and pregnant women [7], and optimising the implementation strategies deployed to ensure high coverage and adherence rates among those with malaria, while limiting use among those with non-malarial febrile illnesses [8].

2 Malaria: The Basics

All malaria is transmitted by female mosquitoes of the genus *Anopheles*. Humans are mainly infected by four species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, although human infections with the monkey malaria parasite, *P. knowlesi* have also been reported recently in the forested regions of Southeast Asia [9]. The majority of all human malaria cases are caused by

P. falciparum and *P. vivax*, although the burden of *P. ovale* and *malariae* are poorly defined. Although almost all severe malaria is caused by *P. falciparum*, severe disease and malaria-related deaths have also been reported with *P. vivax* and *P. knowlesi*.

The sporozoite form of the parasite is inoculated into humans when bitten by an infected female *Anopholes* mosquito. Sporozoites rapidly enter the liver cells where they multiply to form thousands of merozoites. These then enter the bloodstream where they invade red blood cells and multiply to form new merozoites. Infected red blood cells burst, releasing merozoites that infect new red blood cells. This is referred to as the asexual blood stage, the stage of the plasmodial life cycle that causes the clinical signs and symptoms of malaria. Some merozoites that invade the red blood cells develop into gametocytes, the sexual stages of the parasite. Gametocytes are ingested by the mosquito when it takes a blood meal. In the mosquito gut, the gametocytes develop into gametes and fuse to form a zygote. After fertilisation, the zygote transforms into a motile ookinete, which penetrates the mosquito stomach wall and becomes an oocyst. The oocyst divides to produce sporozoites, which move into the salivary glands, from where another human can be infected when the mosquito takes a blood meal (Fig. 1).

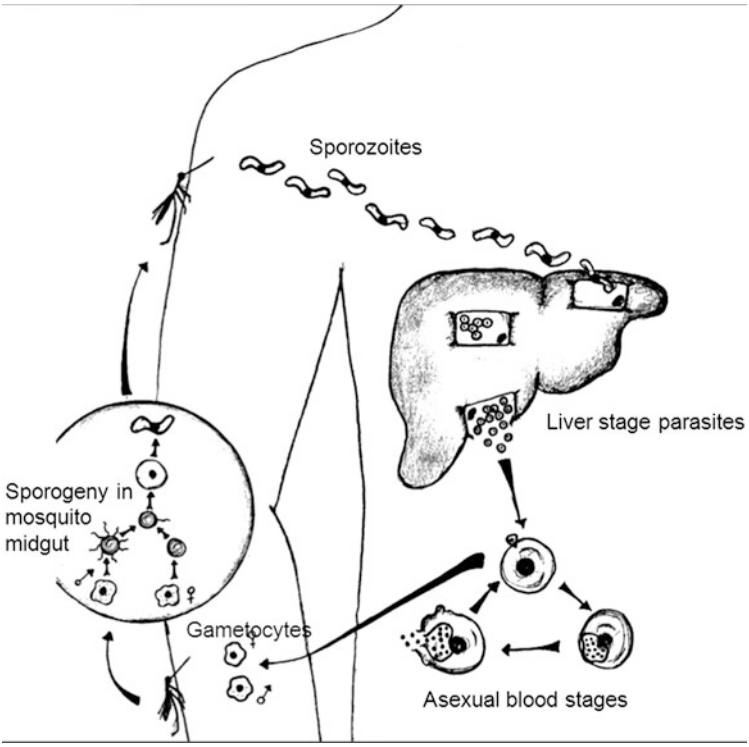


Fig. 1 The lifecycle of the malaria parasite in the human host and *Anopholene* mosquito vector

Malaria transmission rates are determined by the parasite reservoir in a community and the abundance and behaviour of the mosquito vectors [10]. The probability of a mosquito being infected depends on the prevalence, duration and density of viable gametocyte carriage in the human host, although additional immunological factors also affect transmissibility [11]. There are many factors that can lead to an increase in the duration and density of *P. falciparum* gametocyte carriage. Most of these are not well defined, but gametocyte numbers increase with the density and duration of asexual parasitaemia (emphasising the importance of prompt treatment), anaemia and drug resistance [11, 12].

3 Treating Malaria to Prevent Transmission

While eliminating the asexual stages of plasmodial infections is the focus of treatment of individual symptomatic patients, at a population level, limiting the transmission of malaria, and in particular, the transmission of resistant parasites is pivotal for decreasing the community's burden of malaria. In considering antimalarial drug effects on transmissibility, three different components need to be considered (a) activity against asexual stages and early gametocytes, (b) activity against mature infectious gametocytes and (c) sporontocidal effects in the mosquito [13].

Early access to effective treatment of the asexual blood stage can reduce the incidence and prevalence of malaria in a community, although the effects are greater in areas of low transmission where a higher proportion of the infectious reservoir is in non-immune and thus symptomatic individuals, who are more likely to seek antimalarial treatment [13]. However, achieving malaria control and eventually elimination requires a complete parasitological cure, including killing of the parasites in the sexual (gametocyte) stages that are responsible for malaria transmission [11, 14]. *P. falciparum* gametocytes are relatively insensitive to most antimalarials, other than the artemisinins and primaquine [15]. It has been suggested that artemisinins predominantly inhibit gametocyte development, whereas primaquine accelerates gametocyte clearance in *P. falciparum* malaria [16]. ACTs have the advantage of being the only antimalarials currently available that rapidly reduce both asexual and gametocyte stages of *P. falciparum*. When compared with amodiaquine plus sulfadoxine–pyrimethamine treatment, ACTs reduced the duration of gametocyte carriage (quantified using nucleic acid sequence-based amplification) fourfold [17]. The large-scale deployment of ACTs contributed to a marked reduction in the number of malaria cases seen in a number of countries, mostly in areas of low to moderate intensity transmission [18–23].

As the effect of the artemisinins on *P. falciparum* gametocytes is not complete, patients treated with artemisinins can still transmit malaria [24–26]. Mature gametocytes are resistant to almost all of the antimalarial drugs that affect the asexual stages and the only licenced drug that can ensure complete killing of *P. falciparum* gametocytes is primaquine. This 8-aminoquinoline is very effective

in preventing transmission, even when administered as a single dose. The addition of primaquine to artesunate plus sulfadoxine–pyrimethamine in Tanzania, when compared with artesunate plus sulfadoxine–pyrimethamine alone, resulted in a further fourfold reduction of the duration of gametocyte carriage [17], and an even greater reduction in sub-microscopic gametocytaemia [27]. However, primaquine may cause methaemoglobinaemia and haemolysis, which can be severe and occasionally life threatening. Haemolysis occurs most frequently (but not only) in patients with certain glucose-6-phosphate dehydrogenase (G6PD)-deficiency variants, particularly when a prolonged course of treatment is used. Primaquine is contraindicated in pregnancy, lactation, infants and young children and in those with haemolytic anaemia, methaemoglobinaemia or severe G6PD deficiency. As G6PD-deficient variants protect against *P. falciparum* and *vivax* malaria, this abnormality is most prevalent in malaria-endemic areas [6, 28–32]. The key operational question now is whether the benefits of adding primaquine (probably as a single dose) to ACTs in order to further reduce transmission exceed the risks. Unfortunately, there are remarkably limited data available to inform this decision, as summarised by Baird [33]: "Despite more than 50 years of continuous use in millions of people annually as the only drug available for its therapeutic indication, it is not known how primaquine acts or how it should be taken."

Lastly, atovaquone and the antifolate antimalarials reduce transmission by decreasing the formation of sporozoites in the *Anopheline* mosquito. For antifolates, this effect is reduced by antifolate resistance, creating a further transmission advantage for resistant parasites [13]. Also, atovaquone–proguanil offers the further benefit of acting as a causal prophylactic agent, but atovaquone rapidly selects for the *cytochrome b* mutation associated with high-level resistance. The possible role of atovaquone–proguanil alone or in combination with artesunate in attempts to contain or eliminate malaria deserves further study.

4 Antimalarial Resistance: The Major Threat to Malaria Control and Elimination

Parasite resistance to antimalarial medicines is a major threat to achieving malaria control and eventual elimination. Antimalarial resistance in *P. falciparum* parasites results in an enormous public health and economic burden. The rise in malaria-related hospital admissions and malaria mortality across west, east and southern Africa during the 1990s is largely accounted for by the continued use of the cheap monotherapies, chloroquine and sulfadoxine–pyrimethamine, despite widespread high levels of resistance [34–37]. Lower levels of resistance are associated with return of illness, anaemia and increased gametocyte carriage (which fuels malaria transmission, particularly of the resistant parasites) and a higher risk of treatment failure in subsequent infections [38, 39]. Parasite resistance has been documented for all classes of antimalarials, including – in the Southeast Asian epicentre of drug

resistance along the Thai–Cambodian border – the artemisinin derivatives [40, 41]. If the efficacy of the artemisinin derivatives is lost, then effective control and elimination will not be possible with currently available tools [13]. Despite these concerns, the artemisinin-resistance phenotype has been poorly characterised, and the contribution of host factors remains to be defined. The key features of the artemisinin-resistant phenotype are prolonged parasite clearance times, despite apparently adequate drug exposure, and even dose escalation [42, 43]. Although molecular markers for artemisinin resistance remain elusive, a genetic basis for this clinical phenotype has been proposed recently based on its high heritability [44]. The recent declines in the clinical effectiveness of all antimalarial drugs, including the artemisinins, have prompted suggestions to revise the definitions of antimalarial drug resistance to include a category for extensively drug-resistant (XDR) malaria, as this approach has proved useful in tuberculosis for individual patient care and for public health [45].

Antimalarial resistance spreads when parasites are exposed to the selective window of drug concentrations that are sufficient to kill sensitive but not resistant parasites [7] (Fig. 2). Drugs with longer terminal elimination half-lives have the advantage of providing a longer post-treatment prophylactic effect, which appears to be important for their action in intermittent preventive therapy (IPT) in high-risk groups such as pregnant women, infants and young children. However, these long-acting antimalarials have the disadvantage of residual concentrations inhibiting sensitive parasites far longer than resistant parasites, thus fuelling the spread of resistance. The window of selection is prolonged with an increase in resistance or in the terminal elimination half-life (unless these terminal concentrations are too low even to kill sensitive parasites) (Fig. 2).

Antimalarial resistance spreads because gametocyte carriage and infectivity to mosquitoes is consistently higher in patients infected with drug-resistant compared with drug-sensitive parasites. An increase in gametocyte numbers has been identified as the first indication that an antimalarial is beginning to fail and emphasises the need for the treatment policy implemented to include drugs that will kill the sexual stages [12, 13]. Combining antimalarials with differing modes of action is expected to reduce the probability of a resistant (mutant) parasite surviving treatment [46]. Despite their mismatched elimination half-lives, ACTs are preferred to other combination therapies given their potential to reduce malaria transmission – due to their rapid clearance of asexual parasites together with their partial gametocidal activity [6]. The gametocyte-reducing effect of widespread use of artesunate plus mefloquine therapy has resulted not only in the sustained decrease in malaria transmission described above, but also in decreased mefloquine resistance in northwest Thailand, an area of low-intensity malaria transmission notorious for multi-drug resistance [18, 47]. By contrast, the first and only effort to date in Africa documenting the routine large-scale surveillance of temporal changes in resistance after successful implementation of an ACT treatment policy, found that systematic deployment of artesunate plus sulfadoxine–pyrimethamine had not delayed the spread of sulfadoxine–pyrimethamine resistance and may in fact have contributed to the rapid increase in the proportion of parasites carrying quintuple

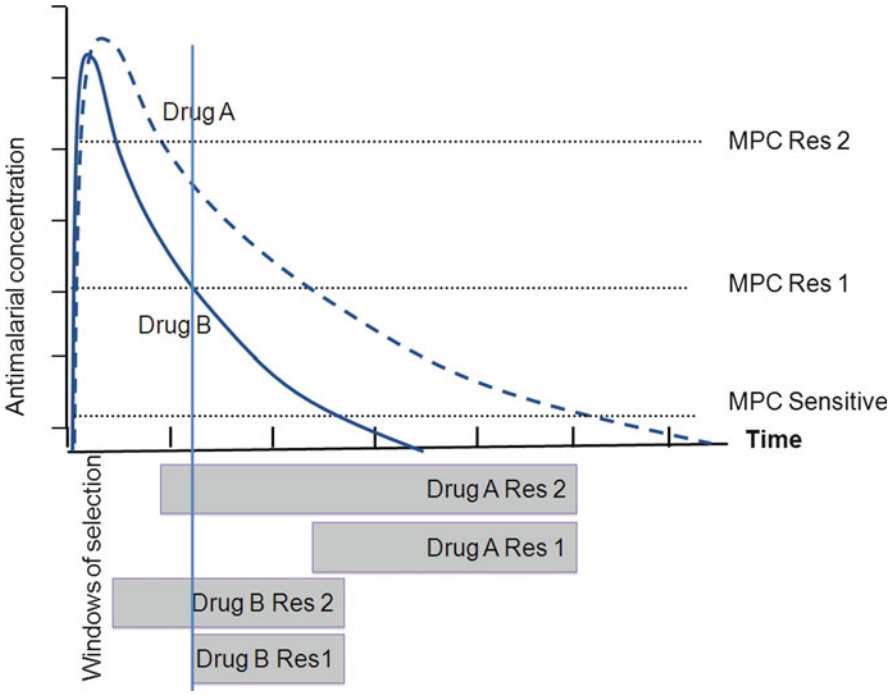


Fig. 2 Resistance selection by drugs with long elimination half-lives. The *curves* show antimalarial drug concentrations over time for Drug A (*dashed line*) and Drug B (*solid line*). The Window of Selection is the time when antimalarial concentrations are sufficient to clear sensitive but not resistant parasites. The three *dotted lines* show hypothetical minimum parasiticidal concentrations (MPCs) needed for clearing sensitive, partially resistant (Res 1) and highly resistant (Res 2) parasites. The duration of the window of selection increases with (a) increasing levels of resistance, so is longer for highly resistant than for partially resistant parasites, and (b) terminal elimination half-life, so is longer for Drug A than for Drug B

dihydrofolate reductase and *dihydropteroate synthetase* resistance markers from 11 to 75% over the 5-year study period [48]. Transmission of sulfadoxine–pyrimethamine resistance occurs intrinsically more readily than with mefloquine, probably since mefloquine resistance confers a survival disadvantage, while this does not appear to be the case with sulfadoxine–pyrimethamine.

A further challenge to limiting the rate of spread of ACT resistance is that expanding ACT access is necessary for reducing malaria morbidity and mortality. To be accessed promptly, ACTs need to be available near the home. With recent efforts to reduce the costs of ACTs dramatically, this is becoming achievable even in the poorest communities. However, such ready access creates creates the opportunity for widespread and indiscriminate use of antimalarials, which exerts a strong selective pressure towards resistant parasites towards high levels of resistance [42]. This could be addressed by limiting ACT use to those with a confirmed malaria diagnosis [6]. While 78 malaria-endemic countries (33 in Africa) have a policy that

patients of all ages with suspected malaria should receive a diagnostic test before treatment, this policy is only implemented in a minority of African cases – but is used in more than 80% of suspected cases outside Africa [23]. Other challenges to the effective targeting of ACTs are that only 38 countries (16 in Africa) are deploying rapid diagnostic tests at a community level and that ACTs continue to be used by those with negative malaria tests [23, 49].

Continued use of artemisinin-based monotherapy is considered a major factor in resistance to the artemisinins emerging and spreading, emphasising the importance of oral artemisinins being used only in combination with an effective longer acting antimalarial. This makes fixed dose artemisinin-based combinations highly preferable to loose tablets or blister-packed combinations [6]. To this end, the WHO recommends the withdrawal of all oral artemisinin-based monotherapies from the market [23, 42]. Others have argued that oral artesunate monotherapies are still needed, but should be reserved for use as a 7-day treatment course for patients with uncomplicated hyperparasitaemia and pregnant women in areas of multi-drug resistance [50].

De novo antimalarial drug resistance is most likely to occur in hyperparasitaemic patients who are non-immune, particularly if their antimalarial drug exposure is inadequate [51]. In hyperparasitaemic patients, parasite populations are larger and recrudescence rates following treatment are high [52]. Drug exposure can be inadequate due to sub-standard antimalarial quality, poor adherence, vomiting, unusual pharmacokinetic behaviour or underdosing [7]. Current antimalarial dosing recommendations are generally based on the lowest effective dose seen in dose-finding studies, which are conducted early in a drug's therapeutic life and thus before parasite resistance has become apparent [7]. Recommending the lowest effective dose, while justified in terms of cost and safety/tolerability, might not be the wisest choice as this is likely to select for resistant parasites. For example, mathematical modelling suggests that, if the recommended 25-mg/kg mefloquine dose had been deployed initially, instead of 15 mg/kg, then mefloquine resistance could have been delayed [53]. Furthermore, the relationship between the drug concentrations actually achieved with the recommended antimalarial dosage regimen and the therapeutic response needs to be reassessed once resistance starts to develop because, by definition, the minimum concentrations required to clear these resistant parasites has increased [7]. Current dose recommendations also almost invariably assume that the same weight-adjusted (milligram per kilogram) dose is effective for all key target population groups [7]. This approach encourages resistance selection, particularly in patients with high parasite burdens and low drug levels [51]. These are often young children or pregnant women who lack immunity and so generally have higher parasite densities, and whose larger apparent volumes of distribution and higher apparent clearance rates result in sub-optimal drug concentrations for many antimalarials [7, 51]. Despite extensive use for four decades, it has only been recognised recently that the currently recommended doses of both sulfadoxine–pyrimethamine and chloroquine achieve substantially lower plasma drug concentrations in young children than in older patients [54, 55]. Children given the recommended dosage regimens are similarly at increased risk of

inadequate exposure to both lumefantrine and piperaquine [56, 57]. Similarly, physiological changes in pregnancy result in decreased exposure to a number of key antimalarial drugs, including the artemisinins, sulfadoxine, lumefantrine and mefloquine [7]; no data on the pharmacokinetics of amodiaquine or piperaquine in pregnancy has been published yet.

To reduce the probability of resistance, quality-assured fixed-dose combination antimalarials should be used, treatment doses need to be optimised on the basis of pharmacokinetic assessments conducted within therapeutic efficacy studies in each key target population and patients with heavy parasite burdens have to be identified and receive sufficient treatment to prevent recrudescence [51].

5 *P. vivax*: A Particular Challenge to Malaria Elimination

The focus of malaria control programmes has, to date, been largely on *P. falciparum* because this is the major cause of severe malaria and malaria mortality, especially in sub-Saharan Africa. However, once elimination becomes the target, *P. vivax* needs to be given much more attention [58]. It has a more widespread distribution and infects 130–435 million people a year amongst a population at risk of approximately 2.85 billion, mostly in Central and Southeast Asia [33, 59]. *P. vivax* can undergo sporogeny in mosquitoes at lower temperatures than *P. falciparum* and forms a latent liver stage, the hypnozoite, which initiates relapses (Fig. 1) [60]. Gametocytes of *P. vivax* appear in the circulation at the same time as the asexual stages and, although killed by the antimalarial drugs that are effective against the asexual blood stages (unlike *P. falciparum*), *P. vivax* transmits well at very low parasite densities, so transmission can already have occurred before a patient has become symptomatic and sought treatment [13]. These factors, together with the low priority given by policy makers, funders and researchers to these infections that have been mislabelled "benign" [61], explain why *P. vivax* malaria is so widespread and is significantly more difficult to control or eliminate than falciparum malaria.

The asexual stages of *P. vivax* are increasingly resistant to chloroquine but remain highly sensitive to the artemisinins [6, 16]. Amodiaquine, mefloquine, piperaquine, lumefantrine, sulfadoxine-pyrimethamine and quinine are also effective in the treatment of chloroquine-resistant asexual blood stages of *P. vivax* [6, 16, 62–64]. ACTs are the preferred treatment in areas where falciparum malaria is also endemic or *P. vivax* is chloroquine resistant [6]. However, ACTs do not provide a radical cure.

Primaquine is the only radically curative drug for *P. vivax* (and *P. ovale*) malaria; it prevents relapse by clearing the hypnozoite stage when given as a 14-day course [65]. This prolonged treatment course compromises adherence and safety, with the main risk being haemolysis (as noted above). Supervision of this long course of therapy markedly reduces the risk of relapse, and almost all reports of primaquine resistant malaria are associated with lack of such supervision [66].

6 Progress Towards Malaria Control and Eventual Elimination

There has been substantial progress in reducing the burden of malaria globally over the last 60 years, with the number of countries that are malaria-free increasing from nine in 1945 to 108 today [58]. More than one-third of the 108 malaria-endemic countries documented reductions in malaria cases of >50% in 2009 compared with 2000, including 11 countries and one area in Africa and 32 countries in other regions [23]. These impressive results occurred in countries that achieved high coverage with their vector control and ACT treatment programmes. These successes have fuelled a wave of optimism that has led to renewed commitments to achieving the ambitious goal of progressively reducing the burden of malaria, leading eventually to global eradication[1], as outlined in the Roll Back Malaria Global Malaria Action Plan. This entails three components (a) effective malaria control[2] to reduce malaria morbidity in the majority of malaria-endemic countries by scaling-up and then sustaining appropriate vector and parasite control interventions, (b) progressive elimination[3] from the margins of malaria transmission, to "shrink the malaria map", and (c) research to bring forward better drugs, diagnostics, insecticides, vaccines and other tools, as well as inform policy and improve operational implementation of effective strategies [58, 67, 68]. Better drugs are needed for elimination-specific indications such as mass treatment, curing asymptomatic infections, curing relapsing liver stages of *P. vivax* and *P. ovale* and preventing transmission [69].

The ACT coverage rates (i.e. the proportion of parasitaemic patients that promptly receives an adequate dose and duration of ACT treatment) need to be high to impact on malaria transmission and the spread of resistance. One of the major deterrents to ensuring widespread access to ACTs is their cost – being tenfold more expensive than chloroquine and sulfadoxine–pyrimethamine monotherapies. The patients and governments that most need ACTs can least afford them [70]. Fortunately, international funding commitments for malaria have increased from around US$ 0.3 billion in 2003 to US$ 1.8 billion in 2010 [23], due to greater commitments by the US President's Malaria Initiative, the World Bank and primarily the emergence of the Global Fund and more recently, its innovative Affordable Medicines Facility for malaria (AMFm). This increased financial, technical and political support is resulting in dramatic scale-up of malaria control interventions in many settings and measurable reductions in malaria burden.

In general, the number of cases fell least in countries with the highest malaria incidence rates, with the notable exceptions of Zanzibar (United Republic of

[1] Malaria eradication is the permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species.

[2] Malaria control is reducing the disease burden to a level at which it is no longer a public health problem.

[3] Malaria elimination is interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e. zero incidence of locally contracted cases.

Tanzania), Zambia, Eritrea, Rwanda and Sao Tome and Principe, that illustrate that dramatic reductions in malaria morbidity and mortality can also be achieved in areas with a high malaria incidence [18–23, 71]. Similar results have also been seen in more limited geographic areas of the high malaria burden countries of Equatorial Guinea (Bioko Island), the Gambia, Kenya and Mozambique [72–75].

There is evidence from Bioko Island (Equatorial Guinea), Kenya, Sao Tome and Principe, Zanzibar and Zambia that large decreases in malaria cases and deaths have been mirrored by steep declines in all-cause deaths in children under 5 years of age [20, 71, 73, 74], suggesting that intensive malaria control in African countries could play an important role in not only achieving the Millennium Development Goal 6 of reducing malaria incidence and death rates, but also the Millennium Development Goal 4 of reducing all-cause childhood mortality by two-thirds by 2015 [76].

In 2009, however, there was evidence of an increase in malaria cases in three countries that had previously reported dramatic reductions in malaria burden (Rwanda, Sao Tome and Principe and Zambia) [23]. These resurgences highlight the fragility of malaria control and the critical importance of sustaining control interventions and surveillance rigorously – particularly in areas that have historically carried a high malaria burden.

At the other end of the malaria transmission intensity spectrum, tangible progress is being made. In 2010, both Morocco and Turkmenistan were certified as having achieved malaria elimination [23]. At least another 27 countries are working towards malaria elimination; nine countries have interrupted transmission and are in the phase of preventing re-introduction of malaria; ten countries are implementing nationwide elimination programmes and eight countries are in the pre-elimination phase [23]. In Botswana, Cape Verde, Namibia, Sao Tome and Principe, South Africa and Swaziland, large initial decreases in the number of malaria cases have been sustained but remain at 10–25% of those reported in 2000 [19, 22, 71]. However, the few remaining cases are proving more difficult to prevent, to detect and treat promptly, and additional interventions are likely to be necessary for further reductions in malaria morbidity to be achieved. Encouraging results of additive benefit are starting to be seen in studies evaluating the combination of indoor residual spraying with insecticide-treated bed-net deployment [77] and of adding primaquine to ACTs [17, 27].

Since the current levels of international financial support for malaria control fall far short of the estimated US$ 6 billion required annually to ensure maximal impact worldwide [56], it seems even less likely that international funding will be sustained for the long haul required to achieve the more expensive and ambitious yet possible goal of malaria eradication. As the risk of malaria decreases, the behaviour of patients, caregivers, healthcare providers and funders become less likely to take the steps needed to reduce the malaria burden further until it is eventually eliminated. Effective information, education and communication campaigns, strong programmes monitoring the impact of malaria control interventions on disease burden, good governance and coherent advocacy (that acknowledges the many demands on limited financial and especially human resources in malaria endemic

countries) are important tools for encouraging ongoing support, once there are only a few locally transmitted malaria cases.

The goals and strategies required to achieve elimination of the parasite from low-transmission settings are very different for those needed for reducing malaria morbidity and mortality in high-transmission settings. In an elimination programme, treatment of a sufficient number of infected subjects in a community to interrupt transmission becomes the primary goal. In order to interrupt transmission, the individuals who are parasitaemic (infected) and – more importantly in terms of elimination – gametocytaemic (infectious) need to be treated even if they are asymptomatic. Two possible approaches to this objective can be adopted – mass screening and treatment of both infected and infectious individuals (regardless of whether nor not they are symptomatic), or mass drug administration (MDA) given to as large a proportion of the population as possible on the grounds that this will cover a higher proportion of those infected. The lack of a rapid diagnostic method that is suitable for field use and sensitive enough for diagnosing the lower limit of parasite and gametocyte densities able to cause and transmit malaria is currently a major obstacle to using mass screening and treatment in malaria elimination.

MDA is the administration of a complete treatment course of antimalarial medicines to every individual in a geographically defined area on a specific day. MDA is not recommended by the World Health Organization, as there is no evidence of long-term benefits in large population groups [6]. An analysis of 19 MDA projects carried out over the period 1932–1999 found only one study in the small island population ($n = 718$) of Aneityum, Vanuatu, where MDA might have contributed to the elimination of *P. falciparum* and *P. vivax* malaria [78, 79]. MDA has been highly effective in reducing parasite prevalence to a very low level, but parasitaemia soon rebounds to its previous level once MDA is stopped, as seen in Garki, Nigeria and Nicaragua [78]. Mass treatment with ACTs alone is unlikely to be sufficient for malaria elimination – and primaquine and/or atovaquone–proguanil may be worth adding. In this context, drug safety should be given priority as drugs are given to a large number of people who are not infected. Thus, more evidence is needed on the risk:benefit profile of atovaquone–proguanil and primaquine to inform mass treatment approaches in the context of malaria elimination programmes [58]. Lessons should also be learnt from the lasting legacy of MDA of chloroquine and pyrimethamine: the rapid selection of resistant parasites.

7 Conclusions

Prompt effective antimalarial treatment is and will remain pivotal in achieving malaria control and eventually elimination. The past decade has seen remarkable progress being made in the fight against malaria. Almost all countries in which *P. falciparum* malaria is endemic have adopted ACT policies. High ACT coverage, together with the scaling-up of effective vector control interventions, has resulted in documented reductions in malaria cases of >50% in 2008 compared with 2000 in

43 of the 108 malaria-endemic countries. Unprecedented levels of financial, technical and political support have made this possible. During this window of opportunity for reducing the burden of malaria globally and possibly eventually eliminating malaria, attention now needs to be focussed on ensuring that countries select treatment policies that not only achieve cure rates >95% [65], but that are also likely to have a prolonged useful therapeutic life, reduce malaria transmission safely and effectively and, where applicable, are also active against *P. vivax*. As important is ensuring optimal targeting, dosing and adherence with these policies.

References

1. Hay C, Guerra A, Tatem A, Noor R, Snow R (2005) The global distribution and population at risk of malaria: past, present, and future. Lancet Infect Dis 4:327–336
2. Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IR, Brooker S, et al (2009) A world malaria map: *Plasmodium falciparum* endemicity in 2007. PLoS Med 6:e1000048. Erratum in: PLoS Med 6
3. Malaney P, Spielman A, Sachs J (2004) The malaria gap. Am J Trop Med Hyg 71:141–146
4. Ward SA, Sevene EJ, Hastings IM, Nosten F, McGready R (2007) Antimalarial drugs and pregnancy: safety, pharmacokinetics, and pharmacovigilance. Lancet Infect Dis 7:136–144
5. Sachs J, Malaney P (2002) The economic and social burden of malaria. Nature 415:680–685
6. World Health Organization (2010). Guidelines for the treatment of malaria. Second Edition. http://www.who.int/malaria/publications/atoz/9789241547925/en/index.html. Accessed 1 July 2010
7. Barnes KI, Watkins WM, White NJ (2008) Antimalarial dosing regimens and drug resistance. Trends Parasitol 24:127–134
8. Malenga G, Palmer A, Staedke S, Kazadi W, Mutabingwa T, Ansah E, Barnes KI, Whitty CJ (2005) Antimalarial treatment with artemisinin combination therapy in Africa. BMJ 331:706–707
9. Kantele A, Jokiranta TS (2011) Review of cases with the emerging fifth human malaria parasite, *Plasmodium knowlesi*. Clin Infect Dis 52:1356–1362
10. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GA (2006) The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. Trends Parasitol 22:424–430
11. Barnes KI, White NJ (2005) Population biology and antimalarial resistance: the transmission of antimalarial drug resistance in *Plasmodium falciparum*. Acta Trop 94:230–240
12. Barnes KI, Little F, Mabuza A, Mngomezulu N, Govere J, Durrheim D, Roper C, Watkins B, White NJ (2008) Increased gametocytemia after treatment: an early parasitological indicator of emerging sulfadoxine-pyrimethamine resistance in falciparum malaria. J Infect Dis 197:1605–1613
13. White NJ (2008) The role of anti-malarial drugs in eliminating malaria. Malar J 7:S8
14. Babiker HA, Schneider P, Reece SE (2009) Gametocytes: insights gained during a decade of molecular monitoring. Trends Parasitol 24:525–530
15. Butcher GA (1997) Antimalarial drugs and the mosquito transmission of Plasmodium. Int J Parasitol 27:975–987
16. Pukrittayakamee S, Chotivanich K, Chantra A, Clemens R, Looareesuwan S, White NJ (2004) Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria. Antimicrob Agents Chemother 48:1329–1334
17. Bousema T, Okell L, Shekalaghe S, Griffin JT, Omar S, Sawa P, Sutherland C, Sauerwein R, Ghani AC, Drakeley C (2010) Revisiting the circulation time of *Plasmodium falciparum*

gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar J 9:136
18. Nosten F, van Vugt M, Price R, Luxemburger C, Thway KL, Broackman A, McGready R, terKuile F, Looareesuwan S, White NJ (2000) Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand. Lancet 356:297–302
19. Barnes KI, Durrheim DN, Little F, Jackson A, Mehta U, Allen E, Dlamini SS, Tsoka J, Bredenkamp B, Mthembu DJ et al (2005) Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa. PLoS Med 2:e330
20. Bhattarai A, Ali AS, Kachur SP, Mårtensson A, Abbas AK, Khatib R, Al-Mafazy AW, Ramsan M, Rotllant G, Gerstenmaier JF et al (2007) Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. PLoS Med 4:e309
21. Otten M, Aregawi M, Were W, Karema C, Medin A, Bekele W, Jima D, Gausi K, Komatsu R, Korenromp E et al (2009) Initial evidence of reduction of malaria cases and deaths in Rwanda and Ethiopia due to rapid scale-up of malaria prevention and treatment. Malar J 8:14
22. Barnes KI, Chanda P, Ab Barnabas G (2009) Impact of the large-scale deployment of artemether/lumefantrine on the malaria disease burden in Africa: case studies of South Africa, Zambia and Ethiopia. Malar J 8:S8
23. World Health Organization (2010). World Malaria Report. http://www.who.int/malaria/world_malaria_report_2010/en/index.html. Accessed 12 May 2011
24. Targett G, Drakeley C, Jawara M, von Seidlein L, Coleman R, Deen J, Pinder M, Doherty T, Sutherland C, Walraven G et al (2001) Artesunate reduces but does not prevent post treatment transmission of *Plasmodium falciparum* to *Anopheles gambiae*. J Infect Dis 183:1254–1259
25. Bousema JT, Schneider P, Gouagna LC, Drakeley CJ, Tostmann A, Houben R, Githure JI, Ord R, Sutherland CJ, Omar SA et al (2006) Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. J Infect Dis 193:1151–1159
26. Okell LC, Drakeley CJ, Ghani AC, Bousema T, Sutherland CJ (2008) Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. Malar J 7:125
27. Shekalaghe S, Drakeley C, Gosling R, Ndaro A, van Meegeren M, Enevold A, Alifrangis M, Mosha F, Sauerwein R, Bousema T (2007) Primaquine clears submicroscopic *Plasmodium falciparum* gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. PLoS One 2:e1023
28. Beutler E, Duparc S; G6PD Deficiency Working Group (2007) Glucose-6-phosphate dehydrogenase deficiency and antimalarial drug development. Am J Trop Med Hyg 77:779–789
29. Leslie T, Briceño M, Mayan I, Mohammed N, Klinkenberg E, Sibley CH, Whitty CJ, Rowland M (2010) The impact of phenotypic and genotypic G6PD deficiency on risk of *Plasmodium vivax* infection: A case-control study amongst Afghan refugees in pakistan. PLoS Med 7: e1000283
30. Coleman MD, Coleman NA (1996) Drug-induced methaemoglobinaemia: treatment issues. Drug Saf 14:394–405
31. Sin DD, Shafran SD (1996) Dapsone- and primaquine-induced methemoglobinemia in HIV-infected individuals. J Acquir Immune Defic Syndr Hum Retrovirol 12:477–481
32. Shekalaghe SA, terBraak R, Daou M, Kavishe R, van den Bijllaardt W, van den Bosch S, Koenderink JB, Luty AJ, Whitty CJ, Drakeley C et al (2010) In Tanzania, hemolysis after a single dose of primaquine coadministered with an artemisinin is not restricted to glucose-6-phosphate dehydrogenase-deficient (G6PD A-) individuals. Antimicrob Agents Chemother 54:1762–1768
33. Baird JK (2007) Neglect of *Plasmodium vivax* malaria. Trends Parasitol 23:533–539
34. Attaran A, Barnes KI, Curtis C, d'Alessandro U, Fanello CI, Galinski MR, Kokwaro G, Looareesuwan S, Makanga M, Mutabingwa T et al (2004) WHO, the Global Fund, and medical malpractice in malaria treatment. Lancet 363:237–240

35. Trape JF (2001) The public health impact of chloroquine resistance in Africa. Am J Trop Med Hyg 64:12–17
36. Trape JF, Pison G, Spiegel A, Enel C, Rogier C (2002) Combating malaria in Africa. Trends Parasitol 18:224–230
37. Zucker JR, Ruebush TK II, Obonyo C, Otieno J, Campbell CC (2003) The mortality consequences of the continued use of chloroquine in Africa: experience in Siaya, Western Kenya. Am J Trop Med Hyg 68:386–389
38. Price R, Nosten F, Simpson JA, Luxemburger C, Phaipun L, ter Kuile F, van Vugt M, Chongsuphajaisiddhi T, White NJ (1999) Risk factors for gametocyte carriage in uncomplicated falciparum malaria. Am J Trop Med Hyg 60:1019–1023
39. Price RN, Simpson JA, Nosten F, Luxemburger C, Hkirjaroen L, ter Kuile F, Chongsuphajaisiddhi T, White NJ (2001) Factors contributing to anaemia in uncomplicated falciparum malaria. Am J Trop Med Hyg 65:614–622
40. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–67. Erratum in. N Engl J Med 361:1714
41. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM; Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620
42. WHO (2010) Global Report on Antimalarial Drug Efficacy and DrugResistance: 2000–2010. WHO, Geneva. http://whqlibdoc.who.int/publications/2010/9789241500470_eng.pd. Accessed 12 May 2011
43. Bethell D, Se Y, Lon C, Tyner S, Saunders D, Sriwichai S, Darapiseth S, Teja-Isavadharm P, Khemawoot P, Schaecher K et al (2011) Artesunate dose escalation for the treatment of uncomplicated malaria in a region of reported artemisinin resistance: a randomized clinical trial. PLoS One 6:e19283
44. Anderson TJ, Nair S, Nkhoma S, Williams JT, Imwong M, Yi P, Socheat D, Das D, Chotivanich K, Day NP, White NJ, Dondorp AM (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in Cambodia. J Infect Dis 201:1326–1330
45. Wongsrichanalai C, Varma JK, Juliano JJ, Kimerling ME, MacArthur JR (2010) Extensive drug resistance in malaria and tuberculosis. Emerg Infect Dis 16:1063–1067
46. White NJ (2004) Antimalarial drug resistance. J Clin Invest 113:1084–1092
47. Brockman A, Price RN, van Vugt M, Heppner DG, Walsh D, Sookto P, Wimonwattrawatee T, Looareesuwan S, White NJ, Nosten F (2000) *Plasmodium falciparum* antimalarial drug susceptibility on the north-western border of Thailand during five years of extensive use of artesunate-mefloquine. Trans R Soc Trop Med Hyg 94:537–544
48. Raman J, Little F, Roper C, Kleinschmidt I, Cassam Y, Maharaj R, Barnes KI (2010) Five years of large-scale *dhfr* and *dhps* mutation surveillance following the phased implementation of artesunate plus sulfadoxine-pyrimethamine in Maputo Province, Southern Mozambique. Am J Trop Med Hyg 82:788–794
49. Lubell Y, Reyburn H, Mbakilwa H, Mwangi R, Chonya S, Whitty CJ, Mills A (2008) The impact of response to the results of diagnostic tests for malaria: cost-benefit analysis. BMJ 336:202–205
50. Nosten F, Ashley E, McGready R, Price R (2006) We still need artesunate monotherapy. BMJ 333:45
51. White NJ, Pongtavornpinyo W, Maude RJ, Saralamba S, Aguas R, Stepniewska K, Lee SJ, Dondorp AM, White LJ, Day NP (2009) Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. Malar J 8:253
52. Luxemburger C, Nosten F, Raimond SD, Chongsuphajaisiddhi T, White NJ (1995) Oral artesunate in the treatment of uncomplicated hyperparasitemic falciparum malaria. Am J Trop Med Hyg 53:522–525

53. Simpson JA, Watkins ER, Price RN, Aarons L, Kyle DE, White NJ (2000) Mefloquine pharmacokinetic-pharmacodynamic models: implications for dosing and resistance. Antimicrob Agents Chemother 44:3414–3424
54. Barnes KI, Little F, Smith PJ, Evans A, Watkins WM, White NJ (2006) Sulfadoxine-pyrimethamine pharmacokinetics in malaria: paediatric dosing implications. Clin Pharmacol Ther 80:582–596
55. Ringwald P, Keundjian A, Same Ekobo A, Basco LK (2000) Chemoresistance of *P. falciparum* in urban areas of Yaounde, Cameroon. Part 1: surveillance of in vitro and in vivo resistance of Plasmodium falciparum to chloroquine from 1994 to 1999 in Yaounde, Cameroon. Trop Med Int Health 5:612–619
56. Checchi F, Piola P, Fogg C, Bajunirwe F, Biraro S, Grandesso F, Ruzagira E, Babigumira J, Kigozi I, Kiguli J et al (2006) Supervised versus unsupervised antimalarial treatment with six-dose artemether-lumefantrine: pharmacokinetic and dosage-related findings from a clinical trial in Uganda. Malar J 5:59
57. Price RN, Hasugian AR, Ratcliff A, Siswantoro H, Purba HL, Kenangalem E, Lindegardh N, Penttinen P, Laihad F, Ebsworth EP et al (2007) Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug resistant malaria. Antimicrob Agents Chemother 51:4090–4097
58. Feachem RGA, The Malaria Elimination Group (2009) Shrinking the malaria map: a guide on malaria elimination for policy makers. The Global Health Group, Global Health Sciences, University of California, San Francisco. http://www.malariaeliminationgroup.org/publications. Accessed 1 July 2010
59. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, Kabaria CW, Tatem AJ, Manh BH, Elyazar IR et al (2010) The international limits and population at risk of *Plasmodium vivax* transmission in 2009. PLoS Negl Trop Dis 4:e774
60. Krotoski WA (1985) Discovery of the hypnozoite and a new theory of malarial relapse. Trans R Soc Trop Med Hyg 79:1–11
61. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM (2007) Vivax malaria: neglected and not benign. Am J Trop Med Hyg 77:79–87
62. Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung RM, Laihad F, Ebsworth EP, Anstey NM, Tjitra E, Price RN (2007) Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: an open-label randomized comparison. Lancet 369:757–765
63. Pukrittayakamee S, Chantra A, Simpson JA, Vanijanonta S, Clemens R, Looareesuwan S, White NJ (2000) Therapeutic responses to different antimalarial drugs in vivax malaria. Antimicrob Agents Chemother 44:1680–1685
64. Arias AE, Corredor A (1989) Low response of Colombian strains of *Plasmodium vivax* to classical antimalarial therapy. Trop Med Parasitol 40:21–23
65. Galappaththy GNL, Omari AAA, Tharyan P (2007) Primaquine for preventing relapses in people with *Plasmodium vivax* malaria. Cochrane Database Syst Rev 2007, Issue 1:CD004389. doi: 10.1002/14651858.CD004389.pub2
66. Baird JK, Hoffman SL (2004) Primaquine therapy for malaria. Clin Infect Dis 39:1336–1345
67. Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH (2009) From malaria control to eradication: the WHO perspective. Trop Med Int Health 14:802–809
68. Roll Back Malaria (2008) The Global Malaria Action Plan for a malaria free world. http://www.rollbackmalaria.org/gmap/toc.pdf. Accessed 4 July 2010
69. The malERA Consultative Group on Drugs (2011) A research agenda for malaria eradication: drugs. PLoS Med 8:e1000402
70. Arrow KJ, Panosian C, and Gelband H (Eds), Institute of Medicine of the National Academies Committee on the Economics of Antimalarial Drugs. Saving lives, buying time: economics of malaria drugs in an age of resistance. The National Academies Press, Washington. http://www.nap.edu/openbook.php?isbn=0309092183. Accessed 17 May 2011

71. World Health Organization (2009) World Malaria Report. http://www.who.int/malaria/world_malaria_report_2009/en/index.html. Accessed 17 Apr 2011
72. Ceesay SJ, Casals-Pascual C, Erskine J, Anya SE, Duah NO, Fulford AJ, Sesay SS, Abubakar I, Dunyo S, Sey O et al (2008) Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. Lancet 372:1545–1554
73. Kleinschmidt I, Schwabe C, Benavente L, Torrez M, Ridl FC, Segura JL, Ehmer P, Nchama GN (2009) Marked increase in child survival after four years of intensive malaria control. Am J Trop Med Hyg 80:882–888
74. O'Meara WP, Bejon P, Mwangi TW, Okiro EA, Peshu N, Snow RW, Newton CR, Marsh K (2008) Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. Lancet 372:1555–1562
75. Sharp BL, Kleinschmidt I, Streat E, Maharaj R, Barnes KI, Durrheim DN, Ridl FC, Morris N, Seocharan I, Kunene S et al (2007) Seven years of regional malaria control collaboration–Mozambique, South Africa, and Swaziland. Am J Trop Med Hyg 76:42–47
76. United Nations Development Programme. Millennium Development Goals. http://www.undp.org/mdg/basics.shtml. Accessed 11 July 2010
77. Kleinschmidt I, Schwabe C, Shiva M, Segura JL, Sima V, Mabunda SJ, Coleman M (2009) Combining indoor residual spraying and insecticide-treated net interventions. Am J Trop Med Hyg 81:519–524
78. von Seidlein L, Greenwood BM (2003) Mass administration of antimalarial drugs. Trends Parasitol 19:790–796
79. Kaneko A, Taleo G, Kalkoa M, Yamar S, Kobayakawa T, Björkman A (2000) Malaria eradication on islands. Lancet 356:1560–1564

4-Aminoquinolines: Chloroquine, Amodiaquine and Next-Generation Analogues

Paul M. O'Neill, Victoria E. Barton, Stephen A. Ward, and James Chadwick

Abstract For several decades, the 4-aminoquinolines chloroquine (CQ) and amodiaquine (AQ) were considered the most important drugs for the control and eradication of malaria. The success of this class has been based on excellent clinical efficacy, limited host toxicity, ease of use and simple, cost-effective synthesis. Importantly, chloroquine therapy is affordable enough for use in the developing world. However, its value has seriously diminished since the emergence of widespread parasite resistance in every region where *P. falciparum* is prevalent. Recent medicinal chemistry campaigns have resulted in the development of short-chain chloroquine analogues (AQ-13), organometallic antimalarials (ferroquine) and the "fusion" antimalarial trioxaquine (SAR116242). Projects to reduce the toxicity of AQ have resulted in the development of metabolically stable AQ analogues (isoquine/*N-tert*-butyl isoquine). In addition to these developments, older 4-aminoquinolines such as piperaquine and the related aza-acridine derivative pyronaridine continue to be developed. It is the aim of this chapter to review 4-aminoquinoline structure–activity relationships and medicinal chemistry developments in the field and consider the future therapeutic value of CQ and AQ.

P.M. O'Neill (✉)
Department of Chemistry, Robert Robinson Laboratories, University of Liverpool, Liverpool L69 7ZD, UK

Department of Pharmacology, MRC Centre for Drug Safety Science, University of Liverpool, Liverpool L69 3GE, UK
e-mail: pmoneill@liverpool.ac.uk

V.E. Barton • J. Chadwick
Department of Chemistry, Robert Robinson Laboratories, University of Liverpool, Liverpool L69 7ZD, UK

S.A. Ward
Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_2,

1 History and Development

Quinine **1**, a member of the *cinchona* alkaloid family, is one of the oldest antimalarial agents and was first extracted from *cinchona* tree bark in the late 1600s. The *cinchona* species is native to the Andean region of South America, but when its therapeutic potential was realised, Dutch and British colonialists quickly established plantations in their south-east Asian colonies. These plantations were lost to the Japanese during World War II, stimulating research for synthetic analogues based on the quinine template, such as the 4-aminoquinoline chloroquine (CQ **2**, Fig. 1) [1].

A thorough historical review of CQ (in honour of chloroquine's 75th birthday) is available elsewhere [2]. In short, CQ was first synthesized in 1934 and became the most widely used antimalarial drug by the 1940s [3]. The success of this class has been based on excellent clinical efficacy, limited host toxicity, ease of use and simple, cost-effective synthesis. Importantly, CQ treatment has always been affordable – as little as USD 0.10 in Africa [4]. However, the value of quinoline-based antimalarials has been seriously eroded in recent years, mainly as a result of the development and spread of parasite resistance [5].

Although much of the current research effort is directed towards the identification of novel chemotherapeutic targets, we still do not fully understand the mode of action and the complete mechanism of resistance to the quinoline compounds, knowledge that would greatly assist the design of novel, potent and inexpensive alternative quinoline antimalarials. The search for novel quinoline-based antimalarials with pharmacological benefits superseding those provided by CQ has continued throughout the later part of the twentieth century and the early part of this century since the emergence of CQ resistance.

Comprehensive reviews on the pharmacology [6] and structure activity relationships [7] have been published previously, so will be only mentioned briefly. It is the aim of this chapter to review developments in the field that have led to the next-generation 4-aminoquinolines in the development "pipeline", in addition to discussion of the future therapeutic value of CQ and amodiaquine (AQ). We will begin with studies directed towards an understanding of the molecular mechanism of action of this important class of drug.

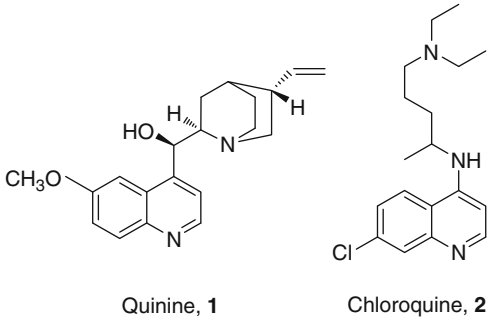


Fig. 1 Quinine **1** and related 4-aminoquinoline antimalarial chloroquine, **2**

2 Mode of Action of Quinoline Antimalarials

The precise modes of action of the quinoline antimalarials are still not completely understood, although various mechanisms have been proposed for the action of CQ and related compounds [8]. Some of the proposed mechanisms would require higher drug concentrations than those that can be achieved in vivo and, therefore, are not considered as convincing as other arguments [9]. Such mechanisms include the inhibition of protein synthesis [10], the inhibition of food vacuole phospholipases [11], the inhibition of aspartic proteinases [12] and the effects on DNA and RNA synthesis [13, 14].

CQ is active against the erythrocytic stages of malaria parasites but not against pre-erythrocytic or hypnozoite-stage parasites in the liver [15] or mature gametocytes. Since CQ acts exclusively against those stages of the intra-erythrocytic cycle during which the parasite is actively degrading haemoglobin, it was assumed that CQ somehow interferes with the parasite-feeding process. Although this is still a matter of some controversy, evidence of proposed mechanisms will be discussed in the following sections.

2.1 Haem–CQ Drug Complexes

To obtain essential amino acids for its growth and division, the parasite degrades haemoglobin within the host red blood cell. Digestion of its food source occurs in an acidic compartment known as the digestive vacuole (DV) (a lysosome-type structure, approximately pH 5). During feeding, the parasite generates the toxic and soluble molecule haem [ferriprotoporphyrin IX, FP Fe (II)] and biocrystallises it at, or within, the surface of lipids to form the major detoxification product haemozoin (Fig. 2) [16].

Slater et al. [17] demonstrated the ability of CQ to inhibit the in vitro FP detoxification in the high micro-molar range. The ability of CQ and a number of other quinoline antimalarial drugs to inhibit both spontaneous FP crystallisation and parasite extract catalysed crystallisation of FP has since been confirmed [18, 19].

Considerable evidence has been presented in recent years that antimalarial drugs such as CQ act by forming complexes with haem (FP Fe (II)) and/or the hydroxo- or aqua complex of haematin (ferriprotoporphyrin IX, Fe (III) FP), derived from parasite proteolysis of host haemoglobin [20–22] (Fig. 2), although the exact nature of these complexes is a matter of debate.

Dorn et al. [23, 24] confirmed that CQ forms a complex with the μ-oxo dimeric form of FP (haematin) with a stoichiometry of 1 CQ: 2 μ-oxo dimers. In other studies, CQ was found to bind to monomeric haem to form a highly toxic haem–CQ complex, which incorporates into the growing dimer chains and terminates the chain extension, blocking further sequestration of toxic haem and disrupting membrane function (Fig. 2) [25, 26].

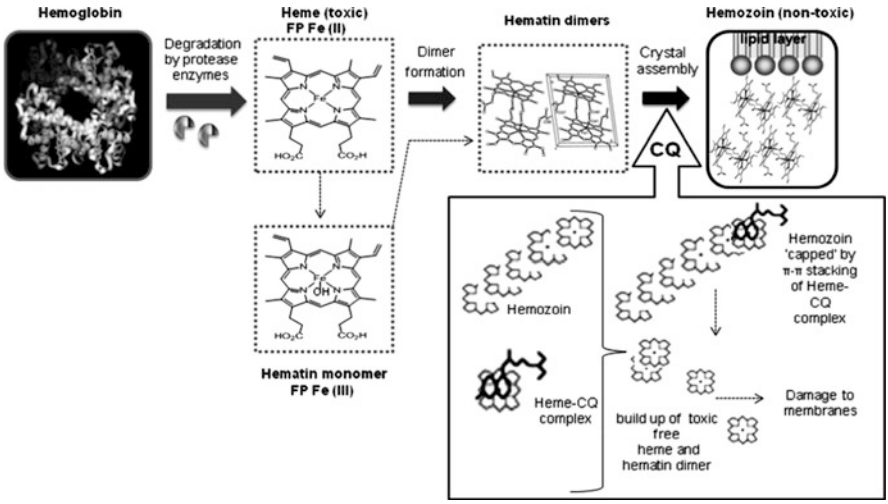


Fig. 2 Degradation of haemoglobin and detoxification mechanisms of the parasite and proposed target of CQ

2.2 Accumulation of CQ in the Acidic Food Vacuole

Due to the weak base properties of CQ and related analogues, their effectiveness has also been shown to be partly dependent upon drug accumulation in the acidic DV. A number of early studies have suggested that CQ accumulation can be explained by an ion-trapping or weak-base mechanism [27, 28]. CQ is a diprotic weak base ($pK_{a1} = 8.1$, $pK_{a2} = 10.2$) and in its unprotonated form, it diffuses through the membranes of the parasitised erythrocyte and accumulates in the acidic DV (pH 5–5.2) [27]. Once inside, the drug becomes protonated and, as a consequence, membrane impermeable and becomes trapped in the acidic compartment of the parasite (Fig. 3).

Various studies have suggested that the kinetics and saturability of CQ uptake are best explained by the involvement of a specific transporter [29, 30] or carrier-mediated mechanism for the uptake of CQ [31]. Another hypothesis by Chou et al. [32] suggests that free haematin (FP) in the DV might act as an intra-vacuolar receptor for CQ. Work by Bray et al. also strongly supports this hypothesis [33].

3 CQ Resistance Development

The first incidences of resistance to CQ were reported in 1957. The reasons for the emergence of resistance are multi-factorial: uncontrolled long-term treatment regimes, travel activity resulting in spread of resistant strains and frequent feeding of mosquitoes from several different hosts, to name but a few [34]. The mechanism by which resistance is acquired is discussed below.

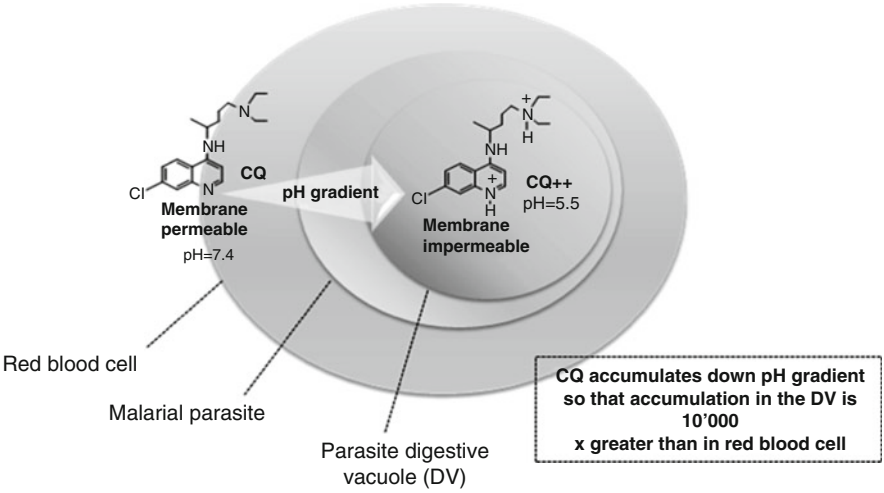


Fig. 3 Ion trapping; diffusion of CQ due to the pH gradient leads to increased concentration of CQ in the DV

3.1 Parasite-Resistance Mechanisms

It was soon proven that the concentration of CQ inside the DV was reduced in parasite-resistant strains. The powerful accumulation mechanism of CQ was therefore less effective, suggesting mutations in transporter proteins in these resistant strains. Resistant isolates also have reduced apparent affinity of CQ–FP binding in the DV, therefore CQ-resistant isolates have evolved a mechanism whereby the access of CQ to FP is reduced [35].

3.1.1 PfCRT

Another characteristic of CQ-resistant isolates is that their phenotype can be partially "reversed" by the calcium channel blocker verapamil so that the isolates become resensitised to CQ [35]. Verapamil was shown to act by increasing the access of CQ to the FP receptor and this effect is considered a phenotypic marker of CQ resistance. The characteristic effects of CQ resistance (reduced CQ sensitivity, reduced CQ uptake and the verapamil effect) have all been attributed to specific amino acid changes in an integral DV membrane protein, the *P. falciparum* chloroquine resistance transporter (PfCRT) [36, 37]. PfCRT mutated at amino acid 76 appears to be central to the chloroquine resistance phenotype. Mutant PfCRT seems to allow movement of drugs out of the DV; therefore blocking of PfCRT by verapamil restores sensitivity.

In brief, there are three proposed models for the resistance mechanism of PfCRT:

- *The partitioning model*: CQ was found to flow out of the DV of CQ-resistant strains much faster that CQ-sensitive strains, by a verapamil-blockable route [38]. Initially, this was attributed to changes in DV pH for CQ-sensitive and CQ-resistant strains. However, it was later shown that CQ-resistant parasites have a similar resting DV pH, and, therefore, must possess a CQ efflux mechanism in the DV membrane, increasing the permeability of a particular form of CQ [39].
- *The channel model*: In this model, mutated PfCRT acts as a channel, providing a leak pathway for the passive diffusion of protonated CQ, allowing it to flow freely from the DV [40, 41].
- *The carrier model*: In this alternate model, mutated PfCRT acts as a carrier, transporting protonated CQ by facilitated diffusion or active transport across the DV membrane [42, 43].

The issue of exactly how PfCRT confers this phenotype has been recently reviewed, although it remains a matter of debate [44].

3.1.2 PfMDR1

A multi-drug resistance homologue in *P. falciparum* (PfMDR1) has also been implicated in CQ resistance. PfMDR1 has been demonstrated to reside in the parasites' DV membrane with its ATP-binding domain facing the cytoplasm [45]. This suggests that PfMDR1 directs drug movement into the DV. Loss of this drug import capability could be advantageous to the parasite when the drug targets the DV. Irrespective of the specifics of MDR1-mediated chloroquine transport, the protein has been shown to contribute to chloroquine resistance. Sanchez et al. functionally expressed a number of different polymorphs of *pfmdr1* (the gene that codes for PfMDR1) in *Xenopus laevis* oocytes in order to characterize the transport properties of PfMDR1 and its interaction with antimalarial drugs. They demonstrated that PfMDR1 does indeed transport CQ and that polymorphisms within PfMDR1 affect the substrate specificity; wild-type PfMDR1 transports CQ, whereas polymorphic PfMDR1 variants from parasite lines associated with resistance apparently are not as efficient [46].

3.2 Recycling of CQ

CQ still remains the treatment of choice in a few geographical areas where it can still be relied upon, although guidelines now instruct the use of combination chemotherapy to slow the development of resistance to the partner drug [47]. In some resistance "hot spots", CQ was completely abandoned for a combination of sulfadoxine–pyrimethamine almost two decades ago. In such cases, there is evidence to suggest that CQ sensitivity can be restored [48]; 8 years after discontinuation of CQ in Malawi, the *pfcrt* T76 mutation [49] had disappeared from nearly

every isolate analysed. Similar observations have been made in Tanzania, South Africa, China and parts of Thailand [50]. These results have given some hope that "drug-cycling" may be an option for the future and CQ combinations may be used effectively again in disease-endemic areas where it was once abandoned [2]. However, the concern with this strategy is that re-selection of resistance mutants is likely to be very rapid.

Ursing et al. have reported that the failure rate of CQ treatment can be decreased by giving the drug twice per day rather than as a once daily treatment regimen [51–53]. Doubling the dosing frequency in this way achieved a high cure rate despite underlying CQ resistance and without any adverse side effects [51]. This increase in efficacy can be explained by the pharmacokinetics of CQ; the second daily dose of CQ acting to raise plasma concentrations to levels where they have activity against resistant parasites [54]. It has also been shown that the use of this type of treatment regimen can stabilize the spread of CQ resistance [53, 55]. One major drawback with this type of double-dose treatment regimen is the narrow therapeutic index for CQ and, in order for such treatment to be widely used, extensive safety re-evaluation would need to be performed in large populations to ensure safety at the population level.

4 Modifications to Improve CQ

CQ, **2** contains a 7-chloroquinoline-substituted ring system with a flexible pentadiamino side chain. The haem-binding template, 7-chloro- and terminal amino group are all important for antimalarial activity, as detailed in Fig. 4.

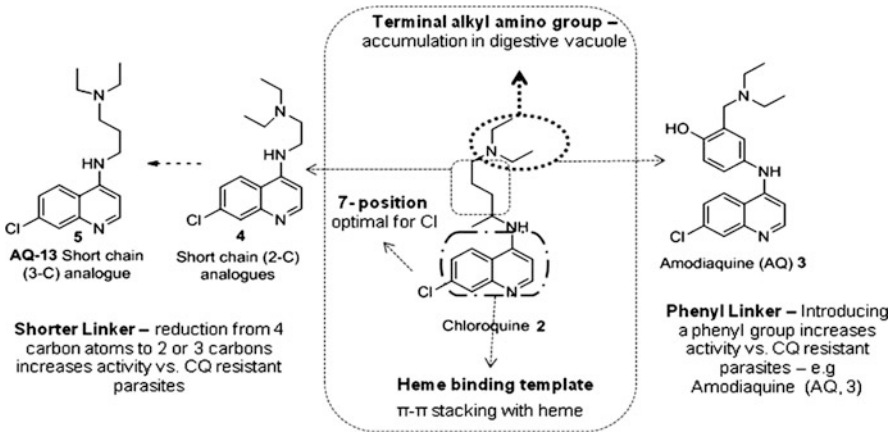


Fig. 4 Exploring the structure–activity relationship (SAR) of CQ: modifications shown led to the development of new analogues AQ (**3**), AQ-13 (**5**) and other short chain analogues (**4**) which have good activities against CQ-resistant strains

Since CQ's discovery, numerous attempts have been made to prepare a superior antimalarial quinolone-based drug. The following section briefly summarizes some of the more important recent advances in the field, with particular emphasis on 4-aminoquinolines that are in clinical and pre-clinical development. For a more in-depth discussion of 4-aminoquinoline analogue development over the last 10 years, Kaur et al. have recently published an extensive review [56].

4.1 Modifications to Overcome Resistance: Short-Chain Analogues

4.1.1 AQ-13

Studies on 4-aminoquinoline structure–activity relationships (SARs) have revealed that 2-carbon side-chain CQ analogues such as **4** retain activity against CQ-resistant *Plasmodium* parasites [57, 58]. Krogstad et al. have synthesized a series of analogues with varying diaminoalkane side chains at the 4-position [57]. Interestingly, compounds with diaminoalkyl side chains shorter than four carbon atoms or longer than seven carbon atoms were active against CQ-susceptible, CQ-resistant, and multi-drug-resistant strains of *P. falciparum* in vitro (IC_{50} values of 40–60 nM against the K1 multi-drug resistant strain) and exhibited no cross-resistance with CQ.

One of these analogues, AQ-13 **5**, a short-chain aminoquinoline antimalarial drug, underwent Phase I clinical trials. The mode of action is suggested to be the same as CQ but the presence of the short linker chain is believed to enable the molecule to circumvent the parasite-resistance mechanism (PfCRT), making **5** active against CQ-resistant parasites.

Preliminary pharmacokinetic studies indicate that AQ-13 has a similar profile to that of CQ [59] and the Phase I clinical trials were positive [60], concluding minimal difference in toxicity compared with CQ. However, since AQ-13 exhibited increased clearance compared with CQ, dose adjustment is required and an initial dose-finding Phase II (efficacy) study of AQ-13 in Mali is planned. Since clinical trials have shown that oral doses of 1,400 and 1,750 mg AQ-13 are as safe as equivalent oral doses of CQ and have similar pharmacokinetics, more recent trials were performed to determine if a 2,100 mg dose of AQ-13 (700 mg per day for 3 days) was safe to include as a third arm in Phase II studies in Mali and to investigate the effects of food (the standardised FDA fatty meal) on the bioavailability and pharmacokinetics of AQ-13. Based on the results, it is proposed to compare the 1,400, 1,700 and 2,100 mg doses of AQ-13 with each other and with Coartem in an initial dose-finding efficacy (Phase II) study of AQ-13 in Mali [61].

A possible drawback with these derivatives is the potential to undergo side-chain dealkylation (for short-chain CQ analogues such as **5** (AQ-13), deethylation is a particular problem in vivo) [62]. This metabolic transformation significantly

reduces the lipid solubility of the drug and significantly increases cross-resistance up to and beyond that seen with CQ [63].

4.1.2 Ferroquine: An Organometallic Antimalarial

Metal complexes have been used as drugs in a variety of diseases [64]. Incorporation of metal fragments into CQ has generally produced an enhancement of the efficacy of CQ with no acute toxicity. Three novel CQ complexes of transition metals (Rh, Ru, Au) have been synthesized (**6**, **7** and **8**, Fig. 5) [65, 66], with the Au–CQ complex **8** in particular, displaying high in vitro activity against the asexual blood-stage of two CQ-resistant *P. falciparum* strains.

Four new ferrocene-CQ analogues were developed by Biot and co-workers, where the carbon chain of CQ was replaced by the hydrophobic ferrocenyl group [67]. Some of the compounds showed potent antimalarial activity in vivo against *P. berghei* and were 22 times more potent against schizonts than CQ in vitro against a drug-resistant strain of *P. falciparum*. The same group reported two new ferrocene-CQ compounds in 1999, one of which (**9**) showed very promising antimalarial activity in vivo against *P. berghei* and in vitro against CQ-resistant strains of *P. falciparum* [68].

Now named ferroquine (SSR-97193, FQ), **9** is the first novel organometallic antimalarial drug candidate to enter clinical trials. A multi-factorial mechanism of action is proposed including the ability to target lipids, inhibit the formation of haemozoin and generate reactive oxygen species [69]. The ferrocene group alone does not have antimalarial activity but possibly utilises the parasites' affinity for iron to increase the probability of encountering the molecule [69, 70]. In addition to its activity against CQ-resistant *P. falciparum* isolates, FQ is also highly effective against drug-resistant *P. vivax* malaria [71]. A Phase II clinical trial in combination with artesunate is to be completed by October 2011 to assess activity in reducing parasitaemia and to explore the pharmacokinetics of ferroquine and its metabolites [72].

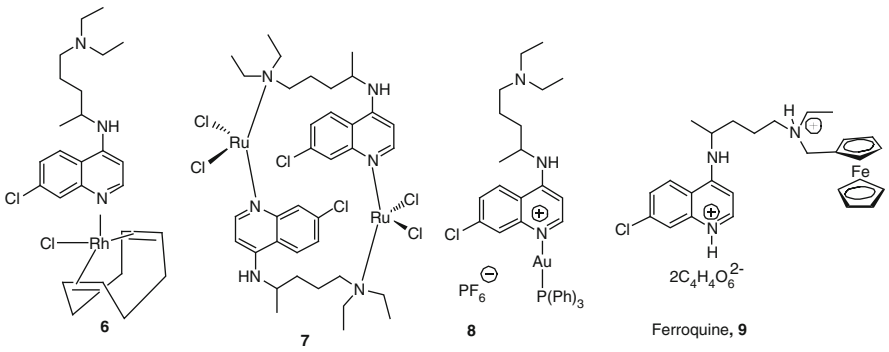


Fig. 5 Organometallic antimalarials

4.1.3 Piperaquine

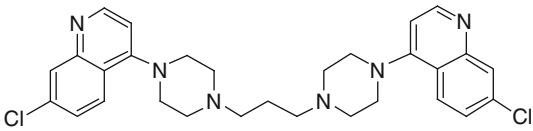
Other notable work in the chloroquine SAR field has involved the preparation of bisquinoline dimers, some of which possess excellent activity against CQ-resistant parasites. This activity against resistant parasites may be explained by their steric bulk, which prevents them from fitting into the binding site of PfCRT. Alternatively, the bisquinolines may be more efficiently trapped inside the DV because of their four positive charges.

Early examples of such agents include bis(quinolyl) piperazines such as piperaquine, **10** (Fig. 6). Piperaquine was first synthesized in the 1960s and used extensively in China for prophylaxis and treatment for the next 20 years. With the development of piperaquine-resistant strains of *P. falciparum* and the emergence of the artemisinin derivatives, its use declined during the 1980s [73].

During the next decade, piperaquine was rediscovered as one of a number of compounds suitable for combination with an artemisinin derivative. The pharmacokinetic properties of piperaquine have now been characterised [74], revealing that it is a highly lipid-soluble drug with a large volume of distribution at steady state, good bioavailability, long elimination half-life and a clearance rate that is markedly higher in children than in adults. The tolerability, efficacy, pharmacokinetic profile and low cost of piperaquine make it a promising partner drug for use as part of an artemisinin combination therapy (ACT).

Initial results were encouraging [73, 75], and Phase III clinical trials were completed in 2009 [76]. A recent report analysing individual patient data analysis of efficacy and tolerability in acute uncomplicated falciparum malaria, from seven published randomised clinical trials conducted in Africa and South East Asia concluded that dihydroartemisinin (DHA)-piperaquine is well tolerated, highly effective and safe [77]. Although not currently registered in the UK, a fixed combination called Duo-cotecxin is registered in China, Pakistan, Cambodia and Myanmar in addition to 18 African countries. Concerns with this combination lie in the fact that the calculated terminal half-life for piperaquine is around 16.5 days [78], compared with that of DHA (approximately 0.5 h) [79]; hence, the development of resistance could be a possibility due to prolonged exposure of piperaquine at sub-therapeutic levels effectively as a monotherapy.

A 1,2,4-trioxolane (RBx11160/Arterolane) has also been recently partnered with piperaquine and progressed to Phase III clinical trials. The clinical trials of RBx11160 alone identified its tendency to degrade relatively rapidly due to high levels of iron (II) in infected red blood cells, leading to a clinical efficacy of 60–70% [80]. The combination with a longer lasting drug such as piperaquine,



Piperaquine, **10**

Fig. 6 Structure of piperaquine **10**

with a completely different mechanism of action, may reduce the possibility of resistance and recrudescence [81]; recent results suggest the combination is highly active, with patients being free from recrudescence on day 28 after treatment [76]. This combination may also offer an advantage over DHA-piperaquine in the sense that the artemisinin-based component of the combination is a totally synthetic 1,2,4-trioxolane. This avoids over-reliance on the natural product artemisinin, whose cost and availability has been shown to fluctuate in recent years [82].

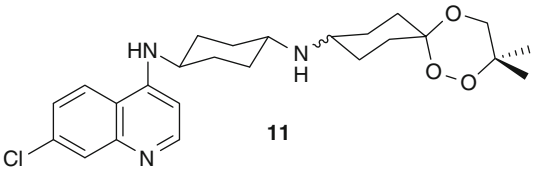
4.1.4 Trioxaquine SAR116242

Combination chemotherapy is now the mainstay of antimalarial treatment; each novel artemisinin-based antimalarial that reaches clinical trials is usually employed in an additional trial with an appropriate partner drug. However, a relatively novel approach is the concept of "covalent biotherapy" – a synthetic hybrid molecule containing two covalently linked pharmacophores [83]. The hybrid is designed to target the parasite by two distinct mechanisms thus circumventing resistance development. The hybrid also has several advantages over multi-component drugs such as:

- Expense – in principle, the risks and costs involved with a hybrid may not be any different when compared with those of a single entity.
- Safety – lower risk of drug–drug adverse interactions.
- Matched pharmacokinetics (i.e. a single entity)

A possible disadvantage, however, is that it is more difficult to adjust the ratio of activities at different targets [84]. Recent examples include trioxaquines developed by Meunier and co-workers, containing a 1,2,4-trioxane (as the artemisinin-based component) covalently bound to a 4-aminoquinoline [85]. These novel trioxaquines were found to be potent against CQ and pyrimethamine-resistant strains, and have improved antimalarial activity compared with the individual components. Several trioxaquines were developed over a number of years culminating in the selection of a drug-development candidate known as SAR116242, **11** (Fig. 7).

The superior antimalarial activity in both CQ-sensitive and CQ-resistant isolates ($IC_{50} = 10$ nM) has been attributed to its dual mechanism of haem alkylation and haemozoin inhibition. In addition, incorporation of a second cyclohexyl ring within the linker that joins the two pharmacophores increased the metabolic stability of this molecule compared with other trioxaquines containing a linear tether [86].



11

SAR116242

Fig. 7 Structure of SAR116242 **11**

The drug was synthesised as a mixture of diastereoisomers, but each diastereoisomer was found to be equipotent in their in vitro antiplasmodial activities and also displayed similar pharmacological profiles. However, it is not clear whether the pharmacokinetics and safety profiles of each individual diasteroisomer are the same. SAR 116242 is undergoing pre-clinical assessment by Sanofi-Aventis to determine its potential as the first "fusion" antimalarial.

4.1.5 Amodiaquine

Amodiaquine **3** (AQ), a phenyl substituted analogue of CQ, was first found to be effective against non-human malaria in 1946. Its mechanism of action is thought to be similar to CQ, but this is again a matter of some controversy [87].

Clinical use of AQ has been severely restricted because of associations with hepatotoxicity and agranulocytosis. Due to this toxicity, WHO withdrew recommendation for the drug as a monotherapy in the early 1990s. The AQ side chain contains a 4-aminophenol group; a structural alert for toxicity, because of metabolic oxidation to a quinoneimine (Fig. 8). Although cross-resistance of CQ and AQ has been documented for 20 years [88], AQ remains an important drug as it is effective against many CQ-resistant strains. Therefore, many drug design projects have since focussed on reducing this toxicity [87].

4.2 Modifications to Reduce Toxicity of AQ

4.2.1 Metabolism of CQ and AQ

CQ is highly lipophilic, as well as being a diacidic base. After oral administration, CQ is rapidly absorbed from the gastrointestinal tract, having a high bioavailability of between 80 and 90%. CQ undergoes *N*-deethylation to give the desethyl

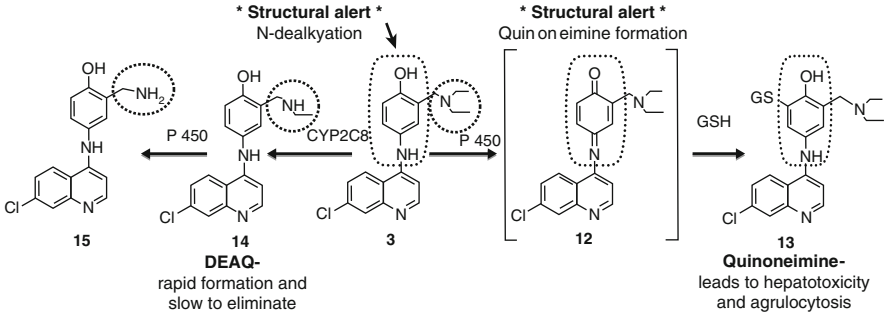


Fig. 8 Metabolism of AQ to toxic quinoneimine and DEAQ metabolites

compound as a major metabolite which has the same activity as CQ against sensitive strains, but reduced activity versus CQ-resistant strains [89].

Upon oral administration, AQ is rapidly absorbed and extensively metabolized. Although AQ has a high absorption rate from the gut due to a large first pass effect, AQ has a low bioavailability and is considered a pro-drug for desethylamodiaquine (DEAQ, **14**) [90]. In contrast to the metabolism of CQ, AQ also produces a toxic quinoneimine metabolite **12** (Fig. 8). The metabolites have been detected in vivo by the excretion of glutathione (GSH) conjugates (such as **13**) in experimental animals [91, 92]. It has been postulated that AQ toxicity involves immune-mediated mechanisms directed against the drug protein conjugates via in vivo bioactivation and covalent binding of the drug to proteins [93].

The main metabolite of AQ is DEAQ **14**, with other minor metabolites being 2-hydroxyl-DEAQ and *N*-bisdesethyl AQ (bis-DEAQ **15**) [94] (Fig. 8). The formation of DEAQ is rapid and its elimination very slow with a terminal half-life of over 100 h [95], as a result the mean plasma concentration of DEAQ is six- to sevenfold higher than the parent drug. Recent studies have established that the main P450 isoform catalysing the *N*-dealkylation of amodiaquine is CYP2C8 [96]. Mutations in PfCRT have been found in resistance isolates and correlate with high-level resistance to the AQ metabolite DEAQ in in vitro tests.

4.2.2 Modification of Metabolic Structural Alerts

Since AQ retains antimalarial activity against many CQ-resistant parasites, the next focus was to make a safer, cost-effective alternative. Initial studies involved the design and synthesis of fluoroamodiaquine (FAQ, **16**, Fig. 9) [97] since this analogue cannot form toxic metabolites by P450-mediated processes and retains substantial antimalarial activity versus CQ-resistant parasites. However, the resulting *N*-desethyl 4′-fluoro amodiaquine metabolite has significantly reduced activity against CQ-resistant parasites [97]. Concerns about cost led to the preparation of

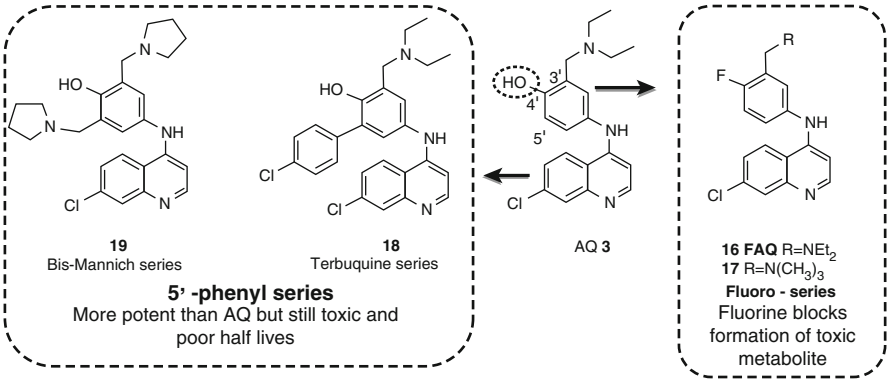


Fig. 9 Modification of structural alerts to reduce toxicity of AQ

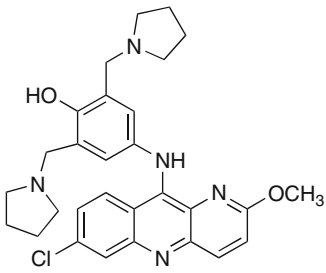
other synthetically accessible analogues; the tebuquine series [98] and the bis-Mannich series [99] (Fig. 9).

Tebuquine (**18**), a biaryl analogue of AQ discovered by Parke-Davis, is significantly more active than AQ and CQ both in vitro and in vivo and has potent antimalarial activity and reduced cross-resistance with CQ [100, 101]. Both the bis-Mannich and terbuquine series were expected to offer advantages over AQ in the sense that they contain Mannich side chains that are more resistant to cleavage to *N*-desalkyl metabolites. A potential drawback with the bis-Mannich class of antimalarial compounds was recognized by Tingle et al. [102]. They demonstrated that such compounds have long half-lives, raising concerns over potential drug toxicity and resistance development. Compounds in the tebuquine series have also been shown to have unacceptable toxicity profiles that is exacerbated by the long half-lives [102].

Pyronaridine

Pyronaridine **20** (Fig. 10) is another member of the class of Mannich-base schizontocides; however, the usual quinoline heterocycle is replaced by an azaacridine. Like AQ **2**, pyronaridine **20** retains the aminophenol substructure which can be oxidised to the respective quinoneimine. Since pyronaridine contains two Mannich-base side chains, it has been suggested that the second Mannich base moiety prevents the formation of the hazardous thiol addition products by sterically shielding the quinoneimine from the attack of the sulphur nucleophile [103].

Pyronaridine **20** was developed and used in China since the 1980s, but has not been registered in other countries. In a clinical study performed in Thailand, high recrudescence has been observed and in vitro assays revealed the presence of pyronaridine-resistant strains [104]. Another study in Africa showed high activity against CQ-resistant field isolates (IC_{50} values of 0.8–17.9 nM) [105]. Data suggest there may be some in vitro cross-resistance or at least cross-susceptibility between pyronaridine **20**, CQ **2** and AQ **3**. The combination of pyronaridine **20** and the artemisinin analogue artesunate (Pyramax) is in clinical development and began Phase III clinical trials in 2006. In terms of safety, pyronaridine-artesunate was well



Pyronaridine **20**

Fig. 10 Structure of pyronaridine **20**

tolerated in Phase II trials. However, a few patients exhibited raised liver enzymes, therefore the risk of toxicity to the liver still needs to be closely monitored [106]. Pyramax was submitted to the European Medicines Agency (EMA) for regulatory approval at the end of March 2010 [107].

Isoquine

An approach to circumvent the facile oxidation of AQ involves the interchange of the 3′-hydroxyl and the 4′-Mannich side-chain function of AQ. This provided a new series of analogues that avoid the formation of toxic quinoneimine metabolites via cytochrome P450-mediated metabolism (Fig. 11) [108].

While several analogues displayed potent antimalarial activity against both CQ-sensitive and resistant strains, isoquine **22** (ISQ), the direct isomer of AQ, displayed potent in vitro antimalarial activity in addition to excellent oral in vivo ED_{50} and ED_{90} activity of 1.6 and 3.7 mg/kg, respectively, against the *P. yoelii* NS strain (compared with 7.9 and 7.4 mg/kg for AQ) [109]. Subsequent metabolism studies in the rat model demonstrated that **22** does not undergo in vivo bioactivation, as evidenced by the lack of glutathione metabolites in the bile. Unfortunately, pre-clinical evaluation displayed unacceptably high first pass metabolism to dealkylated metabolites, which complicated the development and compromised activity against CQ-resistant strains [110].

Since the metabolic cleavage of the *N*-diethylamino-group was an issue, the more metabolically stable *N-tert*-butyl analogue was developed in the hope that this

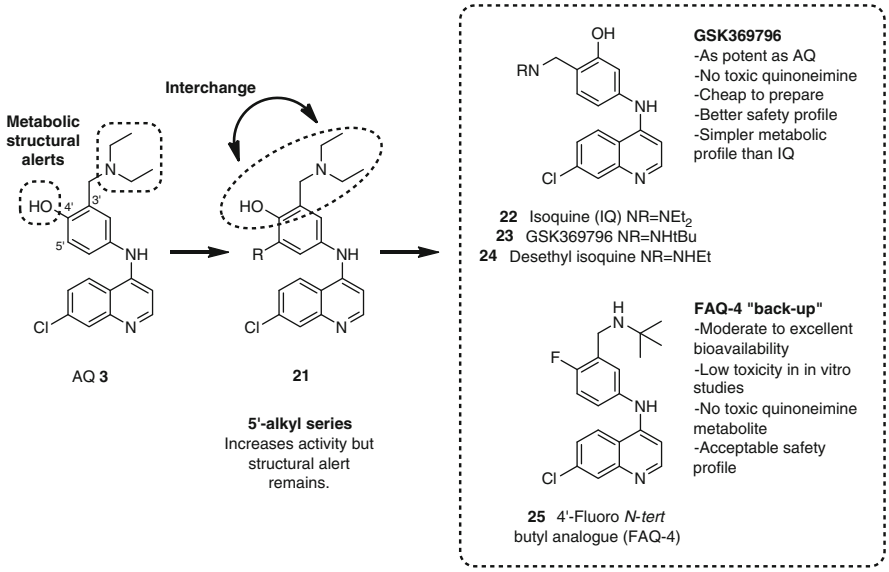


Fig. 11 Modifications of AQ to reduce toxicity of metabolic structural alerts

would lead to a much simpler metabolic profile and enhanced bioavailability. Development of the *N-tert*-butyl analogue **23** (GSK369796) followed (Fig. 11), which has superior pharmacokinetic and pharmacodynamic profiles to isoquine in pre-clinical evaluation studies performed by Glaxo SmithKline pharmaceuticals [110]. In spite of the excellent exposures and near quantitative oral bioavailabilities in animal models, development of **23** has been discontinued due to the inability to achieve exposures at doses considered to demonstrate superior drug safety compared with CQ.

4′-Fluoro-*N-tert*-butylamodiaquine FAQ-4 (**25**) was also identified as a "back-up" candidate for further development studies based on potent activity versus CQ-sensitive and resistant parasites, moderate to excellent oral bioavailability, low toxicity in in vitro studies, and an acceptable safety profile, and this molecule is undergoing formal pre-clinical evaluation [111].

5 The Future of CQ and AQ

5.1 CQ/AQ Next-Generation Candidates in Clinical Development

4-Aminoquinoline-based drug development projects continue to yield promising drug candidates and several molecules have entered into pre-clinical development or clinical trials over the last few years. Projects to reduce resistance development of CQ have resulted in the development of short-chain chloroquine analogues (AQ-13), organometallic antimalarials (ferroquine) and a "fusion" trioxaquine antimalarial (SAR116242). Projects to reduce the toxicity of AQ have resulted in the development of metabolically stable amodiaquine analogues (isoquine/*tert*-butyl isoquine) and aza-acridine derivatives (pyronaridine) (Table 1).

5.2 CQ/AQ Combinations: ACTs and Non-ACTs

The 4-aminoquinolines CQ and AQ have had a revival over the last 20 years due to the development of ACT. Artesunate-amodiaquine (Coarsucam) was approved for the WHO pre-qualification project in October 2008. It is expected to have a 25% share of the ACT market, with another ACT, Coartem (artemether/lumefantrine) taking the remaining 75% [76].

Methylene blue (MB), a specific inhibitor of *P. falciparum* glutathione reductase was the first synthetic antimalarial drug ever used in the early 1900s. Interest in its use as an antimalarial has recently been revived, due to its potential to reverse CQ resistance and its affordability [112]. It is thought that MB prevents the crystallisation of haem to haemozoin in a similar mechanism as the 4-aminoquinolines.

Table 1 Summary of 4-aminoquinolines entering or in clinical trials, modified and updated from recent reviews [4, 76]

Active ingredients (product name)	Partnership	Phase/ status	Strengths	Weakness
Artesunate 50 mg Amodiaquine 135 mg (Coarsucam®)	Sanofi-Aventis, DNDi	Prequalified 2008	• Soluble tablets for paediatric use. • 1 tablet a day – 3 days • WHO prequalified • Three dose strengths • Has 25% of the ACT market	• Resistance to AQ – GI side effects • Not used as prophylactic due to toxic effect of AQ • Reports of resistant strains • No approval yet but WHO prequalified
DHA 10 mg piperaquine 80 mg (Eurartesim™), Artekin, also Duocotexin (fixed dose Holley and Cotect)	Sigma-Tau, MMV, Chongquing, Holley	III	• 1 tablet a day for 3 days • Piperaquine longest half life of all ACTs partners. • Long post-treatment prophylaxic effect • Extensive safety data	• On WHO treatment guidelines but not approved • Long half life of piperaquine could lead to resistance (16.5 days – DHA approximately 0.5 h)
Pyronaridine 60 mg artesunate 20 mg (Pyramax)	Shin Poong, MMV	III	• 1 tablet a day for 3 days • End point achieved in Phase III trials, submitted to EMEA (late 2009) • Clinical data and registration also for *P. vivax*	• Possible hepatotoxicity from pyronaridine – needs to be investigated • Long half life pyronaridine may lead to resistant strains • Paediatric formula in development (2012 release)
Azithromycin 250 mg Chloroquine 150 mg	Pfizer/MMV	III	• Fixed dose combination (four tablets) for prophylactic use during pregnancy • Long post-treatment prophylaxic effect • Extensive safety data • High efficacy in Phase III trials, even in CQ-resistant areas	• Prohibitively expensive for malaria control programmes • Regimen requires partial self-administration • Anti-CQ campaigns in some areas – may be problem with patient compliance
Rbx11160 150 mg Piperaquine 800 mg (Arterolane)	Ranbaxy	II	• No embryotoxicity concern as with artemisinin combinations • Synthetic so costs kept low • Potential activity against artemisinin-resistant strains to be established	• Efficacy concerns (poor activity of Rbx11160as a monotherapy) • As yet no studies in children, or juvenile toxicology data • Phase III India 2009 – no launch until at least 2011

(continued)

Table 1 (continued)

Active ingredients (product name)	Partnership	Phase/ status	Strengths	Weakness
			• Phase III study as a combination planned India 2009	
SSR-97193 (Ferroquine) artesunate	Sanofi-Aventis	II	• Also effective against *P. vivax* chloroquine resistant strains	• Cost of goods for metal based drugs – may be expensive
Methylene blue, chloroquine	Ruprecht-Karls-University, Heidelberg, DSM	II	• Reports of combination with AQ or artesunate planned. • MB/AQ Cost-effective	Methylene blue/chloroquine did not meet WHO criterion of 95% efficacy
AQ-13	Immtech	I	• Similar to CQ in its efficacy and PK	• Very similar structure to CQ-possible parasite could develop resistance very quickly? • AQ-13 exhibits increased clearance compared with CQ therefore higher dose required
N-tert-butyl Isoquine	GSK, MMV	I	• Excellent exposures • Near quantitative bioavailabilites • Superior PK data to ISQ	• *N-tert* discontinued due to problems with inadequate exposure levels • Phase I back-up molecule being evaluated
SAR116242 (Trioxaquine)	Sanofi, Palumed	Preclinical	• Totally synthetic, metabolically stable and cost effective	• Synthetic route produces diastereomers • Molecule has potential to express both established safety concerns of 4-aminoquinolines (narrow TI) and endoperoxides (embryotoxicity, neurotoxicity) requiring careful safety evaluation

MB was entered into clinical trials with CQ as a partner drug but this combination was not sufficiently effective, even at higher doses of MB [113]. More recent trials with AQ or artesunate as a partner drug provided more optimism; MB-artesunate achieved a more rapid clearance of *P. falciparum* parasites than MB–AQ, but MB–AQ displayed the overall highest efficacy. As MB and AQ are both available and affordable, the MB–AQ combination would be an inexpensive non-ACT antimalarial regimen. A larger multi-centre Phase III study is now planned for the near future.

Another non-ACT combination in Phase II clinical trials is azithromycin/chloroquine (AZ/CQ). Azithromycin is a newer member of the family of macrolide antibiotics. This combination has entered Phase III clinical trials and is currently the most promising non-artemisinin-based prophylactic therapy for Intermittent Preventative Treatment in Pregnant Women (IPTp) [76] and a fixed-dose combination tablet of AZ/CQ is being developed specifically for this use. The combination is synergistic against CQ-resistant strains of *P. falciparum* and has already shown efficacy in the treatment of symptomatic malaria in sub-Saharan Africa, an area of high CQ resistance [76]. Both AZ and CQ have demonstrated safety in children and pregnant women over a number of years and azithromycin provides an additional benefit in treating and preventing sexually transmitted diseases [114]. A pivotal study comparing AZ/CQ IPTp with the current adopted therapy sulfadoxine–pyrimethamine IPTp began in October 2010 and is expected to be completed by January 2013 [115].

6 Conclusions

Due to the increasing spread of malaria resistance to drugs such as CQ and AQ, current treatment regimes rely heavily on artemisinin-based therapies. This could lead to an overdependence on artemisinin availability and may influence cost, so it is extremely important that 4-aminoquinoline drug development programmes continue. Costly lessons have been learnt from the loss of sensitivity to one of the most important drugs for malaria treatment and extreme caution is now taken to ensure that with every new antimalarial developed, a partner drug is found and co-administered to reduce the spread of parasite resistance. Increased understanding of 4-aminoquinoline SARs, mechanisms of toxicity and parasite resistance has aided development of what will hopefully be the next generation of 4-aminoquinolines. The future of 4-aminoquinolines relies heavily on strong partnerships between the public health sectors, MMV (Medicines for Malaria Venture) academia and private pharmaceutical/biotechnology companies to yield a continuing pipeline of 4-aminoquinoline candidates, which not only overcome resistance development but also demonstrate increased efficacy compared with CQ. Equally important is a consideration of the safety attributes of this class since the animal toxicities observed in industry standard pre-clinical development of next-generation analogues such as NTB-isoquine (**23**) **8**, in the absence of any prior human

experience, might have precluded the further development of any 4-aminoquinoline and indicates limitations of our current pre-clinical testing strategies to accurately predict human risk in malaria treatment [110].

References

1. Phillipson JD, O'Neill MJ (1986) Novel antimalarial drugs from plants? Parasitol Today 2:355–359
2. Jensen M, Mehlhorn H (2009) Seventy-five years of Resochin in the fight against malaria. Parasitol Res 105:609–627
3. Loeb LF, Clarke WM, Coatney GR, Coggeshall LT, Dieuaide FR, Dochez AR (1946) Activity of a new antimalarial agent, Chloroquine (SN 7618). JAMA 130:1069–1070
4. Wells TN, Poll EM (2010) When is enough enough? The need for a robust pipeline of high-quality antimalarials. Discov Med 9:389–398
5. Winstanley PA, Ward SA, Snow RW (2002) Clinical status and implications of antimalarial drug resistance. Microb Infect 4:157–164
6. Foley M, Tilley L (1998) Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. Pharmacol Ther 79:55–87
7. Egan TJ (2001) Quinoline antimalarials. Expert Opin Ther Patents 11:185–209
8. Tilley L, Loria P, Foley M (2001) Chloroquine and other quinoline antimalarials. In: Rosenthal PJ (ed) Antimalarial chemotherapy: mechanisms of action, resistance and new direction in drug discovery. Humana, Totowa, NJ, pp 87–121
9. Olliaro P (2001) Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther 89:207–219
10. Surolia N, Padmanaban G (1991) Chloroquine inhibits heme-dependent protein synthesis in *Plasmodium falciparum*. Proc Natl Acad Sci USA 88:4786–4790
11. Ginsburg H, Geary TG (1987) Current concepts and new ideas on the mechanism of action of quinoline-containing antimalarials. Biochem Pharmacol 36:1567–1576
12. Vander Jagt DL, Hunsaker LA, Campos NM (1986) Characterization of a hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. Mol Biochem Parasitol 18:389–400
13. Cohen SN, Yielding KL (1965) Inhibition of DNA and RNA polymerase reactions by chloroquine. Proc Natl Acad Sci USA 54:521–527
14. Meshnick SR (1990) Chloroquine as intercalator: a hypothesis revived. Parasitol Today 6:77–79
15. Peters W (1970) Chemotherapy and drug resistance in malaria. Academic, London
16. Egan TJ (2008) Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation. J Inorg Biochem 102:1288–1299
17. Slater AF, Cerami A (1992) Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature 355:167–169
18. Egan TJ, Ross DC, Adams PA (1994) Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment). FEBS Lett 352:54–57
19. Raynes K, Foley M, Tilley L, Deady LW (1996) Novel bisquinoline antimalarials. Synthesis, antimalarial activity, and inhibition of haem polymerisation. Biochem Pharmacol 52:551–559
20. Adams PA, Berman PA, Egan TJ, Marsh PJ, Silver J (1996) The iron environment in heme and heme-antimalarial complexes of pharmacological interest. J Inorg Biochem 63:69–77
21. Egan TJ, Mavuso WW, Ross DC, Marques HM (1997) Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX. J Inorg Biochem 68:137–145

22. Egan TJ, Helder MM (1999) The role of haem in the activity of chloroquine and related antimalarial drugs. Coord Chem Rev 190–192:493–517
23. Vippagunta SR, Dorn A, Matile H, Bhattacharjee AK, Karle JM, Ellis WY, Ridley RG, Vennerstrom JL (1999) Structural specificity of chloroquine-hematin binding related to inhibition of hematin polymerization and parasite growth. J Med Chem 42:4630–4639
24. Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL, Ridley RG (1998) An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. Biochem Pharmacol 55:727–736
25. Sullivan DJ, Gluzman IY, Russell DG, Goldberg DE (1996) On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci USA 93:11865–11870
26. Buller R, Peterson ML, Almarsson O, Leiserowitz L (2002) Quinoline binding site on malaria pigment crystal: a rational pathway for antimalaria drug design. Cryst Growth Des 2:553–562
27. Hawley SR, Bray PG, Park BK, Ward SA (1996) Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine. Mol Biochem Parasitol 80:15–25
28. Geary TG, Divo AD, Jensen JB, Zangwill M, Ginsburg H (1990) Kinetic modelling of the response of *Plasmodium falciparum* to chloroquine and its experimental testing *in vitro*. Implications for mechanism of action of and resistance to the drug. Biochem Pharmacol 40:685–691
29. Ferrari V, Cutler DJ (1991) Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with *Plasmodium falciparum*. Evidence for a drug-importer in chloroquine-sensitive strains. Biochem Pharmacol 42(Suppl):S167–179
30. Ferrari V, Cutler DJ (1991) Kinetics and thermodynamics of chloroquine and hydroxychloroquine transport across the human erythrocyte membrane. Biochem Pharmacol 41:23–30
31. Sanchez CP, Wunsch S, Lanzer M (1997) Identification of a chloroquine importer in *Plasmodium falciparum*. Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. J Biol Chem 272:2652–2658
32. Chou AC, Chevli R, Fitch CD (1980) Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. Biochemistry 19:1543–1549
33. Bray PG, Janneh O, Raynes KJ, Mungthin M, Ginsburg H, Ward SA (1999) Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. J Cell Biol 145:363–376
34. D'Alessandro U, Buttiëns H (2001) History and importance of antimalarial drug resistance. Trop Med Int Health 6:845–848
35. Bray PG, Mungthin M, Ridley RG, Ward SA (1998) Access to hematin: the basis of chloroquine resistance. Mol Pharmacol 54:170–179
36. Sidhu ABS, Verdier-Pinard D, Fidock DA (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 298:210–213
37. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LMB, Sidhu ABS, Naude B, Deitsch KW (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 6:861–871
38. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AMJ, Martin SK, Milhous WK, Schlesinger PH (1987) Efflux of chloroquine from *Plasmodium falciparum* – mechanism of chloroquine resistance. Science 238:1283–1285
39. Hayward R, Saliba KJ, Kirk K (2006) The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. J Cell Sci 119:1016–1025
40. Bray PG, Mungthin M, Hastings IM, Biagini GA, Saidu DK, Lakshmanan V, Johnson DJ, Hughes RH, Stocks PA, O'Neill PM (2006) PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. Mol Microbiol 62:238–251

41. Warhurst DC, Craig JC, Adagu IS (2002) Lysosomes and drug resistance in malaria. Lancet 360:1527–1529
42. Sanchez CP, Stein WD, Lanzer M (2007) Is PfCRT a channel or a carrier? Two competing models explaining chloroquine resistance in *Plasmodium falciparum*. Trends Parasitol 23:332–339
43. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, Kirk K (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science 325:1680–1682
44. Sanchez CP, Dave A, Stein WD, Lanzer M (2010) Transporters as mediators of drug resistance in *Plasmodium falciparum*. Int J Parasitol 40:1109–1118
45. van Es HH, Karcz S, Chu F, Cowman AF, Vidal S, Gros P, Schurr E (1994) Expression of the plasmodial *pfmdr1* gene in mammalian cells is associated with increased susceptibility to chloroquine. Mol Cell Biol 14:2419–2428
46. Sanchez CP, Rotmann A, Stein WD, Lanzer M (2008) Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol Microbiol 70:786–798
47. WHO (2010) Guidelines for the treatment of malaria, 2nd edn. WHO (World Health Organization), Geneva
48. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV (2006) Return of chloroquine antimalarial efficacy in Malawi. New Engl J Med 355:1959–1966
49. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA et al (2001) A molecular marker for chloroquine-resistant falciparum malaria. New Engl J Med 344:257–263
50. Read AF, Huijben S (2009) Evolutionary biology and the avoidance of antimicrobial resistance. Evol Appl 2:40–51
51. Ursing J, Kofoed PE, Rodrigues A, Blessborn D, Thoft-Nielsen R, Bjorkman A, Rombo L (2011) Similar efficacy and tolerability of double-dose chloroquine and artemether-lumefantrine for treatment of *Plasmodium falciparum* infection in guinea-bissau: a randomized trial. J Infect Dis 203:109–116
52. Ursing J, Rombo L, Kofoed PE, Gil JP (2008) Carriers, channels and chloroquine efficacy in Guinea-Bissau. Trends Parasitol 24:49–51
53. Kofoed PE, Ursing J, Poulsen A, Rodrigues A, Bergquist Y, Aaby P, Rombo L (2007) Different doses of amodiaquine and chloroquine for treatment of uncomplicated malaria in children in Guinea-Bissau: implications for future treatment recommendations. Trans R Soc Trop Med Hyg 101:231–238
54. Hand CC, Meshnick SR (2011) Is chloroquine making a comeback? J Infect Dis 203:11–12
55. Ursing J, Schmidt BA, Lebbad M, Kofoed PE, Dias F, Gil JP, Rombo L (2007) Chloroquine resistant *P.falciparum* prevalence is low and unchanged between 1990 and 2005 in Guinea-Bissau: an effect of high chloroquine dosage? Infect Genet Evol 7:555–561
56. Kaur K, Jain M, Reddy RP, Jain R (2010) Quinolines and structurally related heterocycles as antimalarials. Eur J Med Chem 45:3245–3264
57. De D, Krogstad FM, Cogswell FB, Krogstad DJ (1996) Aminoquinolines that circumvent resistance in *Plasmodium falciparum in vitro*. Am J Trop Med Hyg 55:579–583
58. Ridley RG, Hofheinz W, Matile H, Jaquet C, Dorn A, Masciadri R, Jolidon S, Richter WF, Guenzi A, Girometta MA (1996) 4-aminoquinoline analogs of chloroquine with shortened side chains retain activity against chloroquine-resistant *Plasmodium falciparum*. Antimicrob Agents Chemother 40:1846–1854
59. Ramanathan-Girish S, Catz P, Creek MR, Wu B, Thomas D, Krogstad DJ, De D, Mirsalis JC, Green CE (2004) Pharmacokinetics of the antimalarial drug, AQ-13, in rats and cynomolgus Macaques. Int J Toxicol 23:179–189

60. Mzayek F, Deng H, Mather FJ, Wasilevich EC, Liu H, Hadi CM, Chansolme DH, Murphy HA, Melek BH, Tenaglia AN (2007) Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. PLoS Clin Trials 2:e6
61. Mzayek F, Deng HY, Hadi MA, Mave V, Mather FJ, Goodenough C, Mushatt DM, Lertora JJ, Krogstad D (2009) Randomized clinical trial (RCT) with a crossover study design to examine the safety and pharmacokinetics of a 2100 mg dose of AQ-13 and the effects of a standard fatty meal on its bioavailability. Am J Trop Med Hyg 81:S252
62. De D, Krogstad FM, Byers LD, Krogstad DJ (1998) Structure-activity relationships for antiplasmodial activity among 7-substituted 4-aminoquinolines. J Med Chem 41:4918–4926
63. Ward SA, Bray PG, Hawley SR, Mungthin M (1996) Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*. Mol Pharmacol 50:1559–1566
64. Farrel N (1989) Transition metal complexes as drugs and chemotherapeutic agents. Kluwer Academic, Dordrecht
65. Sanchez-Delgado RA, Navarro M, Perez H, Urbina JA (1996) Toward a novel metal-based chemotherapy against tropical diseases. 2. Synthesis and antimalarial activity *in vitro* and *in vivo* of new ruthenium- and rhodium-chloroquine complexes. J Med Chem 39:1095–1099
66. Sanchez-Delgado RA, Navarro M, Perez H (1997) Toward a novel metal-based chemotherapy against tropical diseases. 3. Synthesis and antimalarial activity *in vitro* and *in vivo* of the new gold-chloroquine complex [Au(PPh3)(CQ)]PF6. J Med Chem 40:1937–1939
67. Biot C, Glorian G, Maciejewski LA, Brocard JS, Domarle O, Blampain G, Millet P, Georges AJ, Abessolo H, Dive D (1997) Synthesis and antimalarial activity *in vitro* and *in vivo* of a new ferrocene-chloroquine analogue. J Med Chem 40:3715–3718
68. Biot C, Delhaes L, N'Diaye CM, Maciejewski LA, Camus D, Dive D, Brocard JS (1999) Synthesis and antimalarial activity *in vitro* of potential metabolites of ferrochloroquine and related compounds. Biorg Med Chem 7:2843–2847
69. Dubar F, Khalife J, Brocard J, Dive D, Biot C (2008) Ferroquine, an ingenious antimalarial drug – thoughts on the mechanism of action. Molecules 13:2900–2907
70. Barends M, Jaidee A, Khaohirun N, Singhasivanon P, Nosten F (2007) *In vitro* activity of ferroquine (SSR 97193) against *Plasmodium falciparum* isolates from the Thai-Burmese border. Malar J 6:81
71. Leimanis ML, Jaidee A, Sriprawat K, Kaewpongsri S, Suwanarusk R, Barends M, Phyo AP, Russell B, Renia L, Nosten F (2010) *Plasmodium vivax* susceptibility to ferroquine. Antimicrob Agents Chemother 54:2228–2230
72. Sanofi-Aventis (2000) Dose ranging study of ferroquine with artesunate in african adults and children with uncomplicated *Plasmodium falciparum* malaria (FARM). In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda (MD). http://clinicaltrials.gov/ct2/show/NCT00988507. Accessed 23 May 2011. NLM Identifier: NCT00988507
73. Davis TME, Hung TY, Sim IK, Karunajeewa HA, Ilett KF (2005) Piperaquine – a resurgent antimalarial drug. Drugs 65:75–87
74. Hung TY, Davis TME, Ilett KF, Karunajeewa H, Hewitt S, Denis MB, Lim C, Socheat D (2004) Population pharmacokinetics of piperaquine in adults and children with uncomplicated falciparum or vivax malaria. Br J Clin Pharmacol 57:253–262
75. Hien TT, Dolecek C, Mai PP, Dung NT, Truong NT, Thai LH, An DTH, Thanh TT, Stepniewska K, White NJ (2004) Dihydroartemisinin-piperaquine against multidrug-resistant *Plasmodium falciparum* malaria in Vietnam: randomised clinical trial. Lancet 363:18–22
76. Olliaro P, Wells TNC (2009) The global portfolio of new antimalarial medicines under development. Clin Pharmacol Ther 85:584–595
77. Zwang J, Ashley EA, Karema C, D'Alessandro U, Smithuis F, Dorsey G, Janssens B, Mayxay M, Newton P, Singhasivanon P (2009) Safety and efficacy of dihydroartemisinin-piperaquine in falciparum malaria: a prospective multi-centre individual patient data analysis. PLoS ONE 4:e6358

78. Price RN, Hasugian AR, Ratcliff A, Siswantoro H, Purba HLE, Kenangalem E, Lindegardh N, Penttinen P, Laihad F, Ebsworth EP (2007) Clinical and pharmacological determinants of the therapeutic response to dihydroartemisinin-piperaquine for drug-resistant malaria. Antimicrob Agents Chemother 51:4090–4097
79. Khanh NX, de Vries PJ, Ha LD, van Boxtel CJ, Koopmans R, Kager PA (1999) Declining concentrations of dihydroartemisinin in plasma during 5-day oral treatment with artesunate for falciparum malaria. Antimicrob Agents Chemother 43:690–692
80. Charman SA (2007) Synthetic peroxides: a viable alternative to artemisinins for the treatment of uncomplicated malaria? In: American Society of Tropical Medicine and Hygiene (ASTMH) 56th Annual Meeting, Philadelphia, Pennsylvania, USA, 4–8 Nov 2007
81. Snyder C, Chollet J, Santo-Tomas J, Scheurer C, Wittlin S (2007) *In vitro* and *in vivo* interaction of synthetic peroxide RBx11160 (OZ277) with piperaquine in *Plasmodium* models. Exp Parasitol 115:296–300
82. White NJ (2008) Qinghaosu (Artemisinin): the price of success. Science 320:330–334
83. Meunier B (2008) Hybrid molecules with a dual mode of action: dream or reality? Acc Chem Res 41:69–77
84. Muregi FW, Ishih A (2010) Next-generation antimalarial drugs: hybrid molecules as a new strategy in drug design. Drug Dev Res 71:20–32
85. Benoit-Vical F, Lelievre J, Berry A, Deymier C, Dechy-Cabaret O, Cazelles J, Loup C, Robert A, Magnaval JF, Meunier B (2007) Trioxaquines are new antimalarial agents active on all erythrocytic forms, including gametocytes. Antimicrob Agents Chemother 51:1463–1472
86. Cosledan F, Fraisse L, Pellet A, Guillou F, Mordmuller B, Kremsner PG, Moreno A, Mazier D, Maffrand JP, Meunier B (2008) Selection of a trioxaquine as an antimalarial drug candidate. Proc Natl Acad Sci USA 105:17579–17584
87. O'Neill PM, Bray PG, Hawley SR, Ward SA, Park BK (1998) 4-aminoquinolines – past, present, and future: a chemical perspective. Pharmacol Ther 77:29–58
88. Daily EB, Aquilante CL (2009) Cytochrome P450 2 C8 pharmacogenetics: a review of clinical studies. Pharmacogenomics 10:1489–1510
89. Fu S, Bjorkman A, Wahlin B, Ofori-Adjei D, Ericsson O, Sjoqvist F (1986) *In vitro* activity of chloroquine, the two enantiomers of chloroquine, desethylchloroquine and pyronaridine against *Plasmodium falciparum*. Br J Clin Pharmacol 22:93–96
90. White NJ, Looareesuwan S, Edwards G, Phillips RE, Karbwang J, Nicholl DD, Bunch C, Warrell DA (1987) Pharmacokinetics of intravenous amodiaquine. Br J Clin Pharmacol 23:127–135
91. Jewell H, Maggs JL, Harrison AC, O'Neill PM, Ruscoe JE, Park BK (1995) Role of hepatic metabolism in the bioactivation and detoxication of amodiaquine. Xenobiotica 25:199–217
92. Jewell H, Ruscoe JE, Maggs JL, O'Neill PM, Storr RC, Ward SA, Park BK (1995) The effect of chemical substitution on the metabolic activation, metabolic detoxication, and pharmacological activity of amodiaquine in the mouse. J Pharmacol Exp Ther 273:393–404
93. Clarke JB, Neftel K, Kitteringham NR, Park BK (1991) Detection of antidrug IgG antibodies in patients with adverse drug reactions to amodiaquine. Int Arch Allergy Appl Immunol 95:369–375
94. Churchill FC, Mount DL, Patchen LC, Bjorkman A (1986) Isolation, characterization and standardization of a major metabolite of amodiaquine by chromatographic and spectroscopic methods. J Chromatogr B 377:307–318
95. Laurent F, Saivin S, Chretien P, Magnaval JF, Peyron F, Sqalli A, Tufenkji AE, Coulais Y, Baba H, Campistron G et al (1993) Pharmacokinetic and pharmacodynamic study of amodiaquine and its two metabolites after a single oral dose in human volunteers. Arzneim-Forsch 43:612–616
96. Li XQ, Bjorkman A, Andersson TB, Ridderstrom M, Masimirembwa CM (2002) Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by

CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. J Pharmacol Exp Ther 300:399–407
97. O'Neill PM, Harrison AC, Storr RC, Hawley SR, Ward SA, Park BK (1994) The effect of fluorine substitution on the metabolism and antimalarial activity of amodiaquine. J Med Chem 37:1362–1370
98. O'Neill PM, Willock DJ, Hawley SR, Bray PG, Storr RC, Ward SA, Park BK (1997) Synthesis, antimalarial activity, and molecular modeling of tebuquine analogues. J Med Chem 40:437–448
99. Barlin GB, Ireland SJ, Nguyen TMT, Kotecka B, Rieckmann KH (1994) Potential antimalarials. XXI. Mannich base derivatives of 4-[7-Chloro(and 7-trifluoromethyl) quinolin-4-ylamino]phenols. Aust J Chem 47:1553–1560
100. Peters W, Robinson BL (1992) The chemotherapy of rodent malaria. XLVII. Studies on pyronaridine and other Mannich base antimalarials. Ann Trop Med Parasitol 86:455–465
101. Ward SA, Hawley SR, Bray PG, O'Neill PM, Naisbitt DJ, Park BK (1996) Manipulation of the N-alkyl substituent in amodiaquine to overcome the verapamil-sensitive chloroquine resistance component. Antimicrob Agents Chemother 40:2345–2349
102. Tingle MD, Ruscoe JE, O'Neill PM, Ward SA, Park BK (1998) Effect of disposition of Mannich antimalarial agents on their pharmacology and toxicology. Antimicrob Agents Chemother 42:2410–2416
103. Biagini GA, O'Neill PM, Bray PG, Ward SA (2005) Current drug development portfolio for antimalarial therapies. Curr Opin Pharmacol 5:473–478
104. Looareesuwan S, Kyle DE, Viravan C, Vanijanonta S, Wilairatana P, Wernsdorfer WH (1996) Clinical study of pyronaridine for the treatment of acute uncomplicated falciparum malaria in Thailand. Am J Trop Med Hyg 54:205–209
105. Pradines B, Mabika Mamfoumbi M, Parzy D, Owono Medang M, Lebeau C, Mourou Mbina JR, Doury JC, Kombila M (1999) *In vitro* susceptibility of African isolates of *Plasmodium falciparum* from Gabon to pyronaridine. Am J Trop Med Hyg 60:105–108
106. Nosten FH (2010) Pyronaridine-artesunate for uncomplicated falciparum malaria. Lancet 375:1413–1414
107. Medicines for Malaria Venture. Pyramax dossier submitted to EMA. http://www.mmv.org/achievements-challenges/achievements/pyramax%C2%AE-dossier-submitted-ema?page=0. Accessed 24 May 2011
108. O'Neill PM, Mukhtar A, Stocks PA, Randle LE, Hindley S, Ward SA, Storr RC, Bickley JF, O'Neil IA, Maggs JL (2003) Isoquine and related amodiaquine analogues: a new generation of improved 4-aminoquinoline antimalarials. J Med Chem 46:4933–4945
109. Delarue S, Girault S, Maes L, Debreu-Fontaine MA, Labaeid M, Grellier P, Sergheraert C (2001) Synthesis and *in vitro* and *in vivo* antimalarial activity of new 4-anilinoquinolines. J Med Chem 44:2827–2833
110. O'Neill PM, Park BK, Shone AE, Maggs JL, Roberts P, Stocks PA, Biagini GA, Bray PG, Gibbons P, Berry N (2009) Candidate selection and preclinical evaluation of N-tert-Butyl isoquine (GSK369796), an affordable and effective 4-Aminoquinoline antimalarial for the 21st century. J Med Chem 52:1408–1415
111. O'Neill PM, Shone AE, Stanford D, Nixon G, Asadollahy E, Park BK, Maggs JL, Roberts P, Stocks PA, Biagini G (2009) Synthesis, antimalarial activity, and preclinical pharmacology of a novel series of 4-Fluoro and 4-Chloro analogues of amodiaquine. Identification of a suitable "back-up" compound for N-tert-butyl isoquine. J Med Chem 52:1828–1844
112. Schirmer RH, Coulibaly B, Stich A, Scheiwein M, Merkle H, Eubel J, Becker K, Becher H, Müller O, Zich T (2003) Methylene blue as an antimalarial agent. Redox Rep 8:272–275
113. Meissner PE, Mandi G, Coulibaly B, Witte S, Tapsoba T, Mansmann U, Rengelshausen J, Schiek W, Jahn A, Walter-Sack I (2006) Methylene blue for malaria in Africa: results from a dose-finding study in combination with chloroquine. Malar J 5:84
114. Chico RM, Pittrof R, Greenwood B, Chandramohan D (2008) Azithromycin-chloroquine and the intermittent preventive treatment of malaria in pregnancy. Malar J 7:255

115. Pfizer (2000) Evaluate azithromycin plus chloroquine and sulfadoxine plus pyrimethamine combinations for intermittent preventive treatment of falciparum malaria infection in pregnant women In Africa. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda (MD). http://clinicaltrials.gov/ct2/show/NCT01103063. Accessed 2011 May 23. NLM Identifier: NCT01103063

Cinchona Alkaloids: Quinine and Quinidine

David J. Sullivan

Abstract For 400 years, quinine has been the effective antimalarial. From a pulverized bark, which stopped cyclic fevers, to an easily isolated crystal alkaloid, which launched many pharmaceutical companies, tons of quinine are still purified for medicinal and beverage use. The quest for quinine synthesis pioneered early medicinal dyes, antibacterials, and other drugs. In a specialized *Plasmodium* lysosome for hemoglobin degradation, quinine binds heme, which inhibits heme crystallization to kill rapidly. Although quinine drug resistance was described 100 years ago, unlike chloroquinine or the antifolates that have been rendered ineffective by the spread of resistant mutants, quinine has only a few persistent, resistant parasites worldwide. The artemisinin drugs, superior to quinine for severe malaria, have greatly reduced the use of quinine as an antimalarial. Evidence for prolonged artemisinin parasite clearance times both renews the quest for rapidly parasiticidal drugs for severe malaria and possibly holds a place for quinine.

1 Early History of Quinine

Cinchona bark extracts were identified as early as 1632 to be effective in treating fevers, particularly the tertian fever of malaria [1]. This specific symptomatic management preceded the identification of the etiologic organism of malaria by almost 250 years. Many bacterial organisms were not discovered until the use of microscopy and dyes to contrast them in the late 1800s. Laveran identified the malaria parasite in Algerian soldiers in the 1880s without dyes [2]. His first description was of an exflagellating male gametocyte, later followed by observations of

D.J. Sullivan (✉)
W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Malaria Research Institute, The Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe St. Rm E5628, Baltimore, MD 21205, USA
e-mail: dsulliva@jhsph.edu

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_3,

intraerythrocytic forms containing the malaria pigment hemozoin. Later, Ehrlich in 1891 with the use of methylene blue was able to speciate and treat human *Plasmodium* and put to rest initial doubts of Laveran's descriptions [3, 4].

Despite historical accounts of the Jesuits in Peru curing the Countess of Chinchón with local bark, the countess actually died of yellow fever en route to Europe [5]. There are three other plausible historical accounts of the discovery of the curative properties of Peruvian (Cinchona) bark. One is of Peruvian Indians drinking bark teas to stop shivering while mining in the mountains. Another account relates Indians drinking the bark tea to stop shivering after crossing cold mountain rivers [6, 7]. PCC Garnham relates a third story in the region of Loxa after an earthquake felled cinchona trees into a lake, which grew brown with the bark. This water was curative of malarial fevers [8]. We do know that shipments of bark arrived in Spain in 1636. Soon cinchona bark became known as Jesuit's bark or the Cardinal's bark [5]. In contrast to some historical accounts, Oliver Cromwell may not have died from malaria after refusing to take the Catholic bark, but from septicemia related to kidney stones [9]. The Peruvian bark was much sought after and soon was worth its weight in gold. Other barks were also sampled for fevers. An English physician by the name of Stone described the curative properties of willow bark from which salicylates were later identified [10]. This was the first of many of our present drugs, which trace an origin to quinine [5, 11].

2 Quinine Extraction

There are approximately 90 varieties of Cinchona, a red bark tree, which is included in the madder family of which coffee and gardenias are also members. Different barks of cinchona contain 5–10% quinine [12]. Approximately 1–2 g of pulverized bark, seeped in boiling water for 10 min will yield about 100 mg of active ingredient. In 1820, Pelletier and Caventou identified a straightforward process for crystal purification of the active quinine salt [13]. Pulverized bark was soaked in water and crystal formed from acidified water due to the poor solubility of quinine crystals relative to the other alkaloids in the bark. Unlike others, they made their process freely available. The black residue leftover was not active but the four active alkaloids extracted were quinine and quinidine, cinchoine and cinchoinidine. Much later, Gammie reported a process from India where 100 parts of pulverized powder was mixed with 8 parts caustic soda, 500 parts water and 600 parts fuel and kerosene oil [14]. The alkaloids were absorbed by the oil; the oil was transferred to heated acid water, and then cooled for crystallization. Sulfuric acid was used to make quinine sulfate and hydrochloric acid for quinine hydrochlorate. Even today the process requires 500–1,000 metric tons of bark to produce hundreds of tons of quinine. About half of current twenty-first century production is used for beverages and the rest for medicines. Many pharmaceutical companies such as Boehringer Mannheim (which had a cinchona tree on its original logo) originated by extracting and providing quinine [15]. In addition to malaria, quinine was used in many

Table 1 List of early US Federal Drug Association approvals

Drug	Identifier	NDA number	Company	Date approved	Withdrawn	Trade name	Generic
Oxalic acid	N000001001	000001	John A Millar Co Div Chatham Pharm	9/15/38	2/5/71	Koagamin Inj	Oxalic acid
Quinine	N000027001	000027	1st Texas Pharmaceuticals Inc Sub Scherer Laboratories Inc	9/19/38	10/20/80	Private Formula	Arsenic trioxide 0.5 gr; Strychnine sulfate 0.5 gr; Iron, Reduced 15 gr; Cascara 15 gr; Quinine hydrobromide 30 gr
Quinine	N000077001	000077	Mendez Angel M	7/31/39	7/24/70	Quinarsine Inj	Quinine formate 5 g; Sodium cacodylate 2 g; Urethane 4 g; Amylocaine hydrochloride 1 g
Quinine	N000227003	000227	Modern Drugs Inc	5/2/40	7/24/70	No-Ko Tab	Quinine sulfate 0.75 gr; Belladonna extract 0.05 gr; Camphor 0.25 gr; Capsicum oleoresin 0.25 gr; Sodium salicylate 3 gr
Quinine	N000463001	000463	Portia Laboratories	2/20/39	7/24/70	Mal-Caps	Quinine sulfate 2 gr; Sodium bicarbonate 5 gr
Quinine	N000478001	000478	Joseph Personeni Inc	3/1/39	7/24/70	Treponyl Inj	Quinine bismuth iodide 0.1 gr/Ml
Quinine	N000574001	000574	Sr Seaver and Co	4/8/39	7/24/70	Ryzalen Tab	Quinine sulfate 0.25 gr; Ammonium chloride 0.5 gr; Camphor 0.5 gr; Belladonna extract 0.05 gr; Aconite 0.10 gr; Lime, Iodized 0.33 gr; Phenolphthalein 0.33 gr
Quinine	N001069001	001069	Hoysalb and Co Inc	8/30/39	7/24/70	Kenene Sol	Quinine sulfate 8 gr; Strychnine sulfate 8/64 gr; Ferric citrochloride tincture 24 min/Fl Oz
Quinine	N001180001	001180	1st Texas Pharmaceuticals Inc Sub Scherer Laboratories Inc	7/15/39	8/6/71	Private Formula Cap	Quinine sulfate 2 gr; Bismuth subnitrate 0.1 gr; Charcoal, activated 13/7 gr; Calomel 0.1 gr; Pepsin 4/7 gr
Quinine	N001431001	001431	Standard Products Co	8/25/39	7/24/70	Tonik-Tyme Elx	Quinine phosphate 2 gr/Oz; Pepsin 420 min/Oz; Ferric phosphate 16 gr/Oz; Gentian 5 gr/Oz
Quinine	N001504001	001504	Verard Co	8/30/39	7/24/70	Verard Sol	Quinine sulfate Unk; Hydrogen peroxide Unk
Quinidine	N001975001	001975	Sutliff And Case Co Inc	12/27/39	7/24/70	Quinidine Sul Tab	Quinidine sulfate 3 gr
Chloroquine	N006002002	006002	Sanofi Synthelabo			Aralen Hcl	Chloroquine hydrochloride Eq 40 mg Base/Ml
Primaquine	N008151001	008151	Eli Lilly and Co	8/10/51	8/6/71	Primaquine Tab	Primaquine phosphate 15 mg

grain (gr)= 0.06 grams (g)

remedies for headache, nausea, or as part of a mixture for a general cure-all or analgesic. In the United States, the Federal Drug Association approved quinine in combination with arsenic, amylocaine, belladonna, strychnine, bismuth and iron as part of early twentieth-century popular remedies, as seen in Table 1. The first Federal Drug Association approval for malaria was Mal-Caps in February 1939 by Portia Laboratories with the ingredients quinine sulfate and sodium bicarbonate.

3 Synthesis

The quest for synthesis was long a holy grail of medicinal chemistry, producing many useful drugs and companies along the way. Adolph Strecker in Oslo determined the chemical formula for quinine: $-C_{20}H_{24}N_2O_2$ [16]. William Henry Perkins was trying to synthesize quinine from the oxidation of allyltoluidine ($C_{10}H_{12}N$) and instead produced the color mauve, which was colorfast [17]. This was the first synthesis of a color dye rather than being a plant-derived one. The colored synthetic dye textile industry was begun [5]. Paul Ehrlich is credited with the "magic bullet" concept of specific drugs for microbes, based largely on dyes. Suramin was a dye discovered to be curative for African sleeping sickness [18] and was followed by the synthesis of methylene blue successful for both malaria and African sleeping sickness [19]. Many antipsychotic and antidepressant drugs were discovered after experiments with side-group substitutions on the methylene blue scaffold [20]. In a dye-based rational drug discovery process, the sulfur group was incorporated into Prontosil [21]. This was fortunately first tested in animals where the active sulfa group was cleaved to have an antibacterial effect. This sulfur-based scaffold was the basis for many antibacterial and anticancer therapeutics. The potent antibacterial quinolones were produced during later experiments with chloroquine synthesis [22]. Nalixic acid was not very bioavailable or potent, but addition of the fluorines greatly improved the antibacterial properties of this therapeutic class [23], which also incidentally has weak antimalarial activity first described by Krishna and colleagues with ciprofloxacin [24], which later led to more investigation of the fluoroquinolones [25].

The true synthesis of quinine is a story of deconstruction and reconstruction. Pasteur by acid catalysis and isomerization of quinine, produced *d*-quinotoxine [26]. Rabe and Kindler (1903–1918) later described the synthesis of *d*-quinotoxine back to quinine in a short communication [27]. Prelog and Prostenik first degraded quinotoxine to homomeroquinene, then described the resynthesis of quinotoxine [26, 27]. Woodward and colleagues start from 7-hydroxyisoquinoline and publish the synthesis to homomeroquinene [28]. This completed the synthesis but without any stereochemical resolution. Quinine has four stereogenic sites with a possible 16 different configurations, of which quinine is one. Woodward's student Gilbert Stork completed the full stereochemical synthesis in sufficient yield in 2001 [27]. This was one of the major challenges in medicinal chemistry [26].

4 Mechanism of Action

Quinine and quinidine act rapidly on blood-stage parasites [29]. They are not active against liver stage and gametocytes at pharmacologic relevant doses [30, 31]. Importantly, while the curative properties have saved billions of people, the lack of activity against the gametocytes infective for mosquitoes does not halt the transmission cycle. A present day hope for the artemisinin class of drugs is its relative, but not absolute, potency against the gametocytes [30]. Artemisinin lowers a day-seven gametocyte count, but its population effect on lowering transmission remains to be proven. Despite this deficiency, quinine was successful in mass quinization campaigns, which conquered malaria in temperate Italy at the start of the twentieth century [32].

Early morphologic effects of quinine show digestive vacuolar swelling and pigment clumping [33]. Krishna and colleagues delineated the effects to stages actively degrading hemoglobin from late ring to early schizont stages [29]. Paul Roepe defined high concentrations of quinolines for very short intervals as also being active [34]. Many have postulated additional mechanisms of action for quinine against blood-stage parasites [35–38]. While possible, these additional mechanisms also have to be confined to the stages actively degrading hemoglobin.

The quinolines as a class are now considered to inhibit the process of heme crystal formation in the digestive vacuole [39, 40]. Quinine binds to heme reversibly by π–π interactions with heme [36]. The quinoline nucleus is important for heme binding and the side groups are important for both heme crystal inhibition and digestive vacuole localization as weak bases [41, 42]. Roepe has also described covalent interactions of quinine and quinidine with heme [43, 44]. The abundance and activity of these covalent complexes have not been demonstrated in intraerythrocytic parasites. Stereoisomers like 9-epi-quinine are not active against the parasite which suggests specific transport might be important, as these inactive isomers can bind heme and are equally active in the inhibition of heme crystal formation [35]. Sullivan showed that quinidine has a slower off rate of heme crystals after both chloroquine and quinine were individually bound to crystals in equal amounts in a radiolabeled assay. Chloroquine may have a faster on and off rate but once bound quinidine was slower to be removed from crystals [39, 40, 45]. This differential off rate from insoluble heme crystals complicates models of drug resistance based on transport of soluble drug.

In other cell types, quinine and quinidine inhibit potassium channels and stimulate otic cells to cause tinnitus. Potassium channels have been identified in *Plasmodium* but activity of quinine or quinidine has not been tested, due to a lack of functional characterization [46–48]. In the human *ether-a-go-go* related gene (hERG) channel, quinolines bind and inhibit the channel only when it is open causing a delayed rectification of intracellular potassium [49]. Other drugs that

also inhibit the channel include the tricyclic antidepressants, macrolides like erythromycin and less so azithromycin, antifungal azoles, fluoroquinolines, antihistamines and pentamidine [50, 51]. Many of these classes also exhibit moderate inhibition of *Plasmodium* signifying a decrease in parasitemia by a 10- to a 100-fold amount per 48–72 h cycle rather than the more potent quinolines which decrease by a 100- to almost a 1,000-fold every cycle [52]. Artemisinin is the most rapidly *Plasmodium*-cidal drug with a 10,000-fold drop in parasitemia with treatment. The pharmacodynamics of quinoline action has been partially described [53–56]. Some work has shown that, at low concentrations of quinine (below 500 nM) elimination of parasites is concentration dependent. Above thresholds of 500 nM to 1 μM exposure time regulates parasite clearance in vitro [57]. Most likely, some of both are demonstrated in the killing of *Plasmodium*. The quinolines do accumulate in the digestive vacuole in excess of predictions made by the Henderson–Hasselbach equilibrium based on pH gradients presumably because of either heme or heme crystal binding [35, 39, 58]. Morphologic effects on the parasite by quinine were first described in Dar es Salaam in the late 1800s by Robert Koch. Sometimes in patient blood films shrunken pynotic parasites are visualized at late ring stage with larger digestive vacuoles [33].

5 Structure Activity

None of the alterations to quinine have improved its action against the parasites. The methoxy group of the quinoline ring and the vinyl group of quinuclidine are not required for antimalarial activity. The secondary alcohol group is essential for activity. Reduction of the alcohol group increases toxicity as well as mitigating antimalarial activity [35]. The 9-epiquinine (*8S*, *9S*) and 9-epiquinidine (*8R*, *9R*) stereoisomers were 100 times less potent against sensitive parasites, with IC_{50} values of close to 3,000 nM against clone D-6, but ten times less potent against chloroquine-resistant parasite clone W-2 with IC_{50} values close to 1,000 nM [59–61]. Quinidine (*8R*, *9S*) and dihydroquinidine were slightly more potent than quinine (*8S*, *9R*) and dihydroquinine at 10 nM rather than 30 nM for D-6 and at approximately 60 nM rather than 120 nM for W-2. The dihydroquinolines have a saturated vinyl group. Replacement of the quinuclidine vinyl group with carboxylate greatly reduced activity. In mefloquine, the vinyl-quinuclidine is lost retaining the 4-quinolinemethanol. Mefloquine has equal potency of stereoisomers about the C-11 hydroxy portion [62].

6 Quinine Failure

Drug failure has to be distinguished from *Plasmodium* drug resistance in the case of quinine. The most common cause of quinine drug failure has been inadequate compliance with a full course of therapy [63]. Other causes of apparent drug failure

Table 2 Comparison of quinine and quinidine base and salt weights from formulations

Drug	Molecular weight	Base	Salt	Base/salt ratio	mg Base in 650 mg of salt dose
Quinine	324.4	100	0		
Quinine sulfate	422	100	130	0.77	500
Quinine hydrochloride	360	100	111	0.90	586
Quinine hyrobromide	405	100	125	0.80	520
Quinine ethylcarbonate	396	100	122	0.82	533
Quinine bisulfate	520	100	160	0.63	406
Quinine dihyrochloride	397	100	122	0.82	533
Quinidine gluconate	518	100	160	0.63	406
Quinidine	324	100	0		

include poor quality drug, rare variants of accelerated host metabolism of quinine in spite of adequate dosing, poor absorption from the intestinal tract, underdosing of drug and delay in initiation of treatment such that the disease progresses [63]. Because of the side effects of quinine that include tinnitus, nausea, headache, abdominal pain, and visual defects (so called cinchonism), many people do not finish a complete course, stopping when the symptoms from drug exceed symptoms from disease [64]. Counterfeit drugs, substandard drugs, or variation in drug dose has been associated with the cinchona alkaloids for centuries [65, 66]. Many of the more than 100 species of the trees had different amounts of quinine or quinidine in the bark from 1 to 15%. In the late 1800s and early 1900s, many different manufacturers had diverse quality drugs on the market. Rarely, quinine failure has been documented with inadequate levels despite similar observed dosing in cohorts treated at the same time with the same drug. William Fletcher in the 1920s documented failure in a case given adequate drugs [67]. White and colleagues also documented in a Thailand individual, suboptimum drug levels in spite of adequate dosing that resulted in prolongation of therapy [68]. If a patient is ill, poor absorption from the intestine may result in inadequate levels. The different salts of quinine also do differ substantially in molecular weight. Quinine and quinidine have a molecular weight of 324. At the extremes, quinine bisulfate salt has a molecular weight of 520 with 324 mg of quinine base, while quinine dihydrochloride salt has a molecular weight of 360 with 324 mg of quinine base. The ratio of salt to base can therefore range from 1.7 to 1.2, which can produce confusion and underdosing, if more than 500 mg of quinine is intended but 400 mg of quinine in the bisulfate salt is given. Table 2 illustrates the relative ratios.

7 Quinine Resistance

True drug resistance was first documented in 1910 in German railroad workers in Brazil. Arthur Neiva in a classic report written in German and Portuguese entitled "About the formation of a quinine resistant race of the malaria parasite" describes the setting for the requirement of increasing the dosing for prophylaxis [68]. The

drug was from a reputable manufacturer (Merck). The population was 3,500 railroad workers who also brought families with them. During the preparatory phase, no work was performed during the malaria peak period. Still, 36% of the workers were sick with a high mortality. They worked in deep water and slept in open grass at night. Chagas and Neiva recommended prophylaxis of "Chinin," but the workers refused. Next, enforcement of quinine prophylaxis through controlled administration with the threat of mandatory layoff without compliance was the policy. However, the workers' families did not receive the drug and were constantly sick with malaria and required symptomatic treatment. In the first year, 1/2 of a gram was given every 3 days. Only workers not taking chinin had malaria. In the second season, however, malaria appeared in workers taking chinin. A common pattern was also that workers, on holiday in Rio, showed symptoms of malaria when not taking chinin. The dose was increased to 0.5 g every 2 days during the second season, again with 100% prevention in active workers but increasing number of cases in returning workers from Rio. Neiva called these individuals repositories of adapted parasites. During the peak malaria time of the second season, symptoms appeared in the workers who had not stopped taking chinin. The dose was increased to ½ gram every day, with prevention in the workers, except those taking lesser doses. They concluded that the parasites acquired resistance from the blood of untreated families or returning workers from Rio. The parasites had time to adjust with a subgroup that was constantly exposed to drug during all life stages. In January of 1908, the number of workers increased to 4,000, with regular chinin intake only in railroad workers. Neiva observed highly resistant parasites surviving in both workers taking daily chinin and less frequent doses. The infection rate was 8–10%. Only if there had not been a single interruption of chinin daily were patients symptom-free. Even if workers left the region and stopped taking chinin, malaria was observed 3–30 days later. Resistance was observed in setting of interruptions and where some parasites had been consistently exposed to chinin and still survived. Chinin treatment for the entire population is reasonable if everyone takes medication without interruption. A follow up paper describes the high doses of quinine necessary to treat workers returning to Germany [68]. As many as Thirty-four of 90 patients had *P. falciparum*, with some persistent parasitemias despite 32 grams in 21 days and documentation of drug in urine.

The observation that followed was of specific geographic requirements for increased dosing. Different places in Italy and Sardina required more than 8 times the doses needed for cure of Indian and African falciparum malaria in the 1930s [69]. In recent decades since culturing *P. falciparum* has become possible, a diversity of isolates have widely different concentrations needed to inhibit half of the parasites. A general agreement is an IC_{50} value of 500 nM for a cutoff of quinine-resistant parasites. Unlike chloroquine, which has a clear demarcation between chloroquine sensitive and resistant parasites around 100 nM, quinine has a continuous incremental gradient of IC_{50} values. Some studies report higher IC_{50} values of 800–1,000 nM. A group at the NIH focused on chloroquine resistance, published IC_{50} values to both chloroquine and quinine on almost 100 different laboratory isolates from around the world (Fig. 1) [70]. Only eight isolates were

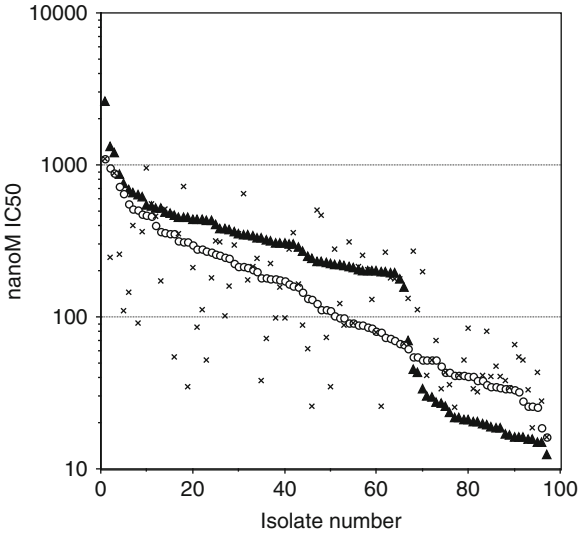


Fig. 1 Comparison of incremental IC_{50} values from more than 100 worldwide laboratory isolates shows a clear separation in chloroquine (*filled triangles*) between 50 and 100 nM. The quinine (*empty circles*) IC_{50} values when also ordered by decreasing values instead show a small step-value difference. Correlation of quinine IC_{50} value paired with the chloroquine IC_{50} value (*cross*) shows that the resistance to either is rarely correlated. The isolate number is a descending rank order number for chloroquine and quinine, except when the quinine value is paired with chloroquine

resistant to quinine, with an incremental drop in IC_{50} values amongst the isolates. Two-thirds were resistant to chloroquine, with a steep shoulder from a sensitive IC_{50} value of less than 30 nM to the resistant IC_{50} value of over 100 nM for chloroquine. There was no correlation of quinine resistance with chloroquine resistance (Fig. 1).

The spread of worldwide clinical quinine resistance has been sporadic and not sustained. A graph of chloroquine resistance as percentage of isolates tested from patient studies with more than 15 subjects depicts high chloroquine resistance in Southeast Asia, increasing chloroquine resistance in Africa but steady low quinine resistance in around 10% of isolates (Fig. 2). This has been the pattern, with quinine resistance being sporadic and staying less than 20% of isolates (Table 3). With increasing use of artemisinin in this century, documentation of quinine resistance has been decreasing. In western Cambodia over the years from 2001 to 2007, the geometric mean IC_{50} value has been 140 nM compared with 90 nM in eastern Cambodia [91]. Furthermore, looking at the trends over the same time span for quinine IC_{50}, an IC_{50} below 300 nM was noted, except for 2007 in western Cambodia. Table 3 and Fig. 2 also show that the percentage of worldwide quinine-resistant isolates in ex vivo drug-sensitivity testing is lower than half.

Quinine is active against the gametocytes of *P. vivax* and *P. malariae* but not *P. falciparum*. In general, *P. vivax* gametocytes are more sensitive to drugs than the

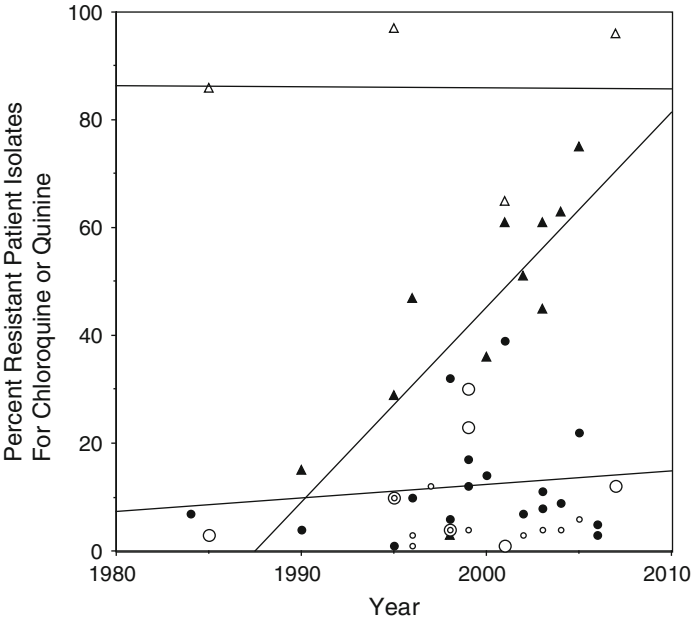


Fig. 2 Quinine-resistant IC_{50} value determined from ex vivo patient isolates not adapted to laboratory culture show that as a percent of a total number, quinine resistance from Africa (*filled circles*), from Southeast Asia (*large empty circle*) or from South America (*small empty circle*), remains approximately 10% over the past 30 years. In contrast, the percent resistant to chloroquine in Southeast Asia (*empty triangle*) was close to 90% over the past 30 years, while in Africa (*filled triangle*) the percentage rapidly climbed from 10% to more than 80%. A single value of 97% chloroquine resistance from South America was not graphed. See Table 3 for study details and references

gametocytes of *P. falciparum*. The avian malaria *P. relictum* also has somewhat drug-resistant gametocytes [67]. In separate reports quinine has a delayed parasite clearance time unique to *P. malariae*. Chloroquine or atebrine would clear parasites in 3–4 days, while quinine would take 6–9 days. Fletcher first analyzed cases in the 1920s with delayed parasite clearance for *P. malariae* (Table 4) [67]. Young and Eyles also noted the delayed parasite clearance with quinine rather than with quinacrine [92]. *P. vivax* was cleared much faster even with higher parasite densities. Delayed parasite clearance kinetics is not a phenotype unique to the artemisinin drugs and *P. falciparum*.

8 Molecular-Resistance Mechanisms

Like other quinolines, quinine resistant parasites have less intracellular drug concentrations. Recent experimental data suggest two phenotypes involved with quinine resistance: lowered drug accumulation [93] and a resistance to toxic effects

Table 3 Quinine and chloroquine resistance from patient studies with IC_{50} value determinations

Region	Country of origin	No. patients	No. with IC_{50} CQ >100 nM	No. with IC_{50} QN >500 nM	No. with IC_{50} MFQ >25 nM	Method	Year isolated	Reference
Africa	Senegal	15		1		RAD	1984	[71]
Africa	Equatorial Guinea	104	16	4		BF	1990	[72]
Africa	Senegal	85	25	1	19	RAD	1995	[73]
Africa	Senegal	161	76	16		RAD	1996	[74]
Africa	Malawi	35	1	2	1	RAD	1998	[75]
Africa	Senegal	51		6		RAD	1999	[76]
Africa	Gabon	65		11		RAD	1999	[77]
Africa	Senegal	70	25	10		LDH	2000	[78]
Africa	Senegal	70	43	27		LDH	2001	[78]
Africa	Senegal	70	36	5		LDH	2002	[78]
Africa	Senegal	70	43	8		LDH	2003	[78]
Africa	Rwanda	74	33	6		RAD	2003	[79]
Africa	Senegal	70	44	6		LDH	2004	[78]
Africa	DRCongo	110	83	24	8	RAD	2005	[80]
Africa	Uganda	196		9		HRPII	2006	[81]
Africa	Madagascar	29		1		RAD	2006	[82]
SEA	Philippine	59	51	2	4	BF	1983	[83]
SEA	Papua New Guinea	31	30	3		BF	1995	[84]
SEA	Thailand	22		5		HRPII	1999	[85]
SEA	Bangladesh	44		13		HRPII	1999	[85]
SEA	Thailand	95		4		RAD	2000	[86]
SEA	Cambodia	293	191	2		RAD	2001	[87]
SEA	Thailand–Burma	26	6	3	12	SYBR Green	2007	[88]
South America	French Guiana	22		2		RAD	1995	[89]
South America	Brazil	30	29	1	0	RAD	1996	[90]
South America	French Guiana	48		5		RAD	1996	[89]

(continued)

Table 3 (continued)

Region	Country of origin	No. patients	No. with IC_{50} CQ >100 nM	No. with IC_{50} QN >500 nM	No. with IC_{50} MFQ >25 nM	Method	Year isolated	Reference
South America	French Guiana	68		8		RAD	1997	[89]
South America	French Guiana	47		2		RAD	1998	[89]
South America	French Guiana	52		2		RAD	1999	[89]
South America	French Guiana	146		3		RAD	2002	[89]
South America	French Guiana	159		6		RAD	2003	[89]
South America	French Guiana	69		3		RAD	2004	[89]
South America	French Guiana	59		3		RAD	2005	[89]

SEA Southeast Asia, *RAD* radioactive hypoxanthine, *HRPII* histidine-rich protein II ELISA, *BF* blood film, *LDH* Lactate dehydrogenase ELISA, *SYBR Green* syber green

Table 4 Parasite clearance times for *P. malariae*, *P. vivax*, and *P. falciparum* among cinchona alkaloids adapted from Fletcher [67]

	Quinine	Quinidine	Cinchonidine
Quartan *P. malariae*			
Number of patients	6	9	1
PCT (days)	6.8	3.7	5
STD PCT (days)	1.7	2.1	
GEOMEAN Parasitemia	793.7	266	333
Par/μl per day reduction	116	70	66
Tertian *P. vivax*			
Number of patients	5	4	3
PCT (days)	2.8	2.5	2.7
STD PCT (days)	1.1	1.0	1.2
GEOMEAN Parasitemia	1,408.8	759.6	584.8
Par/μl per day reduction	503.1	303.9	219.3
Malignant tertian *P. falciparum*			
Number of patients	6	6	5
PCT (days)	2.8	2.3	5.2
STD PCT (days)	0.4	0.8	3.6
GEOMEAN Parasitemia	3,984.2	3,549.5	1,727.5
Par/μl per day reduction	1,406.2	1,521.2	332.2

of the drug on the parasite. Roepe and colleagues show, at similar intracellular drug concentrations, different toxicity on different isolates [34, 94]. Fidock and colleagues have transferred the same chloroquine-resistant transporter (PfCRT) or the sodium–proton exchanger (PfNHE) into two different isolates to show different tolerance to the same extracellular concentrations of parasiticidal quinolines [95]. While the resistance to quinolines involves different transporters and varies between *Plasmodium* species, data are emerging implicating the multidrug resistance transporter, PfMDR1, the multidrug resistance protein, PfMRP, and PfCRT and PfNHE as all being involved in quinoline resistance [93, 96]. The wild type MDR1 transports vinblastine, chloroquine, and quinine [97]. The "resistance" mutations in PfMDR1 do not transport quinine or chloroquine but can still transport vinblastine. The related halofantrine, in contrast, is not transported by wildtype mdr1 but can be effluxed out of *Xenopus* oocytes by mutant PfMDR1. Importantly, quinine can act as an inhibitor of vinblastine transport. Quinine may be acting in a dual role as both inhibitor and substrate of this transporter, compatible with the suggestion that its binding pocket is separate from its transport site. Quinine and mefloquine compete for binding of labeled chloroquine with higher affinity than chloroquine to the PfMDR1 protein [98]. Selection of PfCRT mutations leads to chloroquine resistance and increased sensitivity to quinine [99]. Clinically, the association of quinine resistance with PfHNE is not epidemiologically as strong as chloroquine with PfCRT [100].

9 Toxicity

Quinine received 2.4 million prescriptions in England in 2000–2001 [101]. Toxic overdose is associated with blindness and cardiac toxicity. The maximum daily dose for humans is 33 mg/kg or 2 g for a 60 kg human. Intake of 2–8 g can kill an adult and 900–2,400 mg can kill a child. In a patient (without malaria), levels over 15 mg/L increase risk of blindness and cardiotoxicity [102, 103]. Besides the use of oral charcoal and benzodiazepines, treatment for quinine overdose is supportive. In a case report, complete visual loss, tinnitus and bilateral hearing loss, headache, and diminished taste sensation from quinine have been managed successfully with the calcium antagonist flunarizine and stellate ganglia block [104]. However, most experts report marginal to no success with ganglion block for blindness [105–107]. In mice, the single oral lethal dose 50 is 1,100 mg/kg, rabbits 208 mg/kg, and rhesus monkeys 13 mg/kg [108].

Blackwater fever is a syndrome of hemolysis, fever, and black urine or hemoglobinuria [109]. In the first half of the nineteenth century, it was associated with Europeans residing in the tropics, having little immunity to *P. falciparum* [110]. A rate of 15% in some populations has been reported. Parasitemia at time of presentation was usually not high. While quinine was the most consistent initiator of blackwater fever, it did occur in the setting of no quinine [109]. Acute hemoglobinuria can be caused by high parasitemia in its own right, glucose-6-phosphate dehydrogenase (G6PD) deficiency-induced hemolysis, and drug-related hemolysis, most notably for sulfa drugs and quinine [110]. Largely with the implementation of chloroquine use in the 1950s, the incidence of blackwater fever decreased. Recently, anecdotal cases have been reported in African individuals with and without G6PD deficiency timed with quinine use and history of frequent recurring infections [111]. A recent larger series of 21 cases was documented in Europeans who had lived in sub-Saharan Africa from 1990 to 1999 [112]. Two-thirds had normal G6PD levels and about one-third had low parasitemias. The arylaminoalcohols, quinine, mefloquine, and halofantrine were associated in more than 80% of case presentations [113].

In the use of quinine for leg cramps in the absence of malaria parasites, a quinine-induced thrombocytopenia can occur ("cocktail purpura"). This was first noted in the literature by Dr. Vipan in 1865 in The Lancet in four cases following quinine therapy [114]. The first case was a lady of 50 years prescribed quinine for heart neuralgia. She developed purpura and a bloody discharge at the site of therapeutic chest blistering. Quinine was stopped with resolution of purpura in a few days. The purpura recurred months later with quinine retreatment for dental neuralgia. The second case was a lady with tertian malaria treated with quinine, which developed purpura on the second day. The purpura resolved by day 7 after quinine cessation. In the third case, a boy with general disability and no specific diseases developed purpura a few days following quinine therapy. Despite purpura, the physician continued quinine until bleeding gums developed. The fourth case was a patient taking quinine for 2 weeks for hay fever who developed purpura

3 days after the physician's visit. Dr. Vipan closed with the statement "If these few notes on the effects of quinine prove of any use to the profession or add one drop to the ocean of science, the purpose of the writer will be fully answered" [114].

The mechanism of purpura has been drug-induced antibodies to quinine or quinidine [115]. The precipitating drug exposure can be cocktail purpura from bitter lemon or tonic water containing about 80 mg/L or about 20 mg per cocktail. Quinine, cyclosporine, mitomycin C, and ticlopidine were the most common drugs causing thrombotic thrombocytopenic purpura and hemolytic uremic syndrome [116]. Antibodies bind tightly to drug adherent on platelets on glycoproteins GP IIb–IIIa and/or GP Ib-V–IX [117]. Hemolytic uremic syndrome can also be from antibodies binding to endothelial cells [116, 118, 119]. As much as 82% of the thrombocytopenia cases reported nocturnal leg cramps as the reason for taking quinine [120]. From 1969 to 2000, the FDA received 113 cases of quinine-induced thrombocytopenia (3.6 per year). The cases peaked in 1996 with about 20 decreasing with the FDA elimination of quinine from over-the-counter formularies [121]. After the FDA ban on quinine for leg cramps, the FDA recently reported 38 cases of quinine-associated thrombocytopenia and 2 deaths with the continued use of quinine from the period April 2005 to October 2008 (8.4 cases of thrombocytopenia each year) [122]. A recent Cochrane review for leg cramps and quinine use looked at 23 trials with 1,586 participants, who met the clinical evidence criteria [123]. Quinine at 300 mg per night reduced cramp number by almost 30% from 8.4 cramps per week to 6.3 cramps per week. They found that besides minor gastrointestinal symptoms, serious adverse effects were not greater than placebo up to 60 days of use. Equipoise exists for quinine use in leg cramps.

10 Quinine Pharmacokinetics

The usual adult dose for treating uncomplicated malaria is 10 mg/kg of salt three times a day for 7–14 days. This dosing regimen can be shortened, if quinine is combined with an antibiotic with antimalarial activity, such as clindamycin (10 mg/kg twice daily for 3–7 days) or doxycycline (100 mg once a day for 7 days, if not contraindicated). Dosing after meals decreases the gastric irritation. Totaquine is a mixture of about 75% of the total crystallizable cinchona alkaloids containing about 20% quinine [92, 124]. The cinchona alkaloids are of similar potency to quinine [125, 126]. Quinine and quinidine is usually completely absorbed from the gastrointestinal tract, reaching peak levels in 1–3 h [127]. The elimination half-time increases from healthy subjects (~10 h) to uncomplicated malaria (15 h) to severe malaria (about 20 h) [128]. In the presence of parasitemia, a greater amount of quinine is found in the red blood cell fraction, which quickly decreases. Plasma levels remain elevated because of the increase in the acidic/basic glycoprotein, which is an acute-phase serum protein. The C_{max} after a 20 mg/kg loading dose is 15–16 mg/L. Levels can sometimes reach 20 mg/L in malaria patients but free quinine remains in the nontoxic range below 2 mg/L, with the increase in the acidic/

basic glycoprotein [102]. Only 20% of quinine is removed in the urine with the rest transformed in the liver to 3- and 2-hydroxyquinine by cytochrome P450 3A4, and then to more water-soluble molecules [129]. Of the cytochrome P450s, 2D6 is inhibited by quinine or quinidine via the nitrogen association with aspartate amino acid number 301 [130]. A growing much-needed evidence base is beginning to explore the interaction of the quinolines, like quinine, with the highly active antiretrovirals [131–133]. The ingestion of a liter of tonic water with 80 mg of quinine does not inhibit cytochrome 2D6 [134].

The pharmacodynamic relationship of quinine was studied in 30 adult patients [54]. The mean parasite clearance time was 73 h with a standard deviation of 24 h. Patients with an area under the concentration time curve less than 20 mg/L from 3 to7 days had a 5.3 relative risk increase in recrudescence. The authors determined a minimum parasiticidal concentration of 3.4 mg/L in the plasma and minimal inhibitory concentration of 0.7 mg/L. Treatment for more than 6 days was required to cure patients. In contrast to a 4 log drop in parasites with artemisinins, the parasite reduction was 250-fold per 48 h cycle [54]. These data are in approximate agreement of the mean plasma concentration for the four cinchona alkaloids of 5 mg/L for quinine, 1 mg/L for quinidine, 0.1 mg/L for cinchonine, and 2.5 mg/L for cinchonidine [135].

11 Usage in Severe Malaria

If there has been no previous treatment with quinine or quinidine and a patient is severely ill, then a loading dose of quinine or quinidine is recommended [136], if artesunate is unavailable. For quinine treatment of adults, this would consist of an intravenous infusion of 20 mg/kg of dihydrochloride salt, given over 2–4 h, followed every 8 h by an infusion of 10 mg (salt)/kg given over 2–4 h. Children in Africa can be treated with 20 mg salt/kg as a loading dose, infused as for adults or given by intramuscular injection [137]. If the intramuscular route is used, then the quinine is diluted (1:1, vol:vol with normal saline), and the loading dose is split in two and given to the anterior thigh to minimize local toxicity while preserving appropriate pharmacokinetic profiles. Maintenance doses can be continued by the intramuscular route every 8–12 h. Conversion to the oral route of treatment is recommended as soon as this can be tolerated. If there is multiorgan failure, then a dose reduction is suggested after 48 h of treatment (usually applicable to adults).

Dosing regimens are different for quinidine, for which electrocardiographic monitoring is recommended also because of the greater risk of associated cardiotoxicity [136]. Both drugs require monitoring of glucose for hypoglycemia, which can be persistently severe in pregnancy. This occurs in 50% of pregnant women treated with quinine versus 10% in the nonpregnant population [136].

12 Conclusions

Quinine has been a tremendous success helping to eliminate malaria in mass quinization campaigns in Italy [32]. Through the centuries, quinine has saved countless lives. With the small increase in quinine resistance in Southeast Asia, addition of the antibacterials tetracycline or clindamycin prolonged effectiveness of quinine in severe malaria [138, 139]. Recently, the artemisinin combination therapy and intravenous artesunate have proven superior to quinine for severe malaria [140–143]. Recent reports of artemisinin-associated delayed parasite clearance times in the absence of an increase in clinically relevant IC_{50} values have given many investigators cause for concern regarding the longevity of artemisinin usefulness [144, 145]. In addition to novel antimalarials, returning to the rich chemistry of quinine and its congeners may be able to back up the artemisinins as a rapidly parasitical drug.

Acknowledgments The translation of Arthur Neiva's account of drug resistance from German and Portuguese by Gundula Bosch is most appreciated.

References

1. Bruce-Chwatt LJ (1988) Three hundred and fifty years of the Peruvian fever bark. Br Med J (Clin Res Ed) 296:1486–1487
2. Petithory JC (1995) [On the discovery of the parasite of malaria by A. Laveran: Bone 1878–Constantine 1880]. Hist Sci Med 29:57–62
3. Guttmann P, Ehrlich P (1891) Ueber die Wirkung des Methylenblau bei Malaria. Berlin Klin Wochenschr 28:953–956
4. Vennerstrom JL, Makler MT, Angerhofer CK, Williams JA (1995) Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines. Antimicrob Agents Chemother 39:2671–2677
5. Greenwood D (1992) The quinine connection. J Antimicrob Chemother 30:417–427
6. Guerra F (1977) The introduction of cinchona in the treatment of malaria. Part II. J Trop Med Hyg 80:135–140
7. Guerra F (1977) The introduction of Cinchona in the treatment of malaria. Part I. J Trop Med Hyg 80:112–118
8. Garnham PC (1966) Malaria parasites and other haemosporidia. Blackwell Scientific, Oxford
9. Bruce-Chwatt LJ (1982) Oliver Cromwell's medical history. Trans Stud Coll Physicians Phila 4:98–121
10. Vane JR (2000) The fight against rheumatism: from willow bark to COX-1 sparing drugs. J Physiol Pharmacol 51:573–586
11. Greenwood D (1995) Conflicts of interest: the genesis of synthetic antimalarial agents in peace and war. J Antimicrob Chemother 36:857–872
12. Gramiccia G (1987) Ledger's cinchona seeds: a composite of field experience, chance, and intuition. Parassitologia 29:207–220
13. Delepine M (1951) Joseph Pelletier and Joseph Caventou. J Chem Educ 28:454–461
14. (1890) CXXVIII – Manufacture of quinine in India. Bull Misc Inf (Royal Gardens, Kew) 1890:29–34; www.jstororg/stable/4118253

15. Rocco F (2003) Quinine, malaria and the quest for a cure that changed the world. Perrenial, New York
16. Strecker A (1854) On the constitution of quinine. Lond Edinb Dublin Philos Mag J Sci 8:123–125
17. Johnston WT (2008) The discovery of aniline and the origin of the term "aniline dye". Biotech Histochem 83:83–87
18. Steverding D (2010) The development of drugs for treatment of sleeping sickness: a historical review. Parasit Vectors 3:15
19. Gensini GF, Conti AA, Lippi D (2007) The contributions of Paul Ehrlich to infectious disease. J Infect 54:221–224
20. Frankenburg FR, Baldessarini RJ (2008) Neurosyphilis, malaria, and the discovery of antipsychotic agents. Harv Rev Psychiatry 16:299–307
21. Horlein H (1936) The chemotherapy of infectious diseases caused by protozoa and bacteria: (Section of tropical diseases and parasitology). Proc R Soc Med 29:313–324
22. Lesher GY, Froelich EJ, Gruett MD, Bailey JH, Brundage RP (1962) 1,8-Naphthyridine derivatives: a new class of chemotherapeutic agents. J Med Pharm Chem 91:1063–1065
23. Andriole VT (2005) The quinolones: past, present, and future. Clin Infect Dis 41(Suppl 2): S113–119
24. Krishna S, Davis TM, Chan PC, Wells RA, Robson KJ (1988) Ciprofloxacin and malaria. Lancet 1:1231–1232
25. Mahmoudi N, Ciceron L, Franetich JF, Farhati K, Silvie O, Eling W, Sauerwein R, Danis M, Mazier D, Derouin F (2003) *In vitro* activities of 25 quinolones and fluoroquinolones against liver and blood stage *Plasmodium* spp. Antimicrob Agents Chemother 47:2636–2639
26. Seeman J (2007) The Woodward-Doering/Rabe-Kindler total synthesis of quinine: setting the record straight. Angew Chem Int Ed Engl 46:1378–1413
27. Stork G, Niu D, Fujimoto RA, Koft ER, Balkovec JM, Tata JR, Dake GR (2001) The first stereoselective total synthesis of quinine. J Am Chem Soc 123:3239–3242
28. Woodward RB, Doering WE (1944) The total synthesis of quinine. JACS 66:849
29. ter Kuile F, White NJ, Holloway P, Pasvol G, Krishna S (1993) *Plasmodium falciparum*: *in vitro* studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. Exp Parasitol 76:85–95
30. Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, White NJ (1996) Effects of artemisinin derivatives on malaria transmissibility. Lancet 347:1654–1658
31. Fink E (1974) Assessment of causal prophylactic activity in *Plasmodium berghei yoelii* and its value for the development of new antimalarial drugs. Bull World Health Organ 50:213–222
32. Brown PJ (1997) Malaria, miseria, and underpopulation in Sardinia: the "malaria blocks development" cultural model. Med Anthropol 17:239–254
33. Davies EE, Warhurst DC, Peters W (1975) The chemotherapy of rodent malaria, XXI. Action of quinine and WR 122 (a 9-phenanthrenemethanol) on the fine structure of *Plasmodium berghei* in mouse blood. Ann Trop Med Parasitol 69:147–153
34. Gligorijevic B, Purdy K, Elliott DA, Cooper RA, Roepe PD (2008) Stage independent chloroquine resistance and chloroquine toxicity revealed via spinning disk confocal microscopy. Mol Biochem Parasitol 159:7–23
35. Slater AF (1993) Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum*. Pharmacol Ther 57:203–235
36. Warhurst DC, Craig JC, Adagu IS, Meyer DJ, Lee SY (2003) The relationship of physicochemical properties and structure to the differential antiplasmodial activity of the cinchona alkaloids. Malar J 2:26
37. Ginsburg H, Geary TG (1987) Current concepts and new ideas on the mechanism of action of quinoline-containing antimalarials. Biochem Pharmacol 36:1567–1576

38. Ginsburg H, Golenser J (2003) Glutathione is involved in the antimalarial action of chloroquine and its modulation affects drug sensitivity of human and murine species of *Plasmodium*. Redox Rep 8:276–279
39. Sullivan DJ Jr, Matile H, Ridley RG, Goldberg DE (1998) A common mechanism for blockade of heme polymerization by antimalarial quinolines. J Biol Chem 273:31103–31107
40. Sullivan DJ Jr, Gluzman IY, Russell DG, Goldberg DE (1996) On the molecular mechanism of chloroquine's antimalarial action. Proc Natl Acad Sci USA 93:11865–11870
41. Egan TJ, Mavuso WW, Ross DC, Marques HM (1997) Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX. J Inorg Biochem 68:137–145
42. de Villiers KA, Marques HM, Egan TJ (2008) The crystal structure of halofantrine-ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. J Inorg Biochem 102:1660–1667
43. Leed A, DuBay K, Ursos LM, Sears D, De Dios AC, Roepe PD (2002) Solution structures of antimalarial drug-heme complexes. Biochemistry 41:10245–10255
44. Alumasa JN, Gorka AP, Casabianca LB, Comstock E, de Dios AC, Roepe PD (2011) The hydroxyl functionality and a rigid proximal N are required for forming a novel non-covalent quinine-heme complex. J Inorg Biochem 105:467–475
45. Chong CR, Sullivan DJ Jr (2003) Inhibition of heme crystal growth by antimalarials and other compounds: implications for drug discovery. Biochem Pharmacol 66:2201–2212
46. Ellekvist P, Maciel J, Mlambo G, Ricke CH, Colding H, Klaerke DA, Kumar N (2008) Critical role of a K^+ channel in *Plasmodium berghei* transmission revealed by targeted gene disruption. Proc Natl Acad Sci USA 105:6398–6402
47. Waller KL, Kim K, McDonald TV (2008) *Plasmodium falciparum*: growth response to potassium channel blocking compounds. Exp Parasitol 120:280–285
48. Waller KL, McBride SM, Kim K, McDonald TV (2008) Characterization of two putative potassium channels in *Plasmodium falciparum*. Malar J 7:19
49. Sanchez-Chapula JA, Ferrer T, Navarro-Polanco RA, Sanguinetti MC (2003) Voltage-dependent profile of human ether-a-go-go-related gene channel block is influenced by a single residue in the S6 transmembrane domain. Mol Pharmacol 63:1051–1058
50. Becchetti A, De Fusco M, Crociani O, Cherubini A, Restano-Cassulini R, Lecchi M, Masi A, Arcangeli A, Casari G, Wanke E (2002) The functional properties of the human ether-a-go-go-like (HELK2) K^+ channel. Eur J Neurosci 16:415–428
51. Kuryshev YA, Ficker E, Wang L, Hawryluk P, Dennis AT, Wible BA, Brown AM, Kang J, Chen XL, Sawamura K et al (2005) Pentamidine-induced long QT syndrome and block of hERG trafficking. J Pharmacol Exp Ther 312:316–323
52. White NJ (2004) Antimalarial drug resistance. J Clin Invest 113:1084–1092
53. Le Jouan M, Jullien V, Tetanye E, Tran A, Rey E, Treluyer JM, Tod M, Pons G (2005) Quinine pharmacokinetics and pharmacodynamics in children with malaria caused by *Plasmodium falciparum*. Antimicrob Agents Chemother 49:3658–3662
54. Pukrittayakamee S, Wanwimolruk S, Stepniewska K, Jantra A, Huyakorn S, Looareesuwan S, White NJ (2003) Quinine pharmacokinetic-pharmacodynamic relationships in uncomplicated falciparum malaria. Antimicrob Agents Chemother 47:3458–3463
55. Karbwang J, Harinasuta T (1992) Overview: clinical pharmacology of antimalarials. SE Asian J Trop Med Public Health 23(Suppl 4):95–109
56. Caramello P, Canta F, Cavecchia I, Sergi G, Lipani F, Calleri G, Gobbi F, Di Perri G (2005) Pharmacodynamic analysis of antimalarials used in *Plasmodium falciparum* imported malaria in northern Italy. J Travel Med 12:127–132
57. Mapaba E, Hellgren U, Landberg-Lindgren A, Rombo L (1995) Susceptibility of *Plasmodium falciparum* to quinine in vitro: effects of drug concentrations and time of exposure. Trans R Soc Trop Med Hyg 89:85–89
58. Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X, Ferdig MT (2007) Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine

resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Mol Microbiol 63:270–282
59. Karle JM, Karle IL, Gerena L, Milhous WK (1992) Stereochemical evaluation of the relative activities of the cinchona alkaloids against *Plasmodium falciparum*. Antimicrob Agents Chemother 36:1538–1544
60. Karle JM, Bhattacharjee AK (1999) Stereoelectronic features of the cinchona alkaloids determine their differential antimalarial activity. Bioorg Med Chem 7:1769–1774
61. Brocks DR, Mehvar R (2003) Stereoselectivity in the pharmacodynamics and pharmacokinetics of the chiral antimalarial drugs. Clin Pharmacokinet 42:1359–1382
62. Basco LK, Le Bras J (1992) *In vitro* activity of halofantrine and its relationship to other standard antimalarial drugs against African isolates and clones of *Plasmodium falciparum*. Am J Trop Med Hyg 47:521–527
63. White NJ (1998) Why is it that antimalarial drug treatments do not always work? Ann Trop Med Parasitol 92:449–458
64. Weinke T, Held T, Trautmann M, Rogler G, Mravak S, Alexander M, Pohle HD (1992) Malaria therapy in 452 patients, with special reference to the use of quinine. J Infect 25:173–180
65. Newton PN, Green MD, Fernandez FM (2010) Impact of poor-quality medicines in the 'developing' world. Trends Pharmacol Sci 31:99–101
66. Basco LK (2004) Molecular epidemiology of malaria in Cameroon. XIX. Quality of antimalarial drugs used for self-medication. Am J Trop Med Hyg 70:245–250
67. Fletcher W (1923) Notes on the treatment of malaria with the alkaloids of cinchona. John Bale, London
68. Looareesuwan S, Charoenpan P, Ho M, White NJ, Karbwang J, Bunnag D, Harinasuta T (1990) Fatal *Plasmodium falciparum* malaria after an inadequate response to quinine treatment. J Infect Dis 161:577–580
69. Peters W (1969) Drug resistance in malaria – a perspective. Trans R Soc Trop Med Hyg 63:25–45
70. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC et al (2003) Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 49:977–989
71. Brandicourt O, Druilhe P, Diouf F, Brasseur P, Turk P, Danis M (1986) Decreased sensitivity to chloroquine and quinine of some *Plasmodium falciparum* strains from Senegal in September 1984. Am J Trop Med Hyg 35:717–721
72. Benito A, Roche J, Molina R, Amela C, Alvar J (1995) *In vitro* susceptibility of *Plasmodium falciparum* to chloroquine, amodiaquine, quinine, mefloquine, and sulfadoxine/pyrimethamine in Equatorial Guinea. Am J Trop Med Hyg 53:526–531
73. Pradines B, Rogier C, Fusai T, Tall A, Trape JF, Doury JC (1996) *In vitro* sensitivity of 85 *Plasmodium falciparum* isolates in the Fatick region, Senegal. Med Trop (Mars) 56:141–145
74. Pradines B, Tall A, Parzy D, Spiegel A, Fusai T, Hienne R, Trape JF, Doury JC (1998) *In-vitro* activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial agents. J Antimicrob Chemother 42:333–339
75. Takechi M, Matsuo M, Ziba C, MacHeso A, Butao D, Zungu IL, Chakanika I, Bustos MD (2001) Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility *in vitro* of *P. falciparum* isolates to sulphadoxine-pyremethamine and other antimalarial drugs in Malawian children. Trop Med Int Health 6:429–434
76. Pradines B, Tall A, Rogier C, Spiegel A, Mosnier J, Marrama L, Fusai T, Millet P, Panconi E, Trape JF et al (2002) *In vitro* activities of ferrochloroquine against 55 Senegalese isolates of *Plasmodium falciparum* in comparison with those of standard antimalarial drugs. Trop Med Int Health 7:265–270
77. Pradines B, Fusai T, Daries W, Laloge V, Rogier C, Millet P, Panconi E, Kombila M, Parzy D (2001) Ferrocene-chloroquine analogues as antimalarial agents: *in vitro* activity of

ferrochloroquine against 103 Gabonese isolates of *Plasmodium falciparum*. J Antimicrob Chemother 48:179–184
78. Agnamey P, Brasseur P, de Pecoulas PE, Vaillant M, Olliaro P (2006) *Plasmodium falciparum in vitro* susceptibility to antimalarial drugs in Casamance (southwestern Senegal) during the first 5 years of routine use of artesunate-amodiaquine. Antimicrob Agents Chemother 50:1531–1534
79. Tinto H, Rwagacondo C, Karema C, Mupfasoni D, Vandoren W, Rusanganwa E, Erhart A, Van Overmeir C, Van Marck E, D'Alessandro U (2006) *In-vitro* susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda. Trans R Soc Trop Med Hyg 100:509–514
80. Pradines B, Hovette P, Fusai T, Atanda HL, Baret E, Cheval P, Mosnier J, Callec A, Cren J, Amalvict R et al (2006) Prevalence of *in vitro* resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. J Clin Microbiol 44:2404–2408
81. Nsobya SL, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ (2010) *In vitro* sensitivities of *Plasmodium falciparum* to different antimalarial drugs in Uganda. Antimicrob Agents Chemother 54:1200–1206
82. Andriantsoanirina V, Menard D, Rabearimanana S, Hubert V, Bouchier C, Tichit M, Bras JL, Durand R (2010) Association of microsatellite variations of *Plasmodium falciparum* Na^+/H^+ exchanger (*Pfnhe-1*) gene with reduced *in vitro* susceptibility to quinine: lack of confirmation in clinical isolates from Africa. Am J Trop Med Hyg 82:782–787
83. Smrkovski LL, Buck RL, Alcantara AK, Rodriguez CS, Uylangco CV (1985) Studies of resistance to chloroquine, quinine, amodiaquine and mefloquine among Philippine strains of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 79:37–41
84. Hombhanje FW (1998) In vitro susceptibility of *Plasmodium falciparum* to four antimalarial drugs in the Central Province of Papua New Guinea. P N G Med J 41:51–58
85. Noedl H, Faiz MA, Yunus EB, Rahman MR, Hossain MA, Samad R, Miller RS, Pang LW, Wongsrichanalai C (2003) Drug-resistant malaria in Bangladesh: an *in vitro* assessment. Am J Trop Med Hyg 68:140–142
86. Chaijaroenkul W, Bangchang KN, Mungthin M, Ward SA (2005) *In vitro* antimalarial drug susceptibility in Thai border areas from 1998–2003. Malar J 4:37
87. Lim P, Chim P, Sem R, Nemh S, Poravuth Y, Lim C, Seila S, Tsuyuoka R, Denis MB, Socheat D et al (2005) *In vitro* monitoring of *Plasmodium falciparum* susceptibility to artesunate, mefloquine, quinine and chloroquine in Cambodia: 2001–2002. Acta Trop 93:31–40
88. Chaijaroenkul W, Wisedpanichkij R, Na-Bangchang K (2010) Monitoring of *in vitro* susceptibilities and molecular markers of resistance of *Plasmodium falciparum* isolates from Thai-Myanmar border to chloroquine, quinine, mefloquine and artesunate. Acta Trop 113:190–194
89. Legrand E, Volney B, Meynard JB, Mercereau-Puijalon O, Esterre P (2008) *In vitro* monitoring of *Plasmodium falciparum* drug resistance in French Guiana: a synopsis of continuous assessment from 1994 to 2005. Antimicrob Agents Chemother 52:288–298
90. Cerutti Junior C, Marques C, Alencar FE, Durlacher RR, Alween A, Segurado AA, Pang LW, Zalis MG (1999) Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Brazil using a radioisotope method. Mem Inst Oswaldo Cruz 94:803–809
91. Lim P, Wongsrichanalai C, Chim P, Khim N, Kim S, Chy S, Sem R, Nhem S, Yi P, Duong S et al (2010) Decreased *in vitro* susceptibility of *Plasmodium falciparum* isolates to artesunate, mefloquine, chloroquine, and quinine in Cambodia from 2001 to 2007. Antimicrob Agents Chemother 54:2135–2142
92. Young MD, Eyles DE (1948) The efficacy of chloroquine, quinacrine, quinine and totaquine in the treatment of *Plasmodium malariae* infections (quartan malaria). Am J Trop Med Hyg 28:23–28

93. Sanchez CP, Stein WD, Lanzer M (2008) Dissecting the components of quinine accumulation in *Plasmodium falciparum*. Mol Microbiol 67:1081–1093
94. Cabrera M, Paguio MF, Xie C, Roepe PD (2009) Reduced digestive vacuolar accumulation of chloroquine is not linked to resistance to chloroquine toxicity. Biochemistry 48:11152–11154
95. Nkrumah LJ, Riegelhaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, Wellems TE, Akabas MH, Fidock DA (2009) Probing the multifactorial basis of *Plasmodium falciparum* quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. Mol Biochem Parasitol 165:122–131
96. Ursing J, Zakeri S, Gil JP, Bjorkman A (2006) Quinoline resistance associated polymorphisms in the *pfcrt*, *pfmdr1* and *pfmrp* genes of *Plasmodium falciparum* in Iran. Acta Trop 97:352–356
97. Sanchez CP, Rotmann A, Stein WD, Lanzer M (2008) Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol Microbiol 70:786–798
98. Pleeter P, Lekostaj JK, Roepe PD (2010) Purified *Plasmodium falciparum* multi-drug resistance protein (PfMDR 1) binds a high affinity chloroquine analogue. Mol Biochem Parasitol 173:158–161
99. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol Pharmacol 61:35–42
100. Henry M, Briolant S, Zettor A, Pelleau S, Baragatti M, Baret E, Mosnier J, Amalvict R, Fusai T, Rogier C et al (2009) *Plasmodium falciparum* Na^+/H^+ exchanger 1 transporter is involved in reduced susceptibility to quinine. Antimicrob Agents Chemother 53:1926–1930
101. Langford NJ, Good AM, Laing WJ, Bateman DN (2003) Quinine intoxications reported to the Scottish Poisons Information Bureau 1997–2002: a continuing problem. Br J Clin Pharmacol 56:576–578
102. Taylor WR, White NJ (2004) Antimalarial drug toxicity: a review. Drug Saf 27:25–61
103. Touze JE, Heno P, Fourcade L, Deharo JC, Thomas G, Bohan S, Paule P, Riviere P, Kouassi E, Buguet A (2002) The effects of antimalarial drugs on ventricular repolarization. Am J Trop Med Hyg 67:54–60
104. Gous P, Haus M (1990) Intravenous flunarizine therapy for acute toxicity in malaria. S Afr Med J 77:217
105. Bacon P, Spalton DJ, Smith SE (1988) Blindness from quinine toxicity. Br J Ophthalmol 72:219–224
106. Dyson EH, Proudfoot AT, Prescott LF, Heyworth R (1985) Death and blindness due to overdose of quinine. Br Med J (Clin Res Ed) 291:31–33
107. Dickinson P, Sabto J, West RH (1981) Management of quinine toxicity. Trans Ophthalmol Soc N Z 33:56–58
108. Hill J (1963) Part 2: The antimalarial drugs. In: Schnitzer RJ, Hawking F (eds) Experimental chemotherapy, vol 1. Academic, New York, pp 513–602
109. George CR (2009) Blackwater fever: the rise and fall of an exotic disease. J Nephrol22 (Suppl 14):120–128
110. Bruce-Chwatt LJ (1987) Quinine and the mystery of blackwater fever. Acta Leiden 55:181–196
111. Rogier C, Imbert P, Tall A, Sokhna C, Spiegel A, Trape JF (2003) Epidemiological and clinical aspects of blackwater fever among African children suffering frequent malaria attacks. Trans R Soc Trop Med Hyg 97:193–197
112. Bruneel F, Gachot B, Wolff M, Regnier B, Danis M, Vachon F (2001) Resurgence of blackwater fever in long-term European expatriates in Africa: report of 21 cases and review. Clin Infect Dis 32:1133–1140
113. Van den Ende J, Coppens G, Verstraeten T, Van Haegenborgh T, Depraetere K, Van Gompel A, Van den Enden E, Clerinx J, Colebunders R, Peetermans WE et al (1998) Recurrence of

blackwater fever: triggering of relapses by different antimalarials. Trop Med Int Health 3:632–639
114. Vipan WH (1865) Quinine as a cause of purpura. Lancet 86:37
115. Aster RH (1999) Drug-induced immune thrombocytopenia: an overview of pathogenesis. Semin Hematol 36:2–6
116. Zakarija A, Bennett C (2005) Drug-induced thrombotic microangiopathy. Semin Thromb Hemost 31:681–690
117. Burgess JK, Lopez JA, Berndt MC, Dawes I, Chesterman CN, Chong BH (1998) Quinine-dependent antibodies bind a restricted set of epitopes on the glycoprotein Ib-IX complex: characterization of the epitopes. Blood 92:2366–2373
118. Park YA, Hay SN, King KE, Matevosyan K, Poisson J, Powers A, Sarode R, Shaz B, Brecher ME (2009) Is it quinine TTP/HUS or quinine TMA? ADAMTS13 levels and implications for therapy. J Clin Apher 24:115–119
119. Boehme MW, Werle E, Kommerell B, Raeth U (1994) Serum levels of adhesion molecules and thrombomodulin as indicators of vascular injury in severe *Plasmodium falciparum* malaria. Clin Investig 72:598–603
120. Kojouri K, Vesely SK, George JN (2001) Quinine-associated thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: frequency, clinical features, and long-term outcomes. Ann Intern Med 135:1047–1051
121. Brinker AD, Beitz J (2002) Spontaneous reports of thrombocytopenia in association with quinine: clinical attributes and timing related to regulatory action. Am J Hematol 70:313–317
122. (2010) Safety of quinine. Med Lett Drugs Ther, 52:88
123. El-Tawil S, Al Musa T, Valli H, Lunn MP, El-Tawil T, Weber M (2010) Quinine for muscle cramps. Cochrane Database Syst Rev 12:CD005044
124. Yeager OW, Reider RF, Mc DG (1946) Evaluation of totaquine in treatment of malaria in an endemic malarious area. J Am Pharm Assoc Am Pharm Assoc 35:337
125. Taggart JV, Earle DP Jr et al (1948) Studies on the chemotherapy of the human malarias; the physiological disposition and antimalarial activity of the cinchona alkaloids. J Clin Invest 27:80–86
126. Earle DP Jr, Welch WJ, Shannon JA et al (1948) Studies on the chemotherapy of the human malarias the metabolism of cinchonine in relation to its antimalarial activity. J Clin Invest 27:87–92
127. White NJ (1985) Clinical pharmacokinetics of antimalarial drugs. Clin Pharmacokinet 10:187–215
128. White NJ, Looareesuwan S, Warrell DA, Warrell MJ, Bunnag D, Harinasuta T (1982) Quinine pharmacokinetics and toxicity in cerebral and uncomplicated falciparum malaria. Am J Med 73:564–572
129. Zhao XJ, Yokoyama H, Chiba K, Wanwimolruk S, Ishizaki T (1996) Identification of human cytochrome P450 isoforms involved in the 3-hydroxylation of quinine by human live microsomes and nine recombinant human cytochromes P450. J Pharmacol Exp Ther 279:1327–1334
130. Hutzler JM, Walker GS, Wienkers LC (2003) Inhibition of cytochrome P450 2D6: structure-activity studies using a series of quinidine and quinine analogues. Chem Res Toxicol 16:450–459
131. Soyinka JO, Onyeji CO, Omoruyi SI, Owolabi AR, Sarma PV, Cook JM (2010) Pharmacokinetic interactions between ritonavir and quinine in healthy volunteers following concurrent administration. Br J Clin Pharmacol 69:262–270
132. Soyinka JO, Onyeji CO, Omoruyi SI, Owolabi AR, Sarma PV, Cook JM (2009) Effects of concurrent administration of nevirapine on the disposition of quinine in healthy volunteers. J Pharm Pharmacol 61:439–443
133. Nosten F, McGready R, D'Alessandro U, Bonell A, Verhoeff F, Menendez C, Mutabingwa T, Brabin B (2006) Antimalarial drugs in pregnancy: a review. Curr Drug Saf 1:1–15

134. Donovan JL, DeVane CL, Boulton D, Dodd S, Markowitz JS (2003) Dietary levels of quinine in tonic water do not inhibit CYP2D6 *in vivo*. Food Chem Toxicol 41:1199–1201
135. Taggart JV, Earle DP, Berliner RW, Zubrod CG, Welch WJ, Wise NB, Schroeder EF, London IM, Shannon JA (1948) Studies on the chemotherapy of the human malarias. III. The physiological disposition and antimalarial activity of the cinchona alkaloids. J Clin Invest 27:80–86
136. Krishna S, White NJ (1996) Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. Clin Pharmacokinet 30:263–299
137. Krishna S, Nagaraja NV, Planche T, Agbenyega T, Bedo-Addo G, Ansong D, Owusu-Ofori A, Shroads AL, Henderson G, Hutson A et al (2001) Population pharmacokinetics of intramuscular quinine in children with severe malaria. Antimicrob Agents Chemother 45:1803–1809
138. Watt G, Loesuttivibool L, Shanks GD, Boudreau EF, Brown AE, Pavanand K, Webster HK, Wechgritaya S (1992) Quinine with tetracycline for the treatment of drug-resistant falciparum malaria in Thailand. Am J Trop Med Hyg 47:108–111
139. Pukrittayakamee S, Chantra A, Vanijanonta S, Clemens R, Looareesuwan S, White NJ (2000) Therapeutic responses to quinine and clindamycin in multidrug-resistant falciparum malaria. Antimicrob Agents Chemother 44:2395–2398
140. Sinclair D, Donegan S, Lalloo DG: (2011) Artesunate versus quinine for treating severe malaria. Cochrane Database Syst Rev 3:CD005967
141. Phu NH, Tuan PQ, Day N, Mai NT, Chau TT, Chuong LV, Sinh DX, White NJ, Farrar J, Hien TT (2010) Randomized controlled trial of artesunate or artemether in Vietnamese adults with severe falciparum malaria. Malar J 9:97
142. Lubell Y, Yeung S, Dondorp AM, Day NP, Nosten F, Tjitra E, Abul Faiz M, Yunus EB, Anstey NM, Mishra SK et al (2009) Cost-effectiveness of artesunate for the treatment of severe malaria. Trop Med Int Health 14:332–337
143. Jones KL, Donegan S, Lalloo DG: (2007) Artesunate versus quinine for treating severe malaria. Cochrane Database Syst Rev:CD005967
144. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
145. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F (2010) Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob Agents Chemother 54:1872–1877

8-Aminoquinolines: Primaquine and Tafenoquine

Norman C. Waters and Michael D. Edstein

Abstract 8-Aminoquinolines are an important class of antimalarial drugs because they are effective against the liver stages of *Plasmodium* infections and thus are administered for radical cure and presumptive antirelapse therapy against relapsing malaria. In this chapter, we discuss two 8-aminoquinolines, primaquine and tafenoquine. Primaquine was identified in 1946 and has been used extensively to clear liver-stage parasites, especially those from *Plasmodium vivax*. These can persist in the liver for months, as a dormant form of the parasite (the hypnozoite), which re-emerges much later to cause clinical disease. Tafenoquine, a primaquine analog, is currently under advanced clinical development. Tafenoquine has a much longer elimination half-life compared with primaquine (14 days versus 6 h) and is highly effective both in treating relapses of *P. vivax* malaria and as a causal prophylactic agent against *P. falciparum* and *P. vivax* malaria. A major drawback to the 8-aminoquinolines is their toxicity in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals. We discuss clinical uses, pharmacokinetics and metabolism, safety and tolerability, mechanisms of action and drug resistance for both these drugs.

1 Introduction

The 8-aminoquinolines have a long history, being the first chemotype of synthetic antimalarials when pamaquine was used from the late 1920s [1]. In 1946, the screening of a large number of 8-aminoquinolines identified primaquine as a relatively safe and efficacious compound [2]. Today, additional 8-aminoquinolines have been synthesised as the search for safer and more efficacious compounds

N.C. Waters (✉) • M.D. Edstein
Australian Army Malaria Institute, Gallipoli Barracks, Weary Dunlop Drive, Enoggera, QLD 4051, Australia
e-mail: norman.waters@us.army.mil

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_4,

continues. From these efforts, tafenoquine is now in advanced clinical development and may become a new addition to the arsenal of antimalarial drugs.

The 8-aminoquinolines are effective against the exo-erythrocytic liver stages of the malaria parasite. This is central to preventing relapsing malaria as well as causal prophylaxis for malaria infections. Causal prophylaxis refers to the killing of parasites while they are in the liver, and thus prevents infection of erythrocytes and any signs of clinical disease. The efficacy of 8-aminoquinolines against liver-stage infection is especially valuable in the clearance of *P. vivax* and *P. ovale*, in which latent liver-stage forms known as hypnozoites can persist in the liver for months to years. Relapse infection occurs when the hypnozoites exit dormancy and differentiate into merozoites, which rupture from the hepatocyte to cause a blood-stage infection. In addition to being the only class of drugs with activity against hypnozoites, 8-aminoquinolines are active against gametocytes and thus interfere with malaria transmission.

2 Primaquine

2.1 Chemistry

The chemical name of primaquine is 6-methoxy-8-(4-amino-1-methylbutyl) aminoquinoline and its chemical formula is $C_{15}H_{12}N_3O$, with a molecular weight of 259 (Fig. 1). Primaquine is a racemic mixture composed of D- and L-enantiomers, due to the presence of an asymmetric chiral center. It is water soluble and solutions are stable when protected from light. Primaquine tablets are given in the form of the diphosphate salt containing either 13.2 mg (= ~7.5 mg base) or 26.3 mg (= ~15 mg base).

2.2 Clinical Use

There are three established indications for the use of primaquine: causal prophylaxis for all species of malaria, presumptive antirelapse therapy (terminal prophylaxis or postexposure prophylaxis) for *P. vivax* and *P. ovale* and radical cure of *P. vivax* and *P. ovale* infections. Since the use of primaquine depends on the species of parasite, an understanding of malaria endemicity is necessary for adequate

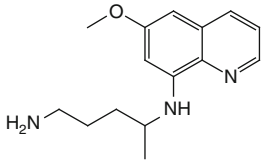


Fig. 1 Structure of Primaquine

prophylaxis use and highlights the importance of diagnostics for appropriate treatment. Primaquine primarily targets the exo-erythrocytic stage of the parasite's life cycle. It is much less effective at killing the asexual blood-stage parasites of *P. falciparum*, as evidenced by in vivo and in vitro studies [3–5]. Asexual blood stages of *P. vivax*, however, are more sensitive to primaquine and administration of a daily dose of 45 mg for 14 days to human subjects demonstrated 80% efficacy in clearing parasitaemia with the primaquine-tolerant Chesson strain of *P. vivax* [6]. In less tolerant strains of *P. vivax*, such as those found in Thailand, a daily dose of 30 mg (0.5 mg/kg) of primaquine was 100% effective at clearing blood-stage parasitaemia [7, 8].

2.3 Chemoprophylaxis

Causal prophylaxis of primaquine is dependent on the dose and the timing of administration. Although primaquine is not currently labeled for use as a causal prophylactic agent, in experimental challenge studies, a single 30-mg primaquine dose given 1 day following infection with *P. falciparum* sporozoites prevented the development of blood-stage parasitaemia. However, lower doses or doses given before or on the day of challenge did not provide adequate protection against *P. falciparum* [3, 9]. Daily primaquine dosing before infection and throughout the exo-erythrocytic development prevented malaria [9, 10]. These studies demonstrate that there is a small therapeutic window for primaquine.

Discrepancy exists regarding the minimal effective dose of primaquine for causal prophylaxis against *Plasmodium* spp. [11, 12]. Several studies support that the most effective dose for primaquine prophylaxis is 30 mg daily. A daily dose of 30 mg primaquine was administered for one year to non-immune Indonesian adults and was found to be 90% and 94% efficacious against *P. vivax* and *P. falciparum*, respectively [13, 14]. Efficacy dropped when an alternate-day dosing scheme of 30 mg primaquine was administered [15]. Similar results were observed in a prophylaxis study of Colombian soldiers, with protective efficacies of 94% (*P. falciparum*) and 85% (*P. vivax*) following 30-mg primaquine daily for 16 weeks [16].

2.4 Presumptive Antirelapse Therapy and Radical Cure

The prevention of *P. vivax* and *P. ovale* relapse infection is unique to primaquine. Primaquine is FDA approved and licensed for presumptive antirelapse therapy and radical cure of *P. vivax* and *P. ovale*. The licensure approval was based on data gathered from US soldiers returning from the Korean War in the 1950s. To prevent *P. vivax* relapse from returning soldiers, a daily dose of 15 mg primaquine for 14 days was used. This dose was used for two reasons; the Korean *P. vivax* strain

infecting US military personnel was efficiently cleared with this dosing regimen and haemolytic anaemia in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a condition more prevalent in African Americans, was less likely to cause a life-threatening condition with this dose [17]. This approved dosing scheme persisted in the face of clinical data suggesting that *P. vivax* strains from Southeast Asia and Oceania were tolerant of a 15-mg dose and therefore higher doses were required [18]. Subsequently, reports from nearly all *P. vivax* endemic areas indicate that the standard daily 15 mg primaquine dose for 14 days has failed to prevent relapses [11, 12].

It has been suggested that the total dose of primaquine may be more important than the particular regimen used as presumptive antirelapse or radical cure therapy [19–21]. For example, a total dose of 420 mg was equally effective if given at 30 mg/day for 14 days or 60 mg/day for 7 days [22]. This observation was supported by a recent evaluation of numerous *P. vivax* antirelapse therapy trials in which total primaquine dose was assessed [23]. Collectively, it was demonstrated that treatment success positively correlated with increasing primaquine dose as a function of body weight. This observation supports recent recommendations of a primaquine therapeutic dose of 0.5 mg/kg daily for 14 days [12]. It was further recognised that additional factors such as geographical-specific relapse rates and tolerance to primaquine should be considered when advocating the most efficacious antirelapse therapy. It should be noted that although higher doses of primaquine have been advocated, US FDA approval is only for the 15 mg/day for 14 days regimen [11].

Poor compliance with the 14-day standard primaquine therapy and treatment studies suggesting total dose is more important than dosing schedule have led to the investigation of regimens using higher doses of primaquine over a shorter time period for radical cure of *P. vivax*. Studies conducted in Vietnam found that 96% of the subjects treated with 200 mg artesunate twice daily for 2 days followed by 22.5 mg primaquine twice daily for 7 days cleared blood and liver stage infection based on a 28-day follow-up period [24]. A study conducted in Thailand investigated the administration of either a 30-mg primaquine daily dose for 7 days versus a 60-mg primaquine daily dose for 7 days for the treatment of *P. vivax*. Both dosing regimens were well tolerated and 28-day relapse rates were 11% and 4%, respectively [25]. Similarly, a study testing the antirelapse efficacy of artesunate followed by various doses of primaquine found that 30 mg twice daily for 7 days was as effective as the standard 14 day regimen of 15 mg daily [26]. The short half-life of artesunate suggests that the higher dose of primaquine was the predominant factor for the relative efficacy. Collectively these results suggest that a 1-week course of primaquine could be effective for the treatment of *P. vivax*.

2.5 *Drug Combinations*

Several reasons exist to investigate partnering drugs with primaquine: to increase efficacy, shorten the 14-day regime to a dosing schedule that will improve

compliance and circumvent the rise of chloroquine-resistant *P. vivax*. For antirelapse or radical cure therapy, primaquine is usually partnered with another antimalarial drug such as chloroquine (25 mg/kg over 3 days). Since primaquine lacks substantial activity against the asexual erythrocytic stages of *P. falciparum* and acts slowly against blood stages of *P. vivax*, a blood schizontocide drug should be administered with primaquine [11]. Early studies suggest that the addition of chloroquine or quinine potentiated the activity of primaquine [27]. A recent report demonstrates that synergy between primaquine and chloroquine may be attributed to the ability of primaquine to increase the accumulation of chloroquine within the parasite [28].

Evaluation of nine different trials that compared a 14-day primaquine plus chloroquine with a 5-day primaquine plus chloroquine regimen concluded that the 14-day primaquine regimen was superior to chloroquine alone or 5-day primaquine plus chloroquine [29]. This evaluation, however, did not take into account the dose of either chloroquine or primaquine. As an alternative to chloroquine plus primaquine for the treatment of vivax malaria, artesunate plus primaquine combinations have been shown to produce markedly shorter parasite and fever clearance times [24, 30]. Although artesunate has no radical curative activity, the rapid action of artemisinins on the blood stages of *P. vivax* is highly beneficial to the patient, in that infection and malaria symptoms are aborted at a much faster rate than with chloroquine. Furthermore, with increasing reports of chloroquine-resistant *P. vivax* malaria in Oceania, Southeast Asia, the Indian subcontinent and the Americas [14], artesunate may be considered a potential replacement for chloroquine for aborting an acute attack of vivax malaria.

2.6 Transmission Blocking

In a few malaria-endemic areas, the addition of a single dose of 45 mg primaquine to the treatment regimen had been advocated to reduce gametocyte burden and thus interfere with the transmission cycle of the malaria parasite. Early studies demonstrated that primaquine is a potent gametocytocidal and sporontocidal agent [31]. Several clinical studies demonstrated a primaquine-dependent reduction in gametocyte clearance times, when administered as a single 0.5-mg/kg dose to artesunate or quinine [32], a single 45-mg dose to chloroquine–sulfadoxine–pyrimethamine [33] and a single 0.75-mg/kg dose to artesunate–sulfadoxine–pyrimethamine [34] as compared with treatment groups receiving the various drug combinations without primaquine.

One study did not observe any significant advantage in adding a single 0.75-mg/kg primaquine dose to artesunate–sulfadoxine–pyrimethamine in reducing the gametocyte burden [35]. Discrepancies may be attributed to the methods and the accuracy of detecting submicroscopic levels of gametocytes, as artesunate possesses gametocytocidal activity. The absence of an additive effect of primaquine is consistent with the suggestion that the most effective way to prevent

gametocytaemia is to clear asexual blood forms [36]. Since artesunate does not kill mature gametocytes [37], unlike primaquine, and treatment with particular antimalarials induces gametocytogenesis [38], additional transmission-blocking studies are required to address the benefit of adding primaquine to treatment regimens. Future studies are essential since ongoing efforts are aimed at eliminating malaria [39].

2.7 *Mechanism of Action*

The mechanism of action by which primaquine exerts its antimalarial activity is largely unknown but the mitochondria may be the biological target of primaquine. Specifically, primaquine accumulates within the mitochondria, resulting in swelling and structural changes within the inner membranes [40–44], thus destroying mitochondrial function [45–47]. Primaquine is quickly metabolised to several reactive intermediates that are responsible for toxicity to erythrocytes (discussed below) and also apparently for antimalarial activity [48, 49]. Several of the active metabolites are structurally similar to naphthoquinones [50]. The antimalarial activity of naphthoquinones, such as atovaquone, is due to inhibition of mitochondrial function [51, 52]. Atovaquone has been shown to collapse the mitochondrial electron membrane potential, resulting in disruption of pyrimidine biosynthesis [53, 54]. Since asexual blood stage parasites rely on glycolysis for their energy source rather than oxidative phosphorylation-generated ATP, a role in pyrimidine biosynthesis would support the essentiality of the mitochondria for asexual growth and explain the blood-stage antimalarial activity of atovaquone. Primaquine, however, is not an effective blood-stage antimalarial against *P. falciparum*. Interestingly, swelling of host cell mitochondria was not observed and hydroxynaphthoquinone and naphthoquinone are approximately 1,000-fold more potent against the plasmodial cytochrome bc_1 complex than the mammalian complex [51]. These selectivity differences are believed to be a result of structural differences within the plasmodial bc_1 complex that increases the affinity for selected antimalarials such as atovaquone and 8-aminoquinolines [55].

The metabolism of primaquine produces reactive intermediates that ultimately results in the accumulation of free radicals, hydrogen peroxides and superoxides which may be responsible for antimalarial activity [56]. Such weak activity of primaquine in vitro may be indicative of the fact that primaquine requires metabolism for antimalarial activity [57]. A similar mode of antimalarial action has been suggested for artemisinins, which are metabolized into free radicals [58]. These free radicals may disrupt oxidation–reduction systems, inactivate specific enzymes or attach to and disrupt biological membranes [59].

Although a generalised mechanism of action has been discussed for *Plasmodium*, it should be acknowledged that different mechanisms may exist depending on the species of *Plasmodium*. For example, primaquine appears to be effective against asexual blood stages of *P. berghei* [43, 60] and *P. vivax* [6–8]; however, it is a poor

inhibitor of *P. falciparum* asexual stages [3]. Additionally, there are discrepancies and inconsistencies with the mechanisms of action of primaquine when compared with that of artemisinins (free radicals and oxidative stress) and atovaquone (collapse mitochondrial electron membrane potential) because the in vitro and in vivo efficiencies and stage specificity of primaquine are clearly different to artemisinins and atovaquone.

2.8 Pharmacokinetics and Metabolism

An oral dose of primaquine is rapidly absorbed, with a mean bioavailability of 96% [61]. Primaquine exhibits linear and first-order kinetics over the dose range of 15–45 mg. The maximum drug concentration (C_{max}) and the time to achieve the maximum concentration (t_{max}) in plasma were 53 ng/mL and 2 h, respectively, following a single dose of 15 mg primaquine to healthy subjects [56]. Primaquine is extensively distributed into body tissues, with an apparent volume of distribution of 200–300 L and a systemic clearance varying between 30 and 40 L/h. The elimination half-life of primaquine is about 6 h [61, 62]. The pharmacokinetic properties of primaquine are comparable between G6PD-normal and G6PD-deficient healthy subjects [63]. Recently, sex-related differences were reported in the pharmacokinetics of primaquine, following multiple dosing of 30 mg primaquine for 14 days, with females having significantly slower clearance (0.31 L/h/kg versus 0.55 L/h/kg) and a lower apparent volume of distribution (3.42 L/kg versus 4.59 L/kg) when compared with males [64]. Further, studies are required to determine whether the increased exposure to primaquine in females leads to increased risk of toxicity compared with males, given the same maintenance dosage.

Excretion studies using ^{14}C-labeled primaquine demonstrated that 64% of the radio label was found in the urine within 143 h after an oral dose [65]. Primaquine is rapidly and completely metabolised, as only 1–4% of the initial compound is found in the urine [61, 66]. Metabolism of primaquine results in the accumulation of numerous unstable intermediates [48]. The major plasma metabolite of primaquine is the inactive carboxyprimaquine but this is thought to be further metabolised as it is not found in urine [65]. Additional metabolites include 5-hydroxyprimaquine, 5,6-dihydroxy-8-aminoquinoline, 6-desmethylprimaquine, 5,6-dihydroxyprimaquine, and 6-methoxy-8-aminoquinoline. It is these later metabolites that are believed to generate oxygen-active species responsible for toxicity of parasite and host cells.

Several different approaches have been investigated to increase the bioavailability and the stability of primaquine. These include different mechanisms of drug administration such as transdermal delivery systems [67], galactose-coated polypropyleneimine nanoparticles as the primaquine vehicle [68] and primaquine encapsulation into liposomes and nanoparticles [69, 70]. These approaches increased stability or exposure time to drug; however, to date, these approaches have not advanced into clinical development to improve the quality of primaquine.

An additional approach used to increase stability and bioavailability is the conjugation of primaquine with amino acids [71] or with polymers of polyaspartamide [72]. The amino acid derivative demonstrated improved stability; however, these conjugates may be readily removed from primaquine via action of aminopeptidases [73]. Polyaspartamide conjugates significantly decreased parasitaemia levels and increased the survival times of mice infected with *P. berghei* compared with untreated or glucosamine-conjugated primaquine-treated mice. Radical cure, however, was not achieved, as all tested mice eventually died [72]. Nevertheless, these approaches support the proposal that modification or conjugation of primaquine and its analogs may be a viable alternative to increase the efficacy of primaquine. Detailed pharmacokinetic studies must be completed to assess the improved stability and bioavailability of these conjugates over the parent compound.

2.9 Safety and Tolerability

The toxicities associated with primaquine and other 8-aminoquinolines are well known [74]. Haemolytic anaemia is the most serious condition induced by primaquine in G6PD-deficient individuals [75, 76]. Erythrocytes, especially those infected with malaria parasites are prone to oxidative stress. Reduced glutathione (GSH) is important in the detoxification of free radicals. Once oxidised to glutathione disulfide (GSSG), GSH levels are reinstated by the activity of glutathione reductase and NAPDH. Because erythrocytes lack mitochondria, the pentose-phosphate pathway is the only source of NADPH. In G6PD-deficient individuals, NADPH levels are inadequate to restore GSH levels resulting in a compromised antioxidant system; thus, the erythrocytes do not have efficient protective mechanisms to handle oxidative stress. Primaquine is rapidly metabolised into hydroxylated intermediates that result in the generation of peroxides, superoxides and hydroxyated free radicals [49]. In G6PD-deficient individuals, erythrocytes are susceptible to these free radicals, which denature haemoglobin to form Heinz bodies that then react with erythrocyte membranes. This process causes premature lysis or subsequent clearing by the spleen [77, 78]. Although administration of primaquine to G6PD-deficient individuals can cause haemolytic anaemia, there are several factors such as polymorphic variation in G6PD alleles, total drug dose, and duration of the treatment that may modulate the severity of the haemolysis. Nevertheless, G6PD deficiency should be evaluated before the administration of primaquine [11]. Primaquine at the approved dosages for radical cure and presumptive antirelapse therapy is safe when administered to individuals with normal G6PD levels.

Methaemoglobinaemia is also a common toxicity associated with primaquine, which can, in some cases, require treatment with methylene blue. Methaemoglobin (MetHb) is an oxidised form of haemoglobin that cannot bind and transport oxygen to various tissues. Normal MetHb levels are less than 1% of total haemoglobin; however, in individuals deficient for G6PD or methaemoglobin reductase (an

NADH-dependent enzyme that converts MetHb to haemoglobin) or under extreme oxidative stress, the levels of MetHb may increase to harmful levels, resulting in cyanosis. Primaquine increases the rate of MetHb formation [79] through oxidative stress via the free-radical metabolites of primaquine. MetHb levels as high as 11% have been reported in healthy Caucasians treated with primaquine [80]. In individuals without anemia, primaquine-induced methaemoglobinaemia, however, is a well-tolerated condition that is alleviated upon the discontinuation of primaquine dosing [11].

Gastrointestinal (GI) discomfort has been associated with primaquine in a dose-dependent manner [80–82]. Symptoms include cramping, nausea, diarrhoea and vomiting. Most of these symptoms are mild and are often avoided, if primaquine is taken with food [80].

2.10 Primaquine Resistance

Experimentally induced primaquine resistance has been developed in *P. berghei* and *P. knowlesi* [83, 84]. These controlled experiments were later supported with field reports that indicated the existence of primaquine-tolerant *P. vivax* [85]. Several reports suggest resistance to standard antirelapse primaquine therapy; however, factors such as noncompliance with the 14-day treatment [12] or inadequate weight-based dose could also explain the observed failures rather than inherited resistance [86]. *P. vivax* strains from Southeast Asia and the Southwest Pacific are more tolerant to primaquine than elsewhere [19]. These tolerant strains, however, can be effectively treated with increased doses of primaquine [11]. Although little evidence exists to support primaquine-resistant exo-erythrocytic stages including hypnozoites, several reports have described multiple relapses of *P. vivax* in military personnel after primaquine treatment [87, 88]. Further well-controlled studies where treatment compliance is known and primaquine is administered in a weight-based dose would help resolve the resistance issue.

3 Tafenoquine

3.1 Historical Development

Originally labeled as WR238605 or SB-252263 and now named tafenoquine, the drug is a new 8-aminoquinoline antimalarial being codeveloped by Glaxo-SmithKline Pharmaceuticals and the US Army as a replacement for primaquine for radical cure of *P. vivax* malaria and as a potential prophylactic agent [89–91]. In an effort to develop less toxic, more active and longer acting 8-aminoquinolines, tafenoquine was first synthesised by the US Army at the Walter Reed Army

Institute of Research in 1979. Although tafenoquine is a primaquine analog, it possesses different physicochemical properties, antimalarial potency and toxicological and pharmacokinetic properties compared with primaquine. In in vitro testing and in vivo preclinical animal models tafenoquine is more active than primaquine. To date, it has been evaluated in more than 2,000 human subjects in clinical studies.

On an equimolar basis, in vitro antimalarial susceptibility studies have shown tafenoquine to exhibit equivalent activity (IC_{50} of 0.7–1.5 μM) to primaquine against culture-adapted chloroquine-sensitive strains, but was considerably more active than primaquine against multidrug-resistant *P. falciparum* lines, with IC_{50} values ranging from 0.06 to 0.3 μM [92]. It is conceivable that tafenoquine's enhanced blood schizontocidal potency compared with primaquine is because it exerts greater oxidative stress on multidrug-resistant parasitised erythrocytes [93]. In contrast to culture-adapted multidrug-resistant *P. falciparum* lines, tafenoquine was only marginally more active than primaquine against wild isolates of *P. falciparum* from central, west and east Africa (mean IC_{50} values of 4.43 μM versus 6.82 μM) [94, 95]. The enantiomers of tafenoquine have similar levels of in vitro antimalarial activity against the drug-sensitive D6 and multidrug-resistant W2, TM90-C2a and TM90-C2b strains of *P. falciparum* (D. K. Kyle personal communication).

In the rodent–*P. berghei* Peters 4-day suppressive test, tafenoquine was about 9 times more active as a blood schizontocide than primaquine against the drug-sensitive *P. berghei* N strain and 4–5 times as active as primaquine against highly resistant chloroquine, mefloquine or halofantrine strains of *P. berghei* [95]. In addition to developing new schizontocidal drugs, the capacity to interrupt malaria transmission is also of great importance. Tafenoquine possesses significant sporontocidal activity against *P. berghei*, with a minimum effective dose of 25 mg/kg that prevents mosquitoes from developing sporozoites [96]. Tafenoquine also has gametocytocidal activity, with a significant reduction in the number of gametocytes in the blood of *P. berghei*-infected mice treated with 25 mg/kg, resulting in a twofold extension of mice survival time [90].

In the rhesus monkey–*P. cynomolgi* model, tafenoquine was effective as a causal prophylactic agent against pre-erythrocytic tissue stages of sporozoite-induced *P. cynomolgi* malaria [97]. The causal prophylactic ED_{50} (50% effective dose) of tafenoquine was 0.125 mg/kg/day or 0.27 μM/kg/day for 3 days, which was 14 times more effective than primaquine, with an ED_{50} of 1 mg/kg/day or 3.86 μM/kg/day for 3 days. Tafenoquine was also a highly effective agent against liver stages of *P. cynomolgi*, with an ED_{50} of 0.172 mg/kg/day or 0.371 μM/kg/day for 7 days and was 7 times more potent than primaquine, with an ED_{50} of 0.712 mg/kg/day or 2.75 μM/kg/day for 7 days [98].

Although developed primarily as an antirelapse agent, tafenoquine has also been found to possess significant blood schizontocidal activity against trophozoite-induced infections in simian–malaria models. Against *P. cynomolgi B* and *P. fragile*, which are recognised as biological counterparts of *P. vivax* and *P. falciparum* infections in humans, respectively [99], tafenoquine at a dose of

3.16 mg/kg/day for 7 days led to a cure for established trophozoite induced infections in monkeys with both these parasites [100]. In contrast, primaquine was only partially curative (25% for *P. cynomolgi B* and 67% for *P. fragile*) at a dose of 10 mg/kg/day for 7 days. Tafenoquine was also effective against blood-induced vivax malaria infections of the chloroquine-resistant AMRU1 strain in the *Aotus* monkey–*P. vivax* model. Parasite clearance of the AMRU1 strain occurred at a dose of 0.3 mg/kg tafenoquine daily for 3 days and cures were achieved at 3 mg/kg daily for 3 days [101].

In addition to tafenoquine's greater in vitro and in vivo antimalarial activities compared with primaquine in preclinical studies, it is less toxic than primaquine. In acute oral toxicity studies, tafenoquine's LD_{50} (50% lethal dose) of 0.78 and 0.64 mM/kg in rats and guinea pigs, respectively, was markedly less toxic than primaquine, with corresponding LD_{50} values of 0.46 and 0.12 mM/kg [98]. In subchronic and chronic studies of tafenoquine (WR 238605 IND #38503), the compound was also found to be less toxic than primaquine. For example, in dog toxicology studies, 3 and 9 mg/kg/day of primaquine orally for 28 days resulted in muscle necrosis, coma and death, whereas tafenoquine up to a maximum tested dose of 16 mg/kg/day for 28 days did not produce these adverse events [102].

3.2 Chemistry

The chemical name for the racemic tafenoquine is (±)-8-[(4-amino-1-methylbutyl) amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy) quinoline succinate. The structural formula for tafenoquine is shown in Fig. 2. Its chemical formula is $C_{24}H_{28}N_3O_3{\cdot}C_4H_6O_4$, with molecular weights of 463 for the base and 581 for the succinate salt. Tafenoquine is an off-white to pink/orange/brown solid powder with a strong phenolic odor. It is poorly soluble in water and stable at room temperature, when stored in amber bottles for at least 10 years. The formulated product of tafenoquine is a hard gelatin capsule containing 250 mg tafenoquine succinate equivalent to 200 mg of the free base. Tafenoquine capsules should be stored below 30°C and protected from light.

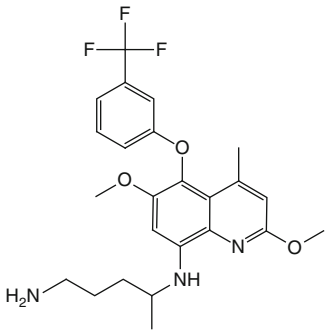


Fig. 2 Structure of Tafenoquine

3.3 Mechanism of Action and Development of Resistance

As already indicated, the exact mechanism of action of 8-aminoquinolines is not well understood. It has been proposed that the blood-stage activity of 8-aminoquinolines may be derived from an oxidative stress mechanism since it is known that primaquine stimulates the hexose monophosphate shunt, increases hydrogen peroxide and MetHb production and decreases glutathione levels in the erythrocyte [93, 103, 104]. Similar to chloroquine, the blood-stage activity of tafenoquine may be through inhibition of haematin polymerisation. In contrast to the inactive primaquine ($IC_{50} > 2{,}500$ μM), tafenoquine (IC_{50} of 16 μM) inhibited haematin polymerisation more efficiently than did chloroquine (IC_{50} of 80 μM) [92]. Other suggested modes of action of tafenoquine include drug-induced mitochondrial dysfunction or inhibition of receptor recycling by endosomes [105, 106].

In vitro studies have also shown a positive correlation between tafenoquine and primaquine ($r^2 = 0.61$) against seven *P. falciparum* lines, with different levels of susceptibility to chloroquine and mefloquine [92]. In contrast, no correlation exists between tafenoquine and either chloroquine or mefloquine, suggesting a lack of cross-resistance between tafenoquine and chloroquine or mefloquine.

3.4 Pharmacokinetics and Metabolism

The pharmacokinetics of tafenoquine has been investigated following both single and multiple oral administration of the drug in healthy subjects. Single-dose studies ranging from 4 to 600 mg tafenoquine have been carried out in 48 healthy males (Caucasian [$n = 20$], African American [$n = 12$] and Hispanic [$n = 16$]) in the fasting state [98]. The absorption half-life of tafenoquine was 1.7 h, suggesting rapid absorption of the compound. However, the t_{max} of 13.8 h implied prolonged absorption of tafenoquine from the gut. Plasma tafenoquine concentrations declined in a mono-exponential manner and the drug was slowly cleared, with an elimination half-life of 14 days. The C_{max} and area under the drug concentration curve of tafenoquine were linear over the doses studied. The tafenoquine concentration–time data were best described by a one-compartment model, with first-order absorption and elimination. Tafenoquine had a low oral clearance (CL/*F*, 5.7 L/h) and a large apparent volume of distribution (*V*/*F*, 2,558 L), suggesting extensive tissue binding. Whole blood concentrations of tafenoquine were 1.8-fold higher than corresponding plasma concentrations, reflecting an accumulation of the drug in erythrocytes, which may contribute to the greater potency of tafenoquine compared with primaquine, which does not concentrate in erythrocytes [61].

The population pharmacokinetics of tafenoquine has also been determined in healthy Thai and Australian soldiers after receiving tafenoquine for malaria prophylaxis. A one-compartment model with first-order absorption and elimination was found to best describe the population pharmacokinetics of tafenoquine. In the

Thai study, 104 soldiers received a loading dose of 400 mg tafenoquine daily for 3 days followed by 400 mg tafenoquine monthly for 5 consecutive months [107]. Blood samples were randomly collected from each soldier on several occasions each month. The population estimates of the first-order absorption rate constant (K_a), CL/F and V/F were 0.69/h, 3.20 L/h and 1,820 L, respectively. The absorption and elimination half-lives were 1.0 h and 16.4 days, respectively. The covariants, age and weight influenced the volume of distribution. The one subject who contracted malaria had a higher plasma clearance, but this was not considered to have sufficient impact to warrant a change in dosing.

In the Australian study, 490 soldiers received a loading dose of 200 mg tafenoquine daily for 3 days followed by a weekly dose of 200 mg tafenoquine for 6 months [108]. Blood samples were collected from each soldier after the last loading dose and then at weeks 4, 8 and 16. Typical values of K_a, CL/F and V/F were 0.24/h, 4.37 L/h and 1,901 L, respectively. The V/F was similar to that reported in the Thai soldiers, but the systemic CL/F was greater (4.37 L/h versus 3.20 L/h). The derived elimination half-life of tafenoquine in the Australian soldiers of 12.7 days was slightly shorter than the 14 and 16 days reported previously in healthy Caucasians, African-Americans and Hispanic subjects [98] and in Thai soldiers [107], respectively, which may partly reflect the fact that the last samples were drawn at only up to 1 week post dose and therefore, the presumed "terminal" phase may have included some components of the distribution phase. The mean values for CL/F and V/F obtained in the fed Australian soldiers were 30–35% lower than values derived in the fasted healthy subjects participating in the single dose escalating study of tafenoquine. A possible explanation for the disparity is that a high-fat meal can increase the oral bioavailability (F) of tafenoquine by up to 40% (A. K. Miller personal communication), which when comparing the two studies would bring the respective CL/F and V/F values into closer agreement after correcting for F.

Limited investigations have been carried out on the metabolism of tafenoquine. In vitro rat liver microsomal studies have identified tafenoquine to be metabolised to aminophenolic compounds that undergo air oxidation to a mixture of quinones and quinoneimines [109]. Similar to primaquine, the metabolism of tafenoquine is difficult to study, because its structure contains several metabolically labile constituent groups, and its intermediates are unstable and possess amphoteric properties [74]. So far, no metabolites of tafenoquine have been identified in either human plasma or urine.

3.5 Safety and Tolerability

In single dose escalating pharmacokinetic studies in healthy subjects, only a few GI side effects such as heartburn, flatulence, vomiting and diarrhoea were seen in those subjects who received the higher doses of 300–600 mg tafenoquine [98]. These side effects were few and were not unexpected, based on past experiences with

primaquine. Methaemoglobinaemia, haemolytic anaemia, thrombocytopenia, or changes in white blood cell counts or electrocardiograms were not observed in the subjects. Because tafenoquine is related to primaquine, it can cause methaemoglobinaemia and haemolytic anaemia in individuals with deficiency of G6PD. Thus, all individuals who receive an 8-aminoquinoline should undergo laboratory testing for confirmation of a normal G6PD status [110]. This is potentially tafenoquine's major drawback for use worldwide as G6PD is one of the most common human genetic polymorphisms. Although malaria patients with anemia may be at greater risk, methaemoglobinaemia generally is not a serious concern when <20% of haemoglobin is in the MetHb form and only rarely will testing for methaemoglobinaemia be indicated on clinical grounds, such as the presence of bluish mucous membranes [111].

In individuals with severe G6PD deficiency, such as the Mediterranean variety, tafenoquine or primaquine should not be used. Even individuals with the low-grade deficiency (A-) variant of G6PD, which is most commonly found in Africa, can be at risk of developing haemolysis when exposed to tafenoquine. In a Kenyan field study, two women who were inadvertently given tafenoquine (400 mg daily for 3 days) experienced a haemolytic reaction when their G6PD deficiency status was incorrectly recorded during screening [112]. One woman, who was later found to be heterozygous for the (A-) G6PD variant, developed intravascular haemolysis and required a 2-unit blood transfusion. Haemolysis did not continue after the acute event, no renal compromise was seen in spite of blackwater urine, and she restored and maintained normal haematologic parameters for 6 months after the event. The other woman, who was later found to be homozygous for the (A-) G6PD variant, remained asymptomatic despite an acute 30 g/L decrease in haemoglobin, which was noticed only because of routine blood tests. She restored her haemoglobin level without intervention.

3.6 Clinical Use

3.6.1 Chemoprophylaxis against *P. falciparum* and *P. vivax* Malaria

The development and spread of multiple drug-resistant *P. falciparum* malaria in many parts of the world highlights the need to develop new, safe, well-tolerated and effective chemoprophylactic agents for travellers and in special risk groups such as military personnel. A long-acting drug that acts on all stages of the malaria parasite could be a significant addition to the limited armamentarium for protecting individuals against malaria infections. Tafenoquine is a long-acting antimalarial and, based on preclinical studies acts on all stages of the parasite, including the pre-erythrocytic stages providing causal prophylactic activity. Table 1 summarises the Phase II and III studies on the safety, tolerability and protective efficacy of tafenoquine in its clinical development.

Table 1 Studies on the safety, tolerability and protective efficacy of tafenoquine

Purpose of study	Study design	TQ Regimen	Subjects	Safety and tolerability	Efficacy
Prophylactic studies					
Prophylactic efficacy against *Pf* in a challenge model [113]	Randomised, placebo-controlled, double-blinded	600 mg	4 Adults	TQ was well tolerated, with only mild, transient headache and diarrhoea reported	3 of 4 subjects protected from developing *Pf* malaria
Minimum effective weekly dose of TQ for prevention of *Pf* malaria in Ghana [114]	Randomised, placebo-controlled, double-blinded, dose-ranging	25, 50, 100 or 200 mg ow for 12 weeks	463 Adults	All regimens were SWT. The four TQ groups demonstrated AE rates comparable to those of the placebo group and showed no evidence of a dose-related effect	Relative to placebo (86/94), the protective efficacies were 32% for 25 mg (58/93), 84% for 50 mg (13/91), 87% for 100 mg (11/94) and 86% for 200 mg (12/91)
Long-term prophylactic activity of TQ against *Pf* in Gabon [110]	Randomised, placebo-controlled, double-blinded	25, 50, 100 or 200 mg od for 3 days	410 (aged 12–20 years)	TQ were well tolerated but abdominal pain was reported more commonly in the TQ groups than in the placebo group. No other symptom such as headache, diarrhea, dizziness and was significantly associated with TQ use	Relative to placebo (14/82), the protective efficacies were 0% for 25 mg (16/79), 80% for 50 mg (3/86), 93% for 100 mg (1/79) and 100% for 200 mg (0/84)
Prophylactic efficacy of TQ against *Pf* in Kenya [112]	Randomised, placebo-controlled, double-blinded	A: LD 400 mg + placebo ow for 13 weeks; B: LD 200 mg + 200 mg ow for 13 weeks; C: LD 400 mg + 400 mg ow for 13 weeks	223 Adults	Reported AEs were similar among the subjects on the four treatment groups. The mean MetHb concentrations in subjects on 200 mg and 400 mg ow were 2.5% and 4.5%, respectively	Relative to placebo (54/59), the protective efficacies were 68% for A (16/54), 86% for B (7/53) and 89% for C (6/57)

(continued)

Table 1 (continued)

Purpose of study	Study design	TQ Regimen	Subjects	Safety and tolerability	Efficacy
Prophylactic activity of TQ against *Pf* and *Pv* malaria in Thailand [115]	Randomised, placebo-controlled, double-blinded	LD 400 mg od for 3 days + 400 mg om for 5 months	205 Thai soldiers	Monthly TQ was SWT. GI complaints (diarrhoea, nausea, or vomiting) were significantly more common in the TQ group than the placebo group	Relative to placebo (30/92), the protective efficacies were 96% against *Pv*, 97% against all species, and 100% against *Pf* (1/96)
Prophylactic trial of TQ in Timor-Leste [116]	Randomised (3:1 to TQ), double-blinded	LD 200 mg od for 3 days + 200 mg ow for 6 months or LD 250 mg od MQ for 3 days + 250 mg MQ ow for 6 months	654 AMP	Both TQ and MQ were well tolerated. In a subset of TQ individuals ($n = 98$), MetHb levels increased by 1.8% and mild vortex keratopathy (phospholipid corneal deposits) was detected in 93% (69/74) of TQ subjects	No diagnoses of malaria occurred for either treatment group in Timor-Leste, but 0.9% (4/462) and 0.7% (1/153) of recipients developed *Pv* infections in the TQ and MQ groups, respectively
Long-term safety of TQ [117]	Randomised (2:1 to TQ), placebo-controlled, double-blinded	LD 200 mg od for 3 days + 200 mg ow for 23 weeks	120 Adults	No effect on night vision or other ophthalmic indices such as colour vision and macular function. After 6 months of dosing, there was no TQ effect on renal function	

Abbreviations: *TQ* tafenoquine, *MQ* mefloquine, *LD* loading dose, *AMP* Australian military personnel, *SWT* safe and well tolerated, *AE* adverse events, *GI* gastrointestinal, *od* once daily, *om once monthly, ow* once weekly, *Pf P. falciparum*, *Pv P. vivax*

3.6.2 Presumptive Antirelapse Therapy and Radical Cure

Tafenoquine was also developed as a potential replacement of primaquine for presumptive antirelapse therapy and radical cure. Table 2 summarises the clinical development of tafenoquine for antirelapse therapy.

3.7 Future Potential

Tafenoquine is a unique antimalarial drug that is active against all stages of *Plasmodium* spp. Although clinical studies of tafenoquine have shown the long-acting 8-aminoquinoline to have comparable efficacy to primaquine for radical cure and presumptive antirelapse therapy, the markedly shorter regime of tafenoquine compared with primaquine (3 days versus 14 days) is more convenient and with improved compliance one could expect the number of relapses of *P. vivax* malaria to decrease markedly. For the treatment of uncomplicated *P. falciparum*, artemisinin-based combination therapies (ACTs) are now recommended for first-line treatment worldwide. Because of tafenoquine's long elimination half-life of 14 days, it could be considered as a partner drug with an artemisinin derivative such as artesunate. Today, however, we have very efficacious and well-tolerated ACTs for the treatment of falciparum malaria [125]. Thus, it may be more prudent to limit the use of tafenoquine to treating *P. vivax* and *P. ovale* infections, and for selected applications, including prophylaxis (short and long-term) for special risk groups such as military personnel.

Furthermore, since tafenoquine possesses both gametocytocidal and sporontocidal activity it is a promising candidate agent for transmission-blocking public health applications. Because of its long half-life, tafenoquine has enormous potential for malaria control and possibly the elimination of the disease. To test this latter concept will be difficult. Perhaps tafenoquine could be evaluated for transmission blocking in an area of low endemicity, with controlled geographical access such as an island. For malaria elimination, tafenoquine could be used in mass drug administration to eliminate residual parasites in an entire population [112] and, thus, would be an excellent drug for the eradication of malaria under the new initiative by the Bill and Melinda Gates Foundation [126].

Before these possible public health applications of tafenoquine can be implemented, a regimen that can safely be given to G6PD-deficient individuals needs to be developed. Alternatively, a field friendly, rapid and inexpensive G6PD test needs to be produced so that the G6PD status of the individual can be ascertained prior to tafenoquine administration. A clinical dose-escalating study in G6PD-deficient subjects is planned to better quantify and characterise the risk of tafenoquine use in this important risk group [117].

Table 2 Studies on the safety, tolerability and efficacy of tafenoquine for anti-relapse therapy

Purpose of study	Study design	Regimen	Subjects	Safety and tolerability	Efficacy – relapse frequency
Presumptive antirelapse therapy					
PNG [118]	Randomised, open-label study	A: 400 mg od TQ for 3 days; B: 7.5 mg tid PQ for 14 days	592 AMP	Increase in mild GI disturbances with TQ vs. PQ	1.9% (7/378) for A and 2.8% (6/214) for B within 12 months after leaving PNG
Timor-Leste [119]	Randomised, open-label study	A/B: 200 mg od/td TQ for 3 days; C: 400 mg od TQ for 3 days; D: 7.5 mg tid PQ for 14 days	925 AMP	GI disturbances in all groups, being twofold higher in females for both treatments [120]. Reduced AEs with reduced dose of TQ	4.9% (20/406) for A, 5.3% (4/75) for B, 11.0% (17/155) for C, and 10.0% (29/289) for D within 12 months after leaving Timor-Leste
Radical cure therapy					
TQ vs. CQ Thailand [121]	Randomised open-label study after CQ treatment (1,500 mg over 3 days)	A: 300 mg od TQ for 7 days; B: 500 mg od TQ for 3 day, repeated after 1 week; C: one dose of 500 mg TQ; D: CQ only	23 adults (completed 2–6 months of follow-up)	TQ was SWT. MetHb values peaked at 13.5%, 14.7%, and 6.4% in treatment groups A–C. Mild, transient AEs consisting of headache and GI in a minority of all patients	0% (0/7) for A, 11.1% (1/9) for B (day 120), 14.3% (1/7) for C (day 112) and 57.1% (4/7) for D (with relapse on days 40, 43, 49 and 84)
TQ vs. PQ Thailand [122]	Randomised open-label study after CQ treatment (1,500 mg over 3 days)	A: 300 mg od TQ for 7 days; B: 600 mg od TQ for 3 days; C: one dose 600 mg TQ; D: no further treatment; E: 15 mg od PQ for 14 days	46 TQ, 10 CQ and 12 CQ + PQ (completed at least 8 weeks of follow-up or had a relapse)	TQ was SWT. AEs on TQ and PQ therapy were generally mild and transient, consisting predominantly of headache, abdominal discomfort or diarrhoea and were more frequent in the TQ groups compared with the PQ group	0% (0/15) for A, 0% (0/15) for B, 6.3% (1/16) for C, 80% (8/10) for D and 25% (3/12) for E. The protective efficacy was 92.6% for CQ + TQ recipients compared with CQ + PQ recipients

TQ alone [123]	Open-label study	800 mg TQ over 3 days	2 AMP returning from PNG	TQ was well tolerated, with one patient experiencing mild diarrhoea	Parasite clearance 3 to 4 days. No recurrence after 2 years
Extended TQ regimen [124]	Open-label study after CQ treatment (1,500 mg over 3 days)	LD 200 mg od TQ for 3 days, plus 200 mg ow TQ for 8 weeks	27 AMP		Patients recruited after 2–4 clinical episodes of *P. vivax* malaria. One patient had a relapse after 6 months of observation

Abbreviations: *TQ* tafenoquine, *PQ* primaquine, *CQ* chloroquine, *LD* loading dose, PNG Papua New Guinea, *AMP* Australian military personnel, *SWT* safe and well tolerated, *AE* adverse events, *GI* gastrointestinal, *od* once daily, *ow* once weekly, *tid thrice daily*

Acknowledgments We thank Professor Dennis Shanks for review and helpful discussions with the manuscript. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official, or as reflecting true views of the United States Department of the Army, the Department of Defense or the Australian Defense Force.

References

1. Peters W (1999) The evolution of tafenoquine–antimalarial for a new millennium? J R Soc Med 92:345–352
2. Alving AS, Pullman TN et al (1948) The clinical trial of 18 analogues of pamaquin (plasmochin) in vivax malaria, Chesson strain. J Clin Invest 27:34–45
3. Arnold J, Alving AS, Hockwald RS, Clayman CB, Dern RJ, Beutler E, Flannagan OL, Jeffery GM (1955) The antimalarial action of primaquine against the blood and tissue stages of falciparum malaria (Panama, P-F-6 strain). J Lab Clin Med 46:391–397
4. Baird JK, Wiady I, Sutanihardja A, Suradi P, Basri H, Sekartuti AE, Fryauff DJ, Hoffman SL (2002) Short report: therapeutic efficacy of chloroquine combined with primaquine against *Plasmodium falciparum* in northeastern Papua, Indonesia. Am J Trop Med Hyg 66:659–660
5. Basco LK, Bickii J, Ringwald P (1999) *In-vitro* activity of primaquine against the asexual blood stages of *Plasmodium falciparum*. Ann Trop Med Parasitol 93:179–182
6. Edgecomb J, Arnold J, Yount E Jr, Alving A, Eichelberger L (1950) Primaquine, SN-13272, a new curative agent in vivax malaria: a preliminary report. Nat Malar Soc 9:285–357
7. Wilairatana P, Silachamroon U, Krudsood S, Singhasivanon P, Treeprasertsuk S, Bussaratid V, Phumratanaprapin W, Srivilirit S, Looareesuwan S (1999) Efficacy of primaquine regimens for primaquine-resistant *Plasmodium vivax* malaria in Thailand. Am J Trop Med Hyg 61:973–977
8. Pukrittayakamee S, Vanijanonta S, Chantra A, Clemens R, White NJ (1994) Blood stage antimalarial efficacy of primaquine in *Plasmodium vivax* malaria. J Infect Dis 169:932–935
9. Powell RD, Brewer GJ (1967) Effects of pyrimethamine, chlorguanide, and primaquine against exoerythrocytic forms of a strain of chloroquine-resistant *Plasmodium falciparum* from Thailand. Am J Trop Med Hyg 16:693–698
10. Arnold J, Alving AS, Hockwald RS, Clayman CB, Dern RJ, Beutler E, Jeffery GM (1954) The effect of continuous and intermittent primaquine therapy on the relapse rate of Chesson strain vivax malaria. J Lab Clin Med 44:429–438
11. Hill DR, Baird JK, Parise ME, Lewis LS, Ryan ET, Magill AJ (2006) Primaquine: report from CDC expert meeting on malaria chemoprophylaxis I. Am J Trop Med Hyg 75:402–415
12. Baird JK, Hoffman SL (2004) Primaquine therapy for malaria. Clin Infect Dis 39:1336–1345
13. Fryauff D, Baird J, Basri H, Sumawinata I, Purnomo RT, Ohrt C, Mouzin E, Church C, Richards A et al (1995) Randomised placebo-controlled trial of primaquine for prophalaxis of falciparum and vivax malaria. Lancet 346:1190–1193
14. Baird JK, Lacy MD, Basri H, Barcus MJ, Maguire JD, Bangs MJ, Gramzinski R, Sismadi P, Krisin LJ et al (2001) Randomized, parallel placebo-controlled trial of primaquine for malaria prophylaxis in Papua, Indonesia. Clin Infect Dis 33:1990–1997
15. Baird JK, Fryauff DJ, Basri H, Bangs MJ, Subianto B, Wiady I, Purnomo LB, Masbar S, Richie TL et al (1995) Primaquine for prophylaxis against malaria among nonimmune transmigrants in Irian Jaya, Indonesia. Am J Trop Med Hyg 52:479–484
16. Soto J, Toledo J, Rodriquez M, Sanchez J, Herrera R, Padilla J, Berman J (1998) Primaquine prophylaxis against malaria in nonimmune Colombian soldiers: efficacy and toxicity. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 129:241–244
17. Alving AS, Arnold J, Robinson DH (1952) Mass therapy of subclinical vivax malaria with primaquine. JAMA 149:1558

18. Jelinek T, Nothdurft HD, Von Sonnenburg F, Loscher T (1995) Long-term efficacy of primaquine in the treatment of vivax malaria in nonimmune travelers. Am J Trop Med Hyg 52:322–324
19. Baird JK, Rieckmann KH (2003) Can primaquine therapy for vivax malaria be improved? Trends Parasitol 19:115–120
20. Schwartz E, Regev-Yochay G, Kurnik D (2000) Short report: a consideration of primaquine dose adjustment for radical cure of *Plasmodium vivax* malaria. Am J Trop Med Hyg 62:393–395
21. Duarte EC, Pang LW, Ribeiro LC, Fontes CJ (2001) Association of subtherapeutic dosages of a standard drug regimen with failures in preventing relapses of vivax malaria. Am J Trop Med Hyg 65:471–476
22. Clyde DF, McCarthy VC (1977) Radical cure of Chesson strain vivax malaria in man by 7, not 14, days of treatment with primaquine. Am J Trop Med Hyg 26:562–563
23. Goller JL, Jolley D, Ringwald P, Biggs BA (2007) Regional differences in the response of *Plasmodium vivax* malaria to primaquine as anti-relapse therapy. Am J Trop Med Hyg 76:203–207
24. Dao NV, Cuong BT, Ngoa ND, le Thuy TT, The ND, Duy DN, Dai B, Thanh NX, Chavchich M, Rieckmann KH et al (2007) Vivax malaria: preliminary observations following a shorter course of treatment with artesunate plus primaquine. Trans R Soc Trop Med Hyg 101:534–539
25. Pukrittayakamee S, Imwong M, Chotivanich K, Singhasivanon P, Day NP, White NJ (2010) A comparison of two short-course primaquine regimens for the treatment and radical cure of *Plasmodium vivax* malaria in Thailand. Am J Trop Med Hyg 82:542–547
26. Krudsood S, Tangpukdee N, Wilairatana P, Phophak N, Baird JK, Brittenham GM, Looareesuwan S (2008) High-dose primaquine regimens against relapse of *Plasmodium vivax* malaria. Am J Trop Med Hyg 78:736–740
27. Alving AS et al (1955) Potentialtion of the curative action of primaquine in vivax malaria by quinine and chloroquine. J Lab Clin Med 46:301–306
28. Bray PG, Deed S, Fox E, Kalkanidis M, Mungthin M, Deady LW, Tilley L (2005) Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum*. Biochem Pharmacol 70:1158–1166
29. Galappaththy GN, Omari AA, Tharyan P (2007) Primaquine for preventing relapses in people with *Plasmodium vivax* malaria. Cochrane Database Syst Rev CD004389
30. Silachamroon U, Krudsood S, Treeprasertsuk S, Wilairatana P, Chalearmrult K, Mint HY, Maneekan P, White NJ, Gourdeuk VR, Brittenham GM et al (2003) Clinical trial of oral artesunate with or without high-dose primaquine for the treatment of vivax malaria in Thailand. Am J Trop Med Hyg 69:14–18
31. Rieckmann KH, McNamara JV, Frischer H, Stockert TA, Carson PE, Powell RD (1968) Gametocytocidal and sporontocidal effects of primaquine and of sulfadiazine with pyrimethamine in a chloroquine-resistant strain of *Plasmodium falciparum*. Bull World Health Organ 38:625–632
32. Pukrittayakamee S, Chotivanich K, Chantra A, Clemens R, Looareesuwan S, White NJ (2004) Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria. Antimicrob Agents Chemother 48:1329–1334
33. Lederman ER, Maguire JD, Sumawinata IW, Chand K, Elyazar I, Estiana L, Sismadi P, Bangs MJ, Baird JK (2006) Combined chloroquine, sulfadoxine/pyrimethamine and primaquine against *Plasmodium falciparum* in Central Java, Indonesia. Malar J 5:108
34. Shekalaghe S, Drakeley C, Gosling R, Ndaro A, van Meegeren M, Enevold A, Alifrangis M, Mosha F, Sauerwein R, Bousema T (2007) Primaquine clears submicroscopic *Plasmodium falciparum* gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. PLoS One 2:e1023
35. El-Sayed B, El-Zaki SE, Babiker H, Gadalla N, Ageep T, Mansour F, Baraka O, Milligan P, Babiker A (2007) A randomized open-label trial of artesunate- sulfadoxine-pyrimethamine

with or without primaquine for elimination of sub-microscopic *P. falciparum* parasitaemia and gametocyte carriage in eastern Sudan. PLoS One 2:e1311
36. Suputtamongkol Y, Chindarat S, Silpasakorn S, Chaikachonpatd S, Lim K, Chanthapakajee K, Kaewkaukul N, Thamlikitkul V (2003) The efficacy of combined mefloquine-artesunate versus mefloquine-primaquine on subsequent development of *Plasmodium falciparum* gametocytemia. Am J Trop Med Hyg 68:620–623
37. Kumar N, Zheng H (1990) Stage-specific gametocytocidal effect *in vitro* of the antimalaria drug qinghaosu on *Plasmodium falciparum*. Parasitol Res 76:214–218
38. Dunyo S, Milligan P, Edwards T, Sutherland C, Targett G, Pinder M (2006) Gametocytaemia after drug treatment of asymptomatic *Plasmodium falciparum*. PLoS Clin Trials 1:e20
39. White NJ (2008) The role of anti-malarial drugs in eliminating malaria. Malar J 7(Suppl 1):S8
40. Lanners HN (1991) Effect of the 8-aminoquinoline primaquine on culture-derived gametocytes of the malaria parasite *Plasmodium falciparum*. Parasitol Res 77:478–481
41. Beaudoin RL, Aikawa M (1968) Primaquine-induced changes in morphology of exoerythrocytic stages of malaria. Science 160:1233–1234
42. Boulard Y, Landau I, Miltgen F, Ellis DS, Peters W (1983) The chemotherapy of rodent malaria. XXXIV. Causal prophylaxis Part III: Ultrastructural changes induced in exo-erythrocytic schizonts of *Plasmodium yoelii yoelii* by primaquine. Ann Trop Med Parasitol 77:555–568
43. Howells RE, Pters W, Fullard J (1970) The chemotherapy of rodent malaria. 13. Fine structural changes observed in the eryhrocytic stages of *Plasmodium berghei berghei* following exposureto primaquine and menoctone. Ann Trop Med Parasitol 64:203–207
44. Aikawa M, Beaudoin RL (1970) *Plasmodium fallax*: high-resolution autoradiography of exoerythrocytic stages treated with Primaquine *in vitro*. Exp Parasitol 27:454–463
45. Peters W, Ellis D, Boulard Y, Landau I (1984) The chemotherapy of rodent malaria XXXVI. Part IV. The activity of a new 8-aminoquinoline, WR 225,448 against exo-erythrocytic schizonts of *Plasmodium yoelii yoelii*. Ann Trop Med Parasitol 78:467–478
46. Rotman A (1975) Genetics of a primaquin-resistant yeast. J Gen Microbiol 89:1–10
47. Krungkrai J, Burat D, Kudan S, Krungkrai S, Prapunwattana P (1999) Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite, *Plasmodium falciparum*. Southeast Asian J Trop Med Public Health 30:636–642
48. Idowu OR, Peggins JO, Brewer TG (1995) Side-chain hydroxylation in the metabolism of 8-aminoquinoline antiparasitic agents. Drug Metab Dispos 23:18–27
49. Vale N, Moreira R, Gomes P (2009) Primaquine revisited six decades after its discovery. Eur J Med Chem 44:937–953
50. Grewal RS (1981) Pharmacology of 8-aminoquinolines. Bull World Health Organ 59:397–406
51. Fry M, Pudney M (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566 C80). Biochem Pharmacol 43:1545–1553
52. Fry M, Beesley JE (1991) Mitochondria of mammalian Plasmodium spp. Parasitology 102:17–26
53. Srivastava IK, Rottenberg H, Vaidya AB (1997) Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J Biol Chem 272:3961–3966
54. Painter HJ, Morrisey JM, Mather MW, Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 446:88–91
55. Vaidya AB, Lashgari MS, Pologe LG, Morrisey J (1993) Structural features of *Plasmodium* cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol Biochem Parasitol 58:33–42
56. Fletcher KA, Barton PF, Kelly JA (1988) Studies on the mechanisms of oxidation in the erythrocyte by metabolites of primaquine. Biochem Pharmacol 37:2683–2690

57. Greenberg J, Taylor DJ, Josephson ES (1951) Studies on *Plasmodium gallinaceum in vitro* II. The effects of some 8-aminoquinolines against the erythrocytic parasites. J Infect Dis 88:163–167
58. Meshnick SR (1998) Artemisinin antimalarials: mechanisms of action and resistance. Med Trop 58:13–17
59. McChesney JD (1981) Considerations about the structure-activity relationships of 8- aminoquinoline antimalarial drugs. Bull World Health Organ 59:459–462
60. Peters W, Irare SG, Ellis DS, Warhurst DC, Robinson BL (1984) The chemotherapy of rodent malaria, XXXVIII. Studies on the activity of three new antimalarials (WR 194,965, WR 228,258 and WR 225,448) against rodent and human malaria parasites (*Plasmodium berghei* and *P. falciparum*). Ann Trop Med Parasitol 78:567–579
61. Mihaly GW, Ward SA, Edwards G, Nicholl DD, Orme ML, Breckenridge AM (1985) Pharmacokinetics of primaquine in man. I. Studies of the absolute bioavailability and effects of dose size. Br J Clin Pharmacol 19:745–750
62. Ward SA, Mihaly GW, Edwards G, Looareesuwan S, Phillips RE, Chanthavanich P, Warrell DA, Orme ML, Breckenridge AM (1985) Pharmacokinetics of primaquine in man. II. Comparison of acute vs chronic dosage in Thai subjects. Br J Clin Pharmacol 19:751–755
63. Bangchang KN, Songsaeng W, Thanavibul A, Choroenlarp P, Karbwang J (1994) Pharmacokinetics of primaquine in G6PD deficient and G6PD normal patients with vivax malaria. Trans R Soc Trop Med Hyg 88:220–222
64. Binh VQ, Chinh NT, Thanh NX, Cuong BT, Quang NN, Dai B, Travers T, Edstein MD (2009) Sex affects the steady-state pharmacokinetics of primaquine but not doxycycline in healthy subjects. Am J Trop Med Hyg 81:747–753
65. Mihaly GW, Ward SA, Edwards G, Orme ML, Breckenridge AM (1984) Pharmacokinetics of primaquine in man: identification of the carboxylic acid derivative as a major plasma metabolite. Br J Clin Pharmacol 17:441–446
66. Greaves J, Evans DA, Gilles HM, Fletcher KA, Bunnag D, Harinasuta T (1980) Plasma kinetics and urinary excretion of primaquine in man. Br J Clin Pharmacol 10:399–404
67. Mayorga P, Deharo E, Landau I, Couarraze G (1997) Preliminary evaluation of primaquine activity on rodent malaria model after transdermal administration. Parasite 4:87–90
68. Nishi KK, Jayakrishnan A (2007) Self-gelling primaquine-gum arabic conjugate: an injectable controlled delivery system for primaquine. Biomacromolecules 8:84–90
69. Singh KK, Vingkar SK (2008) Formulation, antimalarial activity and biodistribution of oral lipid nanoemulsion of primaquine. Int J Pharm 347:136–143
70. Stensrud G, Sande SA, Kristensen S, Smistad G (2000) Formulation and characterisation of primaquine loaded liposomes prepared by a pH gradient using experimental design. Int J Pharm 198:213–228
71. Vangapandu S, Sachdeva S, Jain M, Singh S, Singh PP, Kaul CL, Jain R (2004) 8-Quinolinamines conjugated with amino acids are exhibiting potent blood-schizontocidal antimalarial activities. Bioorg Med Chem 12:239–247
72. Rajic Z, Kos G, Zorc B, Singh PP, Singh S (2009) Macromolecular prodrugs. XII. Primaquine conjugates: synthesis and preliminary antimalarial evaluation. Acta Pharm 59:107–115
73. Borissova R, Lammek B, Stjarnkvist P, Sjoholm I (1995) Biodegradable microspheres. 16. Synthesis of primaquine-peptide spacers for lysosomal release from starch microparticles. J Pharm Sci 84:249–255
74. Brueckner RP, Ohrt C, Baird JK, Milhous WK (2001) 8-aminoquinolines. In: RP J (eds) Antimalarial chemotherapy: mechanisms of action, resistance and new directions. Humana, Totowa, NJ, pp 123–151
75. Cappellini MD, Fiorelli G (2008) Glucose-6-phosphate dehydrogenase deficiency. Lancet 371:64–74
76. Beutler E, Duparc S (2007) Glucose-6-phosphate dehydrogenase deficiency and antimalarial drug development. Am J Trop Med Hyg 77:779–789

77. Beutler E, Dern RJ, Alving AS (1955) The hemolytic effect of primaquine. VI. An *in vitro* test for sensitivity of erythrocytes to primaquine. J Lab Clin Med 45:40–50
78. Beutler E, Dern RJ, Flanagan CL, Alving AS (1955) The hemolytic effect of primaquine. VII. Biochemical studies of drug-sensitive erythrocytes. J Lab Clin Med 45:286–295
79. Srivastava P, Singh S, Jain GK, Puri SK, Pandey VC (2000) A simple and rapid evaluation of methemoglobin toxicity of 8-aminoquinolines and related compounds. Ecotoxicol Environ Saf 45:236–239
80. Clayman C, Arnold J, Hockwald R, Yount E Jr, Edgecomb J, Alving A (1952) Toxicity of primaquine in Caucasians. JAMA 149:1563–1568
81. Clyde DF (1981) Clinical problems associated with the use of primaquine as a tissue schizontocidal and gametocytocidal drug. Bull World Health Organ 59:391–395
82. Taylor WR, White NJ (2004) Antimalarial drug toxicity: a review. Drug Saf 27:25–61
83. Prakash S, Chakrabarti AK, Choudhury DS (1961) Studies on *Plasmodium berghei* Vincke and Lips, 1948. XXXI. Selection of a primaquine resistant strain. Indian J Malariol 15:115–122
84. Peters W (1966) Drug responses of mepacrine- and primaquine-resistant strains of *Plasmodium berghei* Vincke and Lips, 1948. Ann Trop Med Parasitol 60:25–30
85. Arnold J, Alvinig AS, Clayman CB (1961) Induced primaquine resistance in vivax malaria. Trans R Soc Trop Med Hyg 55:345–350
86. Baird JK (2007) A rare glimpse at the efficacy of primaquine. Am J Trop Med Hyg 76:201–202
87. Kitchener S (2002) Malaria in the Australian Defence Force associated with the InterFET peacekeeping operation in East Timor. Mil Med 167:iii–iv
88. Spudick JM, Garcia LS, Graham DM, Haake DA (2005) Diagnostic and therapeutic pitfalls associated with primaquine-tolerant *Plasmodium vivax*. J Clin Microbiol 43:978–981
89. Crockett M, Kain KC (2007) Tafenoquine: a promising new antimalarial agent. Expert Opin Investig Drugs 16:705–715
90. The GlaxoSmithKline (GSK) Clinical Study Register. http://www.gsk-clinicalstudyregister.com. Accessed 23 Apr 2010
91. Medicines for Malaria Venture Project Portfolio. http://www.mmv.org/research-development/project-portfolio/tafenoquine. Accessed 23 Apr 2010
92. Vennerstrom JL, Nuzum EO, Miller RE, Dorn A, Gerena L, Dande PA, Ellis WY, Ridley RG, Milhous WK (1999) 8-aminoquinolines active against blood stage *Plasmodium falciparum in vitro* inhibit hematin polymerization [In Process Citation]. Antimicrob Agents Chemother 43:598–602
93. Vennerstrom JL, Eaton JW (1988) Oxidants, oxidant drugs, and malaria. J Med Chem 31:1269–1277
94. Pradines B, Mamfoumbi MM, Tall A, Sokhna C, Koeck JL, Fusai T, Mosnier J, Czarnecki E, Spiegel A, Trape JF et al (2006) *In vitro* activity of tafenoquine against the asexual blood stages of *Plasmodium falciparum* isolates from Gabon, Senegal, and Djibouti. Antimicrob Agents Chemother 50:3225–3226
95. Peters W, Robinson BL, Milhous WK (1993) The chemotherapy of rodent malaria. LI. Studies on a new 8-aminoquinoline, WR 238,605. Ann Trop Med Parasitol 87:547–552
96. Coleman RE, Clavin AM, Milhous WK (1992) Gametocytocidal and sporontocidal activity of antimalarials against *Plasmodium berghei* ANKA in ICR Mice and *Anopheles stephensi* mosquitoes. Am J Trop Med Hyg 46:169–182
97. Heisy GE, Milhous WK, Hansuklarita P, Theoharides AD, Schuster BG, Davidson DE (1988) Radical curative properties of Tafenoquine (WR 238605, SB252263), Abstr. No. 323. The American Society of Tropical Medicine and Hygiene, Washington DC, p 217
98. Brueckner RP, Lasseter KC, Lin ET, Schuster BG (1998) First-time-in-humans safety and pharmacokinetics of WR 238605, a new antimalarial. Am J Trop Med Hyg 58:645–649
99. Coatney GE, Collins WE, Warren M, Contacos PG (1971) Primate malarias. Department of Health and Welfare, US Govt Publication, Washington, DC

100. Puri SK, Dutta GP (2003) Blood schizontocidal activity of WR 238605 (Tafenoquine) against *Plasmodium cynomolgi* and *Plasmodium fragile* infections in rhesus monkeys. Acta Trop 86:35–40
101. Obaldia N 3rd, Rossan RN, Cooper RD, Kyle DE, Nuzum EO, Rieckmann KH, Shanks GD (1997) WR 238605, chloroquine, and their combinations as blood schizonticides against a chloroquine-resistant strain of *Plasmodium vivax* in *Aotus* monkeys. Am J Trop Med Hyg 56:508–510
102. Lee CC, Kinter LD, Heiffer MH (1981) Subacute toxicity of primaquine in dogs, monkeys, and rats. Bull World Health Organ 59:439–448
103. Augusto O, Weingrill CL, Schreier S, Amemiya H (1986) Hydroxyl radical formation as a result of the interaction between primaquine and reduced pyridine nucleotides. Catalysis by hemoglobin and microsomes. Arch Biochem Biophys 244:147–155
104. Baird JK, Davidson DJ, Decker JJ (1986) Oxidative activity of hydroxylated primaquine analogs. Non-toxicity to glucose-6-phosphate dehydrogenase-deficient human red blood cells *in vitro*. Biochem Pharmacol 35:1091–1098
105. Hiebsch RR, Raub TJ, Wattenberg BW (1991) Primaquine blocks transport by inhibiting the formation of functional transport vesicles. Studies in a cell-free assay of protein transport through the Golgi apparatus. J Biol Chem 266:20323–20328
106. van Weert AW, Geuze HJ, Groothuis B, Stoorvogel W (2000) Primaquine interferes with membrane recycling from endosomes to the plasma membrane through a direct interaction with endosomes which does not involve neutralisation of endosomal pH nor osmotic swelling of endosomes. Eur J Cell Biol 79:394–399
107. Edstein MD, Kocisko DA, Brewer TG, Walsh DS, Eamsila C, Charles BG (2001) Population pharmacokinetics of the new antimalarial agent tafenoquine in Thai soldiers. Br J Clin Pharmacol 52:663–670
108. Charles BG, Miller AK, Nasveld PE, Reid MG, Harris IE, Edstein MD (2007) Population pharmacokinetics of tafenoquine during malaria prophylaxis in healthy subjects. Antimicrob Agents Chemother 51:2709–2715
109. Idowu OR, Peggins JO, Brewer TG, Kelley C (1995) Metabolism of a candidate 8-aminoquinoline antimalarial agent, WR 238605, by rat liver microsomes. Drug Metab Dispos 23:1–17
110. Lell B, Faucher JF, Missinou MA, Borrmann S, Dangelmaier O, Horton J, Kremsner PG (2000) Malaria chemoprophylaxis with tafenoquine: a randomised study. Lancet 355:2041–2045
111. Shanks GD, Kain KC, Keystone JS (2001) Malaria chemoprophylaxis in the age of drug resistance. II. Drugs that may be available in the future. Clin Infect Dis 33:381–385
112. Shanks GD, Oloo AJ, Aleman GM, Ohrt C, Klotz FW, Braitman D, Horton J, Brueckner R (2001) A new primaquine analogue, tafenoquine (WR 238605), for prophylaxis against *Plasmodium falciparum* malaria. Clin Infect Dis 33:1968–1974
113. Brueckner RP, Coster T, Wesche DL, Shmuklarsky M, Schuster BG (1998) Prophylaxis of *Plasmodium falciparum* infection in a human challenge model with WR 238605, a new 8-aminoquinoline antimalarial. Antimicrob Agents Chemother 42:1293–1294
114. Hale BR, Owusu-Agyei S, Fryauff DJ, Koram KA, Adjuik M, Oduro AR, Prescott WR, Baird JK, Nkrumah F, Ritchie TL et al (2003) A randomized, double-blind, placebo-controlled, dose-ranging trial of tafenoquine for weekly prophylaxis against *Plasmodium falciparum*. Clin Infect Dis 36:541–549
115. Walsh SD, Eamsila C, Sasiprapha T, Sangkharomya S, Khaewsathien P, Supakalin P, Tang DB, Jarasrumgsichol P, Chuenchitra SC, Edstein MD, et al (2004) Randomized, double-blind, placebo controlled evaluation of monthly tafenoquine (WR 238605) for *Plasmodium falciparum* and *P. vivax* malaria in Royal Thai Army soldiers. J Inf Dis 190:1456-1463
116. Nasveld PE, Edstein MD, Reid M, Brennan L, Harris IE, Kitchener SJ, Leggat PA, Pickford P, Kerr C, Ohrt C et al (2010) Randomized, double-blind study of the safety, tolerability, and efficacy of tafenoquine versus mefloquine for malaria prophylaxis in nonimmune subjects. Antimicrob Agents Chemother 54:792–798

117. Leary KJ, Riel MA, Roy MJ, Cantilena LR, Bi D, Brater DC, van de Pol C, Pruett K, Kerr C, Veazey JM Jr et al (2009) A randomized, double-blind, safety and tolerability study to assess the ophthalmic and renal effects of tafenoquine 200 mg weekly versus placebo for 6 months in healthy volunteers. Am J Trop Med Hyg 81:356–362
118. Nasveld P, Kitchener S, Edstein M, Rieckmann K (2002) Comparison of tafenoquine (WR238605) and primaquine in the post-exposure (terminal) prophylaxis of vivax malaria in Australian Defence Force personnel. Trans R Soc Trop Med Hyg 96:683–684
119. Elmes NJ, Nasveld PE, Kitchener SJ, Kocisko DA, Edstein MD (2008) Comparison of three different dose regimens of tafenoquine versus primaquine for post exposure prophylaxis of vivax malaria in the South West Pacific. Trans Roy Soc Trop Med Hyg 102:1095–1101
120. Edstein MD, Nasveld PE, Kocisko DA, Kitchener SJ, Gatton ML, Rieckmann KH (2007) Gender differences in gastrointestinal disturbances and plasma concentrations of tafenoquine in healthy volunteers after tafenoquine administration for post-exposure vivax malaria prophylaxis. Trans R Soc Trop Med Hyg 101:226–230
121. Walsh DS, Looareesuwan S, Wilairatana P, Heppner DG Jr, Tang DB, Brewer TG, Chokejindachai W, Viriyavejakul P, Kyle DE, Milhous WK et al (1999) Randomized dose-ranging study of the safety and efficacy of WR 238605 (Tafenoquine) in the prevention of relapse of *Plasmodium vivax* malaria in Thailand. J Infect Dis 180:1282–1287
122. Walsh DS, Wilairatana P, Tang DB, Heppner DG Jr, Brewer TG, Krudsood S, Silachamroon U, Phumratanaprapin W, Siriyanonda D, Looareesuwan S (2004) Randomized trial of 3-dose regimens of tafenoquine (WR238605) versus low-dose primaquine for preventing *Plasmodium vivax* malaria relapse. Clin Infect Dis 39:1095–1103
123. Nasveld P, Kitchener S (2005) Treatment of acute vivax malaria with tafenoquine. Trans R Soc Trop Med Hyg 99:2–5
124. Kitchener S, Nasveld P, Edstein MD (2007) Tafenoquine for the treatment of recurrent *Plasmodium vivax* malaria. Am J Trop Med Hyg 76:494–496
125. Nosten F, White NJ (2007) Artemisinin-based combination treatment of falciparum malaria. Am J Trop Med Hyg 77:181–192
126. Roberts L, Enserink M (2007) Malaria. Did they really say ... eradication? Science 318:1544–1545

Other 4-Methanolquinolines, Amyl Alcohols and Phentathrenes: Mefloquine, Lumefantrine and Halofantrine

Francois Nosten, Penelope A. Phillips-Howard, and Feiko O. ter Kuile

Abstract This chapter describes mefloquine, pyronaridine, halofantrine, piperaquine and lumefantrine under the broader title of the 4-methanolquinolines, amyl alcohols and phentathrenes. We provide a brief resume of each drug, in terms of their chemical properties, formulae, pharmacokinetics, clinical indications for use, and their efficacy and safety. Recognizing the limited number of antimalarials available, and in the developmental pipeline, attention is focussed on describing the history of each drug and how their indications have evolved as data on safety in human populations accumulates over time, and how patterns of use have changed with growing multiple drug resistance. Their combined use with the artemisinin derivatives is briefly described and readers are recommended to consult other chapters in this book which more fully describe such combinations.

F. Nosten (✉)
Nuffield Department of Clinical Medicine, Centre for Tropical Medicine, Churchill Hospital, University of Oxford, Oxford OX3 7LJ, UK

Shoklo Malaria Research Unit, PO Box 46, 68/30 Baan Tung Road, Mae Sot, Tak Province 63110, Thailand

Mahidol University, Bangkok, Thailand
e-mail: SMRU@tropmedres.ac

P.A. Phillips-Howard
Centre for Public Health, Liverpool John Moores University, Liverpool, Henry Cotton Campus, 15–21 Webster Street, L3 2ET Liverpool, UK
e-mail: p.phillips-howard@ljmu.ac.uk

F.O. ter Kuile
Malaria Epidemiology Unit Child and Reproductive Health Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK
e-mail: terkuile@liverpool.ac.uk

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_5,

1 Mefloquine

1.1 Structure and Action

Mefloquine hydrochloride is a 4-quinolinemethol derivative synthesised as a structural analogue (2-aryl substituted chemical) of quinine. Its full chemical name is (R*, S*)-(±)-α-2-piperidinyl-2,8-bis (trifluoromethyl)-4-quinolinemethanol hydrochloride. Its formula is $C_{17}H_{16}F_6N_2O$ (Fig. 1). Mefloquine was discovered by the Experimental Therapeutics Division of the Walter Reed Army Institute of Research (WRAIR) in the 1970s for chemoprophylaxis (250 mg weekly) and therapy (15–25 mg/kg) and was approved by the U.S. Food and Drug Administration in 1989.

Mefloquine is a blood schizonticide, active against the erythrocytic stages of *Plasmodium falciparum* and *P. vivax*, with no effect on the exoerythrocytic (hepatic) stages of the parasite, and with limited information of its effect on *P. ovale, P. malariae* and *P. knowlesi*. Studies indicate mefloquine interferes with the transport of haemoglobin and other substances from the host erythrocyte to the food vacuole of the malaria parasite, causing swelling and cytotoxicity [1]. Mefloquine strongly inhibits endocytosis in the D10 strain of *P. falciparum* using several lines of evidence: a reduction in haemoglobin levels in the parasite as assessed by Western blotting, decreased levels of accumulation of biotinylated dextran by the parasite in preloaded erythrocytes, significantly lower concentrations of fluorescent dextran in the food vacuole, and a reduced percentage of parasites with multiple transport vesicles [2].

Mefloquine is a chiral molecule; it has two asymmetric carbon atoms and exists in two racemic forms (erythro and threo), each of which is composed of a pair of optical isomers, i.e. (±)-erythro-enantiomers and the (±)-threo-epimers. Clinically, the racemic mixture of the erythro-enantiomers is used [3]. Unlike other antimalarial drugs such as chloroquine, halofantrine, and lumefantrine, there is stereoselectivity

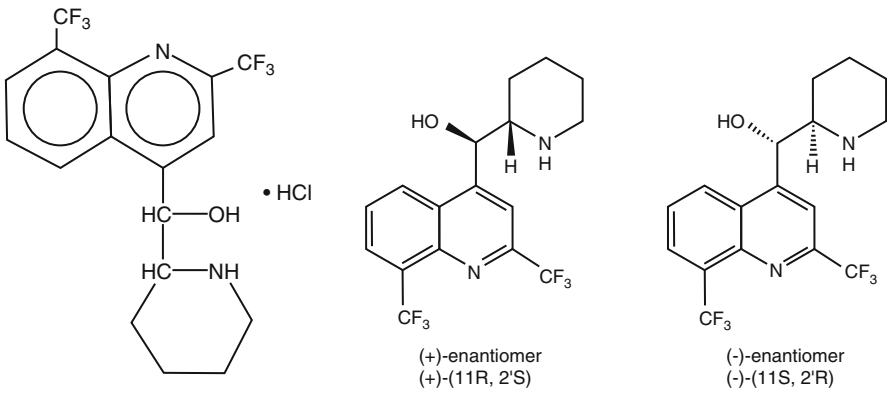


Fig. 1 Structure of mefloquine hydrochloride and its two enantiomers

in its antimalarial activity, with the (+)-isomer ~1.7 times more potent than the (−)-isomer in vitro [4, 5].

1.2 Pharmacokinetics

Mefloquine is moderately well absorbed orally and extensively distributed and is 98% bound to plasma proteins. Splitting 25 mg/kg mefloquine into 2 or 3 doses given 16–24 h apart reduces vomiting, improves oral bioavailability and the therapeutic response in the treatment of acute falciparum malaria [6]. Food increases its bioavailability by up to 40%. The parent compound is metabolized by the cytochrome P450 enzyme CYP3A4 to two major metabolites: carboxy- and hydroxyl-mefloquine, which are inactive against *P. falciparum*. Mefloquine is eliminated slowly and has a terminal elimination half-life of ~3 weeks in volunteers and 2 weeks in patients. Total clearance, which is essentially hepatic, is 30 ml/min in volunteers. A steady-state plasma concentration of 1,000–2,000 μg/l is reached after 7–10 weeks following weekly 250 mg prophylaxis and it is therefore recommended to start medication at least 2 weeks before travel. There are stereoselective differences in their pharmacokinetics and the ability of the mefloquine enantiomers to cause certain adverse effects [5]. In humans, the plasma concentration of the (−) enantiomer is approximately threefold higher than the (+) enantiomer, reflecting the stereoselectivity in the clearance and volume distribution [5, 7–11]. Co-administration with artemisinin does not appear to influence mefloquine enantiomer pharmacokinetics [12].

1.3 Clinical Use

One tablet of 250 mg mefloquine hydrochloride per week (adult dose; equivalent to 228 mg of the free base) has been used for prophylaxis in travellers, including for young children and pregnant women. The limited data available on the use of mefloquine in human pregnancy are reassuring and do not indicate an increased teratogenic risk [13]. A retrospective study of 208 women on the Thai–Burmese border treated with mefloquine found an increased risk of stillbirths [14], however, this finding was not confirmed in a large prospective trial of mefloquine prophylaxis in Malawian pregnant women [15] and remains unexplained. As a treatment, it is now mainly used in combination with artesunate, a water-soluble artemisinin derivative. It is available as a loose combination (as part of a blister pack containing both drugs), and as a new fixed-dose combination developed with support from the Drugs for Neglected Diseases Initiative (DNDi) and produced by Farmanguinhos/Fiocruz, Brazil and Cipla, India. The treatment dose is 25 mg/kg of mefloquine and 12 mg/kg of artesunate given as 8.3 and 4 mg/kg/day over 3 days, respectively. Tablets of the new fixed-dose combination come in adult and child “strengths”,

with several co-blistered formulations of the loose combinations made by different manufacturers. Mefloquine is also being explored for a new indication as intermittent preventive therapy (IPT) against malaria. Two trials evaluating the role of mefloquine as IPT in infants (IPTi) [16] and in pregnant women (IPTp) [17] found mefloquine to be very effective, but the low tolerability limited its acceptance for use as IPTi. Further IPTp studies are ongoing in five countries in Africa with the lower 15 mg/kg dose in pregnancy.

1.4 Resistance

Most experience with mefloquine as monotherapy and later in combination with the artemisinin derivatives has been gained from areas of multiple drug resistance in Southeast Asia, such as on the Thai–Burmese (Myanmar) border. Thailand was the first country to use mefloquine for first-line treatment of acute malaria. From 1985 to 1990, it was recommended in combination with sulfadoxine and pyrimethamine, as "MSP" in a fixed-dose combination, at a single dose of ~15/30/1.5 mg/kg, providing a 98% cure rate after its introduction in 1985, however, this dropped to <50% in children by 1990 [18]. Because of high levels of existing parasite resistance to SP, and lack of additional therapeutic efficacy over mefloquine alone, the SP component was dropped [18, 19] and replaced by mefloquine monotherapy; initially at a single dose of 15 mg/kg. High levels of treatment failure with this dose [18, 20] prompted 25 mg/kg dosing [21–23], split (750 and 500 mg, 16–24 h apart) to reduce vomiting [24]. Within 8 years, mefloquine monotherapy failure rates on the Thai–Burmese border reached 60% and, following extensive testing, the combination of mefloquine 25 mg/kg with artesunate 12 mg/kg given over 3 days (MAS_3) became the new standard therapy [25]. This therapy, combined with early diagnosis and use of insecticide treated nets, reduced *P. falciparum* malaria incidence, and halted, and later reversed the progression of mefloquine resistance [26–28]. The combination offered a potential public health solution for multiple drug-resistant *P. falciparum*, and allowed time for the development of other new drugs [1].

In vivo resistance to mefloquine, mediated mainly by an increase in gene copy number and expression of the *P. falciparum* multi-drug resistance (MDR) gene-1 (*pfmdr1*), a gene encoding a parasite-transport protein [29, 30], has been confirmed. This has been reported on the borders of Thailand with Burma (Myanmar) and Cambodia, in the western provinces of Cambodia, the eastern states of Burma (Myanmar) and its border with China, along the Laos and Burma borders, the adjacent Thai–Cambodian border and in southern Vietnam. It is likely the initial deployment of low dose of mefloquine may have encouraged resistance. Theoretical evidence suggests that initial use of higher doses, preferably in combination with an artemisinin derivative, is less likely to lead to resistance [31].

1.5 Tolerability

Preclinical studies demonstrate mefloquine to be safe and effective [32], and extensive clinical experience to date supports this. Nevertheless, widespread deployment of mefloquine for treatment and prophylaxis has been hampered by concerns about its tolerability. Side effects following treatment are common; they are usually mild and restricted to dizziness/vertigo and gastro-intestinal disturbances [24]. Vomiting after mefloquine is a problem in young children, but can be mitigated by splitting the dose over 2 or 3 days, and by fever reduction [24, 33]. In older children and adults, mild neuro-psychiatric events (headache, dizziness, insomnia and vivid dreams) are reported in ~25% of patients treated with 25 mg/kg mefloquine.

Mefloquine is also associated with a self-limiting acute neuropsychiatric syndrome manifest by encephalopathy, convulsions or psychosis [34–36], apparent in international travellers taking 250 mg mefloquine each week for prophylaxis [35, 37]. Mefloquine is thus contraindicated for prophylaxis in patients with active depression, a recent history of depression, generalised anxiety disorder, psychosis, or schizophrenia or other major psychiatric disorders, or with a history of convulsions [38]. These contraindications are prevalent in 9–10% of the military [39] and civilian [40] populations presenting for malaria chemoprophylaxis [41], but are not documented in endemic populations. While the mechanism is not yet fully understood, neuropsychiatric events have been demonstrated to be associated with dose in humans [42, 43]. The rates in travellers are estimated to be 1:10,000 persons, equally frequent with chloroquine prophylaxis, but higher than in similar populations that used other forms of prophylaxis [35, 44]. The incidence following treatment doses is 1:1,000 in Asian patients [45], 1:200 in Caucasian or African patients with uncomplicated malaria and 1:20 in patients recovering from severe malaria [46]. Previous history of psychiatric illness or epilepsy is a risk factor. Females and individuals of low body mass index are also at apparent greater risk. Neuropsychiatric reactions are more common if mefloquine was used in the previous 2 months, and thus should not be used to treat recrudescent infections within 2 months of treatment.

Total (racemic) concentrations of mefloquine are ~30-fold higher in brain than in plasma [47]. In man, approximately threefold higher concentrations of the (−)-enantiomer is observed in plasma, and 1.5-fold higher in brain, but post-mortem studies demonstrated stereoselective brain penetration, greater for the (+)-enantiomer, with (−) and (+) concentrations at ~23- versus 56-fold higher in the brain's white matter compared with plasma (the reverse is found in rat models where the penetration of the (−)-enantiomer is greater than that of its antipode) [7, 47–49]. There is a growing body of evidence on the mechanisms of possible neurotoxicity (see reviews [50] and [41]). The high level of accumulation of mefloquine in brain tissue may be associated with direct neurotoxic damage and cell death, with binding to neuroreceptors and cholinesterases, inhibition of sarco (endo)plasmic reticulum Ca^{2+} ATPase (SERCA) activity and interference with

cellular Ca^{2+} homeostasis and reductions in central nervous system efflux in individuals possessing certain (human) MDR1 polymorphisms [50].

1.6 The Future

Using mefloquine as a scaffold, WRAIR has constructed a library of 200 potential next generation quinoline methanol compounds to identify leads that possess biological properties consistent with the target product profile for malaria chemoprophylaxis but less susceptible to passage across the blood–brain barrier (to reduce adverse neurological effects) [51, 52]. During a programme to examine the biochemical basis of side effects, investigators discovered that the (−)-(R,S)-enantiomer is a potent adenosine A2A receptor antagonist, resulting in a programme to develop novel adenosine A2A antagonists for the management of Parkinson's disease [53, 54]. Mefloquine is effective against JC virus and is reported to have successfully treated progressive multifocal leuko-encephalopathy (a progressive, usually fatal, demyelinating disease caused by the JC virus) [55, 56]. Mefloquine is undergoing in vitro and in vivo studies to evaluate its effectiveness for the treatment of helminth infections [57], including those caused by *Schistosoma* [58], *Clonorchis* and *Paragonimus* [59].

2 Pyronaridine

2.1 Structure and Action

Pyronaridine is a Mannich base with a pyronaridine nucleus synthesised from mepacrine (9 amino acridine). Its formula is $C_{29}H_{32}ClN_5O_2$ (Fig. 2). It was synthesised in 1970 in China and is available as a free base and as a tetraphosphate, the salt used in current formulations. It was used in China as a monotherapy in the 1980s and 1990s but has now been developed as a combination therapy with

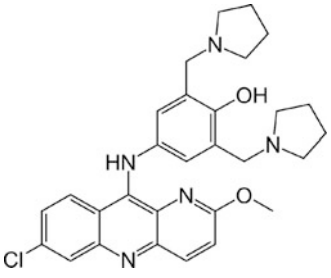


Fig. 2 Structure of pyronaridine

artesunate by Shin Poong Pharmaceutical (Korea) and Medicines for Malaria Venture (MMV, Switzerland).

Pyronaridine is active against asexual forms of *Plasmodium* by forming complexes with ferriprotoporphyrin IX. Growth studies of *P. falciparum* K1 in culture demonstrate the ability of pyronaridine to inhibit in vitro β-haematin formation, to form a complex with a stoichiometry of 1:2, to enhance haematin-induced red blood cell lysis, and to inhibit glutathione-dependent degradation of haematin [60]. However, observations that pyronaridine exerted this mechanism of action in situ, based on showing antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, and quinine) that inhibit β-haematin formation, were equivocal. Interestingly pyronaridine is also active against young gametocytes (stage II and III) in vitro [61], although recent clinical studies did not detect a difference in gametocyte carriage following treatment with the fixed combination artesunate–pyronaridine when compared with artemether– lumefantrine [62]. The compound is also a poor substrate and an inhibitor of the Permeability glycoprotein (P-gp) ATP-dependant transporter, a product of the human multidrug resistance-1 (MDR1;ABCB1) gene that influences the passage of many drugs across epithelial barriers. The P-gp-mediated efflux could attenuate oral absorption of the drug when the luminal concentration falls; however, this is likely to play a minimal role in the initial absorption of the drug.

2.2 Pharmacokinetics

The pharmacokinetic properties of pyronaridine are not well characterised. The drug is readily absorbed from the small intestine following oral administration and is widely distributed in most tissues [63]. The peak value of the drug in the blood is reached at around 8 h post-administration, and it shows a poor permeability across the blood–brain barrier. Pyronaridine concentrates preferentially in infected red blood cells and its distribution and elimination are influenced by age and disease status. It is eliminated slowly, with the half-life currently estimated at 18 days in patients with malaria [64]. Improvements to the assay that measures blood concentrations are likely to reveal a longer half-life.

2.3 Resistance

Resistance is known to have developed in *P. falciparum* when the drug was used in China but the molecular mechanism is unknown. High recurrence rates were noted in early clinical trials in Thailand [65] and cross-resistance with chloroquine was suggested in vitro [66]. Pyronaridine is not used as a single agent anymore but only in combination with artesunate to prevent the emergence of de novo resistance.

3 Halofantrine

3.1 Structure, Action and Resistance

Halofantrine belongs to the phenanthrene methanol group and was developed by WRAIR in the 1960s and then by Smith Klein, now GlaxoSmithKlein. It is a small chiral molecule and the chemical formula is $C_{26}H_{30}Cl_2F_3NO$ (Fig. 3). The oral formulation is in tablets containing 250 mg of hydrochloride salt. Like other quinoline derivatives, the mode of action of halofantrine appears to be in the inhibition of the formation of β-haematin crystals but the precise mechanism of action is unclear. Recently it was shown that halofantrine forms complexes with ferriprotoporphyrin IX and that the inhibition of the haemozoin formation occurs principally at the lipid–aqueous interface, an environment more compatible with the crystal structure of halofantrine–ferriprotoporphyrin IX [67]. Halofantrine is poorly and erratically absorbed due to its low solubility. Absorption is enhanced by fat co-administration. The principal active metabolite is *N*-desbutyl-halofantrine. The terminal half-life in patients with malaria is ~4.7 days, making it a relatively rapidly eliminated antimalarial when compared with other drugs of the same group. As halofantrine was withdrawn from use due to the discovery of halofantrine cardiotoxicity (see below), there are few studies on the development of resistance to this drug. Molecular studies have demonstrated that mutations in *pfmdr1* result in altered halofantrine transport, suggesting a role for this efflux transport mechanism in resistance to this drug [68]. Cross-resistance with mefloquine was shown in clinical studies in South East Asia [69] and it is likely that the drug shares common resistance mechanisms with mefloquine and lumefantrine due to their chemical similarities.

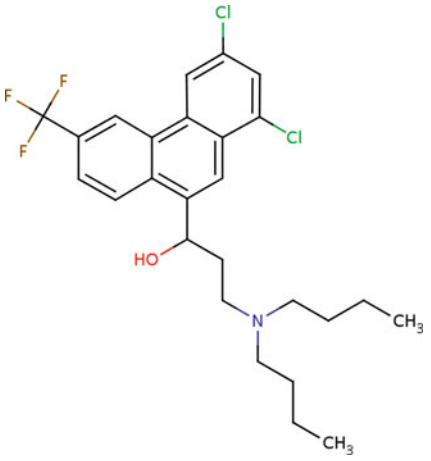


Fig. 3 Structure of halofantrine

3.2 Tolerability

Initially, halofantrine looked like a promising drug for the treatment of uncomplicated falciparum infections caused by chloroquine-resistant parasites. It rapidly cleared parasites and was well tolerated. The first report of the cardiotoxicity of halofantrine in 1993 [70], came as a surprise since the drug had been developed in full compliance with GCP standards. Halofantrine and its principal quinidine-like metabolite have a Class III effect on cardiac repolarization [71]. It causes a dose-dependent blockade of the I_{kr} channel (through hERG) by binding to the open or inactivated state. This translates on an ECG to a marked prolongation of the QT interval and is more marked when halofantrine is given after mefloquine, probably as a result of the inhibition of the slow delayed rectifier potassium channel I_{ks} [72]. This QT prolongation, seen at therapeutic doses, increases the risk of the potentially fatal Torsade de Pointes and since the first report, several sudden deaths have been related to the drug. Because of this, halofantrine has been withdrawn in many countries and from international guidelines on the treatment of malaria.

4 Piperaquine

4.1 Structure and Action

Piperaquine is a bis-amino 4 quinoline synthesised more than 50 years ago by Rhone-Poulenc (France). It was abandoned and rediscovered in China by the Shanghai Research Institute of Pharmaceutical Industry. The drug was used on a large scale (140,000,000 doses) for prophylaxis and treatment of chloroquine resistant *P. falciparum* between 1978 and 1994, but resistance developed in the 1990s. Piperaquine is a lipophilic compound and its chemical formula is $C_{29}H_{32}Cl_2N_6$ (Fig. 4). The full mechanism of action is unknown but piperaquine concentrates in the parasite food vacuole and inhibits the dimerisation of haematin by binding. Recent work by Warhurst and colleagues has shed more light on the possible mode of action [73]. The high activity of piperaquine against chloroquine-resistant falciparum could be explained by its high lipid accumulation ratio (LAR) leading to an increase in β-haematin inhibition in vacuolar lipids where the crystals of haemozoin are produced. The drug may also act by blocking efflux from the food

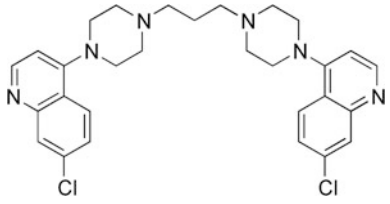


Fig. 4 Structure of piperaquine

vacuole by hydrophobic interaction with the parasite chloroqine-resistance transporter, *pfcrt* [73].

4.2 Pharmacokinetics

Piperaquine is lipophilic and exhibits considerable inter-individual variability in pharmacokinetics. It accumulates preferentially in infected red blood cells and this affects the plasma/blood concentration ratio. Like chloroquine, it has a large apparent volume of distribution and a slow elimination. The terminal elimination half-life is probably longer than previously thought and could exceed 30 days [74]. This is valuable for ensuring a prolonged post-treatment prophylactic effect and when the drug is used for IPT. In children, studies have shown that, dose adjustment may be needed [75]. Likewise, pregnant women may have lower piperaquine exposure than non-pregnant women, and the dosage in pregnancy may also need to be adjusted. Piperaquine has two major metabolites: a carboxylic acid and a mono-*N*-oxidated piperaquine product.

4.3 Resistance

Resistance to piperaquine is known to have developed in vivo in China when it was used as monotherapy but there is no indication that it has spread elsewhere. There is no specific molecular marker of resistance to piperaquine and the role of the *P. falciparum* transport proteins *pfmdr1* and *pfcrt* remains unclear [76, 77]. In clinical use, piperaquine is now used in a fixed combination with dihydroartemisinin (DHA) developed by Holleypharm (China) and Sigma-Tau (Italy) in partnership with MMV (Switzerland). DHA-piperaquine is one of the most promising artemisinin-based combination therapies (ACTs) in the antimalarial armamentarium.

5 Lumefantrine

5.1 Structure and Action

Lumefantrine is a racemic 2,4,7,9-substituted fluorine derivative belonging to the arylamino-alcohol group of antimalarials with a molecular structure reminiscent of halofantrine. It was originally synthesised by the Academy of Military Sciences in Beijing (PRC) in the 1980s. Its chemical formula is $C_{30}H_{32}Cl_3NO$ (Fig. 5). It is insoluble in water and the two enantiomers have equal antimalarial activities.

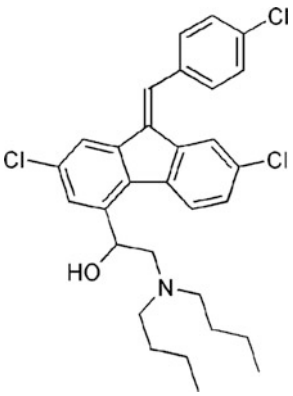


Fig. 5 Structure of lumefantrine

Lumefantrine is active against *P. falciparum* and *P. vivax* asexual stages but not against pre-erythrocytic liver stages, including hynozoites, or against gametocytes. The mode of action of lumefantrine is not known precisely but by similarity of structure with the antimalarials of the same group, it is assumed that lumefantrine kills parasites by inhibiting the polymerisation of heme.

5.2 Pharmacokinetics

The pharmacokinetics of lumefantrine have been extensively described in various populations (European, Asian and African) in adults, children and also in pregnant women. The drug is slowly absorbed (time to peak concentrations is approximately 6 h) and metabolised to desbutyl-lumefantrine via CYP3A4 but largely eliminated as parent compound via the liver in faeces and urine. The absorption is dose limited, so the total daily dose must be given on two separate occasions in order to be absorbed, the first serious impediment to observance [78]. The terminal elimination half-life is approximately 4.5 days, reminiscent of halofantrine and much shorter than mefloquine or piperaquine. Lumefantrine is highly lipophilic and has a low and variable bioavailability. This is a major contributor to the observed inter-individual variability in its kinetics. The relative fraction of the dose absorbed is also highly variable between patients and between doses. This is probably explained by the combined effects of illness on intestinal mobility, increased food intake with recovery and decreasing parasitaemia. Co-administration of food (or some fat) has a marked effect on the absorption of lumefantrine and this is the second major impediment to observance. Recent studies have shown that as little as 1.2 g of fat was needed for optimum lumefantrine absorption [71]. Unfortunately, this was not taken into account when the paediatric formulation of artemether–lumefantrine (Coartem) was developed. Initially, a 4-dose regimen of Coartem was recommended based on the initial Chinese trials. However this resulted in low cure rates in Thailand [79] but it helped in defining the lumefantrine exposure–cure

rate relationship. The most determinant factors of cure were found to be the initial parasite load and the Area Under the plasma concentration Curve (AUC) of lumefantrine. A useful surrogate of the AUC is the day 7 lumefantrine concentration. A plasma lumefantrine concentration of 280 ng/ml was found to be a useful discriminating cut-off to determine subsequent risk of recrudescence [80] and, in the absence of resistance, a day 7 concentration of 500 ng/ml would be expected to cure >90% of patients [81]. After too many years of delay, the 6-dose regimen became universally recommended. For pregnancy, however, this standard regimen is associated with lower plasma concentrations because of the increased volume of distribution and faster elimination, which will lead to treatment failures [82]. Modelling suggests that longer courses are needed to achieve lumefantrine concentrations comparable to that in non-pregnant patients [75].

5.3 Resistance

Resistance to lumefantrine can be readily obtained in vitro and in animal models. In vitro, single-nucleotide polymorphisms in the *pfmdr1* gene have been associated with increased IC_{50} values for lumefantrine [83]. An increase in *pfmdr1* copy number also resulted in decreased in vitro susceptibility and increased risk of failure in patients receiving the 4-dose regimen [80]. Interestingly, resistance to lumefantrine in *P. falciparum* could be associated with the loss of chloroquine resistance (i.e. the loss of the *pfcrt* K76T mutation) [84]. Lumefantrine is not used as a single drug but only in combination with artemether (see Coartem). Its future depends on controlling the emergence of resistance to the artemisinin derivatives and/or to the emergence of resistance to lumefantrine itself or through cross resistance with mefloquine.

References

1. Olliaro P (2001) Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther 89:207–219
2. Hoppe HC, van Schalkwyk DA, Wiehart UI, Meredith SA, Egan J, Weber BW (2004) Antimalarial quinolines and artemisinin inhibit endocytosis in *Plasmodium falciparum*. Antimicrob Agents Chemother 48:2370–2378
3. Sweeney TR (1981) The present status of malaria chemotherapy: mefloquine, a novel antimalarial. Med Res Rev 1:281–301
4. Karle JM, Olmeda R, Gerena L, Milhous WK (1993) *Plasmodium falciparum*: role of absolute stereochemistry in the antimalarial activity of synthetic amino alcohol antimalarial agents. Exp Parasitol 76:345–351
5. Brocks DR, Mehvar R (2003) Stereoselectivity in the pharmacodynamics and pharmacokinetics of the chiral antimalarial drugs. Clin Pharmacokinet 42:1359–1382

6. Simpson JA, Price R, ter Kuile F, Teja-Isavatharm P, Nosten F, Chongsuphajaisiddhi T, Looareesuwan S, Aarons L, White NJ (1999) Population pharmacokinetics of mefloquine in patients with acute falciparum malaria. Clin Pharmacol Ther 66:472–484
7. Gimenez F, Pennie RA, Koren G, Crevoisier C, Wainer IW, Farinotti R (1994) Stereoselective pharmacokinetics of mefloquine in healthy Caucasians after multiple doses. J Pharm Sci 83:824–827
8. Martin C, Gimenez F, Bangchang KN, Karbwang J, Wainer IW, Farinotti R (1994) Whole blood concentrations of mefloquine enantiomers in healthy Thai volunteers. Eur J Clin Pharmacol 47:85–87
9. Hellgren U, Jastrebova J, Jerling M, Krysen B, Bergqvist Y (1996) Comparison between concentrations of racemic mefloquine, its separate enantiomers and the carboxylic acid metabolite in whole blood serum and plasma. Eur J Clin Pharmacol 51:171–173
10. Bourahla A, Martin C, Gimenez F, Singhasivanon V, Attanath P, Sabchearon A, Chongsuphajaisiddhi T, Farinotti R (1996) Stereoselective pharmacokinetics of mefloquine in young children. Eur J Clin Pharmacol 50:241–244
11. Kerb R, Fux R, Morike K, Kremsner PG, Gil JP, Gleiter CH, Schwab M (2009) Pharmacogenetics of antimalarial drugs: effect on metabolism and transport. Lancet Infect Dis 9:760–774
12. Svensson US, Alin H, Karlsson MO, Bergqvist Y, Ashton M (2002) Population pharmacokinetic and pharmacodynamic modelling of artemisinin and mefloquine enantiomers in patients with falciparum malaria. Eur J Clin Pharmacol 58:339–351
13. Phillips-Howard PA, Steffen R, Kerr L, Vanhauwere B, Schildknecht J, Fuchs E, Edwards R (1998) Safety of mefloquine and other antimalarial agents in the first trimester of pregnancy. J Travel Med 5:121–126
14. Nosten F, Vincenti M, Simpson J, Yei P, Thwai KL, de Vries A, Chongsuphajaisiddhi T, White NJ (1999) The effects of mefloquine treatment in pregnancy. Clin Infect Dis 28:808–815
15. Steketee RW, Wirima JJ, Slutsker L, Roberts JM, Khoromana CO, Heymann DL, Breman JG (1996) Malaria parasite infection during pregnancy and at delivery in mother, placenta, and newborn: efficacy of chloroquine and mefloquine in rural Malawi. Am J Trop Med Hyg 55(1 Suppl):24–32
16. Gosling RD, Gesase S, Mosha JF, Carneiro I, Hashim R, Lemnge M, Mosha FW, Greenwood B, Chandramohan D (2009) Protective efficacy and safety of three antimalarial regimens for intermittent preventive treatment for malaria in infants: a randomised, double-blind, placebo-controlled trial. Lancet 374:1521–1532
17. Briand V, Bottero J, Noel H, Masse V, Cordel H, Guerra J, Kossou H, Fayomi B, Ayemonna P, Fievet N et al (2009) Intermittent treatment for the prevention of malaria during pregnancy in Benin: a randomized, open-label equivalence trial comparing sulfadoxine-pyrimethamine with mefloquine. J Infect Dis 200:991–1001
18. Nosten F, ter Kuile F, Chongsuphajaisiddhi T, Luxemburger C, Webster HK, Edstein M, Phaipun L, Thew KL, White NJ (1991) Mefloquine-resistant falciparum malaria on the Thai-Burmese border. Lancet 337:1140–1143
19. Nosten F, Price RN (1995) New antimalarials. A risk-benefit analysis. Drug Saf 12:264–273
20. White NJ (1992) Antimalarial drug resistance: the pace quickens. J Antimicrob Chemother 30:571–585
21. ter Kuile FO, Nosten F, Thieren M, Luxemburger C, Edstein MD, Chongsuphajaisiddhi T, Phaipun L, Webster HK, White NJ (1992) High-dose mefloquine in the treatment of multidrug-resistant falciparum malaria. J Infect Dis 166:1393–1400
22. Fontanet AL, Johnston DB, Walker AM, Rooney W, Thimasarn K, Sturchler D, Macdonald M, Hours M, Wirth DF (1993) High prevalence of mefloquine-resistant falciparum malaria in eastern Thailand. Bull World Health Organ 71:377–383
23. Fontanet AL, Johnston BD, Walker AM, Bergqvist Y, Hellgren U, Rooney W (1994) Falciparum malaria in eastern Thailand: a randomized trial of the efficacy of a single dose of mefloquine. Bull World Health Organ 72:73–78

24. ter Kuile FO, Nosten F, Luxemburger C, Kyle D, Teja-Isavatharm P, Phaipun L, Price R, Chongsuphajaisiddhi T, White NJ (1995) Mefloquine treatment of acute falciparum malaria: a prospective study of non-serious adverse effects in 3673 patients. Bull World Health Organ 73:631–642
25. Ter Kuile FO, Teja-Isavatharm P, Edstein MD, Keeratithakul D, Dolan G, Nosten F, Phaipun L, Webster HK, White NJ (1994) Comparison of capillary whole blood, venous whole blood, and plasma concentrations of mefloquine, halofantrine, and desbutyl-halofantrine measured by high-performance liquid chromatography. Am J Trop Med Hyg 51:778–784
26. Nosten F, van Vugt M, Price R, Luxemburger C, Thway KL, Brockman A, McGready R, ter Kuile F, Looareesuwan S, White NJ (2000) Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. Lancet 356:297–302
27. Carrara VI, Sirilak S, Thonglairuam J, Rojanawatsirivet C, Proux S, Gilbos V, Brockman A, Ashley EA, McGready R, Krudsood S et al (2006) Deployment of early diagnosis and mefloquine-artesunate treatment of falciparum malaria in Thailand: the Tak Malaria Initiative. PLoS Med 3:e183
28. Carrara VI, Zwang J, Ashley EA, Price RN, Stepniewska K, Barends M, Brockman A, Anderson T, McGready R, Phaiphun L et al (2009) Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS One 4:e4551
29. Price R, Robinson G, Brockman A, Cowman A, Krishna S (1997) Assessment of *pfmdr 1* gene copy number by tandem competitive polymerase chain reaction. Mol Biochem Parasitol 85:161–169
30. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ et al (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 364:438–447
31. Simpson JA, Watkins ER, Price RN, Aarons L, Kyle DE, White NJ (2000) Mefloquine pharmacokinetic-pharmacodynamic models: implications for dosing and resistance. Antimicrob Agents Chemother 44:3414–3424
32. Palmer KJ, Holliday SM, Brogden RN (1993) Mefloquine: a review of its antimalarial activity, pharmacokinetic properties and therapeutic efficacy. Drugs 45:430–475
33. Ashley EA, McGready R, Hutagalung R, Phaiphun L, Slight T, Proux S, Thwai KL, Barends M, Looareesuwan S, White NJ et al (2005) A randomized, controlled study of a simple, once-daily regimen of dihydroartemisinin-piperaquine for the treatment of uncomplicated, multi-drug-resistant falciparum malaria. Clin Infect Dis 41:425–432
34. Bem JL, Kerr L, Stuerchler D (1992) Mefloquine prophylaxis: an overview of spontaneous reports of severe psychiatric reactions and convulsions. J Trop Med Hyg 95:167–179
35. Phillips-Howard PA, ter Kuile FO (1995) CNS adverse events associated with antimalarial agents. Fact or fiction? Drug Saf 12:370–383
36. WHO (1991) Review of central nervous system adverse events related to the antimalarial drug, mefloquine (1985–1990). World Health Organization, Geneva
37. Schlagenhauf P, Steffen R, Lobel H, Johnson R, Letz R, Tschopp A, Vranjes N, Bergqvist Y, Ericsson O, Hellgren U et al (1996) Mefloquine tolerability during chemoprophylaxis: focus on adverse event assessments, stereochemistry and compliance. Trop Med Int Health 1:485–494
38. Roche Laboratories Inc (2008) Lariam® (mefloquine hydrochloride) Complete Product Information. US Package Insert, Nutley, NJ
39. Nevin RL, Pietrusiak PP, Caci JB (2008) Prevalence of contraindications to mefloquine use among USA military personnel deployed to Afghanistan. Malar J 7:30
40. Hill DR (1991) Pre-travel health, immunization status, and demographics of travel to the developing world for individuals visiting a travel medicine service. Am J Trop Med Hyg 45:263–270

41. Nevin RL (2009) Epileptogenic potential of mefloquine chemoprophylaxis: a pathogenic hypothesis. Malar J 8:188
42. Taylor WR, White NJ (2004) Antimalarial drug toxicity: a review. Drug Saf 27:25–61
43. Baird JK (2005) Effectiveness of antimalarial drugs. N Engl J Med 352:1565–1577
44. Weinke T, Trautmann M, Held T, Weber G, Eichenlaub D, Fleischer K, Kern W, Pohle HD (1991) Neuropsychiatric side effects after the use of mefloquine. Am J Trop Med Hyg 45:86–91
45. Luxemburger C, Nosten F, ter Kuiile F, Frejacques L, Chongsuphajaisiddhi T, White NJ (1991) Mefloquine for multidrug-resistant malaria. Lancet 338:1268
46. Nguyen TH, Day NP, Ly VC, Waller D, Mai NT, Bethell DB, Tran TH, White NJ (1996) Post-malaria neurological syndrome. Lancet 348:917–921
47. Pham YT, Nosten F, Farinotti R, White NJ, Gimenez F (1999) Cerebral uptake of mefloquine enantiomers in fatal cerebral malaria. Int J Clin Pharmacol Ther 37:58–61
48. Gimenez F, Gillotin C, Basco LK, Bouchaud O, Aubry AF, Wainer IW, Le Bras J, Farinotti R (1994) Plasma concentrations of the enantiomers of halofantrine and its main metabolite in malaria patients. Eur J Clin Pharmacol 46:561–562
49. Pham YT, Regina A, Farinotti R, Couraud P, Wainer IW, Roux F, Gimenez F (2000) Interactions of racemic mefloquine and its enantiomers with P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT. Biochim Biophys Acta 1524:212–219
50. Toovey S (2009) Mefloquine neurotoxicity: a literature review. Travel Med Infect Dis 7:2–6
51. Milner E, McCalmont W, Bhonsle J, Caridha D, Carroll D, Gardner S, Gerena L, Gettayacamin M, Lanteri C, Luong T et al (2010) Structure-activity relationships amongst 4-position quinoline methanol antimalarials that inhibit the growth of drug sensitive and resistant strains of *Plasmodium falciparum*. Bioorg Med Chem Lett 20:1347–1351
52. Milner E, McCalmont W, Bhonsle J, Caridha D, Cobar J, Gardner S, Gerena L, Goodine D, Lanteri C, Melendez V et al (2010) Anti-malarial activity of a non-piperidine library of next-generation quinoline methanols. Malar J 9:51
53. Weiss SM, Benwell K, Cliffe IA, Gillespie RJ, Knight AR, Lerpiniere J, Misra A, Pratt RM, Revell D, Upton R et al (2003) Discovery of nonxanthine adenosine A2A receptor antagonists for the treatment of Parkinson's disease. Neurology 61:S101–S106
54. Gillespie RJ, Adams DR, Bebbington D, Benwell K, Cliffe IA, Dawson CE, Dourish CT, Fletcher A, Gaur S, Giles PR et al (2008) Antagonists of the human adenosine A2A receptor. Part 1: Discovery and synthesis of thieno[3,2-d]pyrimidine-4-methanone derivatives. Bioorg Med Chem Lett 18:2916–2919
55. Gofton TE, Al-Khotani A, O'Farrell B, Ang LC, McLachlan RS (2011) Mefloquine in the treatment of progressive multifocal leukoencephalopathy. J Neurol Neurosurg Psychiatry 82:452–455
56. Brickelmaier M, Lugovskoy A, Kartikeyan R, Reviriego-Mendoza MM, Allaire N, Simon K, Frisque RJ, Gorelik L (2009) Identification and characterization of mefloquine efficacy against JC virus *in vitro*. Antimicrob Agents Chemother 53:1840–1849
57. Keiser J, Utzinger J (2010) The drugs we have and the drugs we need against major helminth infections. Adv Parasitol 73:197–230
58. Holtfreter MC, Loebermann M, Klammt S, Sombetzki M, Bodammer P, Riebold D, Kinzelbach R, Reisinger EC (2011) *Schistosoma mansoni*: schistosomicidal effect of mefloquine and primaquine *in vitro*. Exp Parasitol 127:270–276
59. Xiao SH, Xue J, Li-Li X, Zhang YN, Qiang HQ (2010) Effectiveness of mefloquine against *Clonorchis sinensis* in rats and *Paragonimus westermani* in dogs. Parasitol Res 107:1391–1397
60. Auparakkitanon S, Chapoomram S, Kuaha K, Chirachariyavej T, Wilairat P (2006) Targeting of hematin by the antimalarial pyronaridine. Antimicrob Agents Chemother 50:2197–2200
61. Chavalitshewinkoon-Petmitr P, Pongvilairat G, Auparakkitanon S, Wilairat P (2000) Gametocytocidal activity of pyronaridine and DNA topoisomerase II inhibitors against multi-drug-resistant *Plasmodium falciparum in vitro*. Parasitol Int 48:275–280

62. Tshefu AK, Gaye O, Kayentao K, Thompson R, Bhatt KM, Sesay SS, Bustos DG, Tjitra E, Bedu-Addo G, Borghini-Fuhrer I et al (2010) Efficacy and safety of a fixed-dose oral combination of pyronaridine-artesunate compared with artemether-lumefantrine in children and adults with uncomplicated *Plasmodium falciparum* malaria: a randomised non-inferiority trial. Lancet 375:1457–1467
63. Park SH, Pradeep K (2010) Absorption, distribution, excretion, and pharmacokinetics of 14 C-pyronaridine tetraphosphate in male and female Sprague-Dawley rats. J Biomed Biotechnol 2010:590707
64. Wattanavijitkul T. (2010) Population pharmacokinetics of pyronaridine in the treatment of malaria. Ph.D. dissertation, University of Iowa. http://ir.uiowa.edu/etd/622. Accessed 15 Apr 2011
65. Looareesuwan S, Kyle DE, Viravan C, Vanijanonta S, Wilairatana P, Wernsdorfer WH (1996) Clinical study of pyronaridine for the treatment of acute uncomplicated falciparum malaria in Thailand. Am J Trop Med Hyg 54:205–209
66. Elueze EI, Croft SL, Warhurst DC (1996) Activity of pyronaridine and mepacrine against twelve strains of *Plasmodium falciparum in vitro*. J Antimicrob Chemother 37:511–518
67. de Villiers KA, Marques HM, Egan TJ (2008) The crystal structure of halofantrine-ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. J Inorg Biochem 102:1660–1667
68. Sanchez CP, Rotmann A, Stein WD, Lanzer M (2008) Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol Microbiol 70:786–798
69. ter Kuile FO, Dolan G, Nosten F, Edstein MD, Luxemburger C, Phaipun L, Chongsuphajaisiddhi T, Webster HK, White NJ (1993) Halofantrine versus mefloquine in treatment of multidrug-resistant falciparum malaria [see comments]. Lancet 341:1044–1049
70. Nosten F, ter Kuile F, Luxemburger C, Woodrow C, Kyle DE, Chongsuphajaisiddhi T, White NJ (1993) Cardiac effects of antimalarial treatment with halofantrine. Lancet 341:1054–1056
71. Ashley EA, Stepniewska K, Lindegardh N, Annerberg A, Kham A, Brockman A, Singhasivanon P, White NJ, Nosten F (2007) How much fat is necessary to optimize lumefantrine oral bioavailability? Trop Med Int Health 12:195–200
72. Tie H, Walker BD, Singleton CB, Valenzuela SM, Bursill JA, Wyse KR, Breit SN, Campbell TJ (2000) Inhibition of HERG potassium channels by the antimalarial agent halofantrine. Br J Pharmacol 130:1967–1975
73. Warhurst DC, Craig JC, Adagu IS, Guy RK, Madrid PB, Fivelman QL (2007) Activity of piperaquine and other 4-aminoquinoline antiplasmodial drugs against chloroquine-sensitive and resistant blood-stages of *Plasmodium falciparum*. Role of beta-haematin inhibition and drug concentration in vacuolar water- and lipid-phases. Biochem Pharmacol 73:1910–1926
74. Tarning J, Lindegardh N, Annerberg A, Singtoroj T, Day NP, Ashton M, White NJ (2005) Pitfalls in estimating piperaquine elimination. Antimicrob Agents Chemother 49:5127–5128
75. Tarning J, Ashley EA, Lindegardh N, Stepniewska K, Phaiphun L, Day NP, McGready R, Ashton M, Nosten F, White NJ (2008) Population pharmacokinetics of piperaquine after two different treatment regimens with dihydroartemisinin-piperaquine in patients with *Plasmodium falciparum* malaria in Thailand. Antimicrob Agents Chemother 52:1052–1061
76. Briolant S, Henry M, Oeuvray C, Amalvict R, Baret E, Didillon E, Rogier C, Pradines B (2010) Absence of association between piperaquine *in vitro* responses and polymorphisms in the *pfcrt*, *pfmdr1*, *pfmrp*, and *pfnhe* genes in *Plasmodium falciparum*. Antimicrob Agents Chemother 54:3537–3544
77. Muangnoicharoen S, Johnson DJ, Looareesuwan S, Krudsood S, Ward SA (2009) Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin *in vitro*. Antimicrob Agents Chemother 53:1362–1366
78. Ashley EA, Stepniewska K, Lindegardh N, McGready R, Annerberg A, Hutagalung R, Singtoroj T, Hla G, Brockman A, Proux S et al (2007) Pharmacokinetic study of artemether-lumefantrine given once daily for the treatment of uncomplicated multidrug-resistant falciparum malaria. Trop Med Int Health 12:201–208

79. van Vugt M, Wilairatana P, Gemperli B, Gathmann I, Phaipun L, Brockman A, Luxemburger C, White NJ, Nosten F, Looareesuwan S (1999) Efficacy of six doses of artemether-lumefantrine (benflumetol) in multidrug-resistant *Plasmodium falciparum* malaria. Am J Trop Med Hyg 60:936–942
80. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S et al (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin Infect Dis 42:1570–1577
81. White NJ, van Vugt M, Ezzet F (1999) Clinical pharmacokinetics and pharmacodynamics and pharmacodynamics of artemether-lumefantrine. Clin Pharmacokinet 37:105–125
82. McGready R, Tan SO, Ashley EA, Pimanpanarak M, Viladpai-Nguen J, Phaiphun L, Wustefeld K, Barends M, Laochan N, Keereecharoen L et al (2008) A randomised controlled trial of artemether-lumefantrine versus artesunate for uncomplicated *Plasmodium falciparum* treatment in pregnancy. PLoS Med 5:e253
83. Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F (2005) Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob Agents Chemother 49:2180–2188
84. Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu AB, Bray PG, Ward SA (2004) Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol Cell 15:867–877

Antifolates: Pyrimethamine, Proguanil, Sulphadoxine and Dapsone

Alexis Nzila

Abstract The inhibition or disruption of folate metabolism remains an attractive target for the discovery of new antimalarial drugs. The importance of this pathway was proved in the 1940s with the discovery of the triazine proguanil. Proguanil is converted in vivo to the active metabolite, cycloguanil, an inhibitor of the dihydrofolate reductase enzyme. Proguanil has mainly been used for prophylaxis and currently is used in combination with atovaquone (Malarone®) for this purpose. Pyrimethamine was discovered based on its similarity to cycloguanil, and has been combined with the sulpha drug sulphadoxine. This combination of pyrimethamine/sulphadoxine has been the drug of choice to replace chloroquine in the treatment of uncomplicated malaria. However, resistance to pyrimethamine/sulphadoxine is now common, and its use is now restricted to the treatment of malaria in pregnancy, and "intermittent preventive treatment." Efforts are under way to discover and develop new antifolates. In this chapter, I summarize our knowledge of folate metabolism in the malarial parasite, and discuss the role and place of antifolates in the treatment of malaria and new strategies of folate disruption as a drug target.

1 Folate Biochemistry

Antifolates block the synthesis or conversion of folate derivatives. Folate derivatives are important cellular cofactors for the production of deoxythymidylate (dTMP) and, thus, synthesis of DNA. In mammals and plants, the folate pathway also generates the amino acid methionine, mediates the metabolism of histidine, glutamic acid and serine and controls the initiation of protein synthesis in mitochondria through formylation of methionine [1, 2]. Thus, rapidly dividing cells, such as cancer cells

A. Nzila (✉)
Biological studies Group, Department of Chemistry King Fayhd University of Petroleum and Minerals, PO Box 468, Dharan 31261, Saudi Arabia
e-mail: alexisnzila@kfupm.edu.sa

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_6,

and malaria parasites, rely on the availability of folates. The antifolate methotrexate has been in use for over 60 years as an anticancer drug, and currently, disruption of folate metabolism is central in anticancer chemotherapy [2].

Plasmodium falciparum relies completely on the de novo synthesis pathway of dTMP and is unable to salvage pyrimidine from the exogenous medium. Thus, the folate pathway is critical to the parasite's survival [3]. Figure 1 summarizes the folate biochemical pathway in *P. falciparum* [4]. The important enzymes, with regard to antifolate activity, are dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Indeed, all antifolates currently in use target these two enzymes. Pyrimethamine and proguanil are the archetypal DHFR inhibitors (Fig. 2a). Drugs that target DHPS are sulpha-based and they include sulphonamide (sulphadoxine) and sulfone (dapsone) (Fig. 2b). In the past, attempts were made to use the DHPS inhibitors alone as antimalarial agents [5]. However, this approach was abandoned because of their low efficacy and unacceptable toxicity. The interest in this class of antifolates was fostered when it was demonstrated that they synergized with DHFR inhibitors [6], leading to their use in combination.

2 Combination with Proguanil

2.1 Proguanil with the Antifolate Dapsone

Proguanil was the first antifolate against malaria to be discovered [7]. This drug is metabolized to its triazine form, cycloguanil, an inhibitor of DHFR [8] (Fig. 2a). It has been used alone as a prophylactic agent against malaria [9]. However, selection of mutations in DHFR, as the result of pyrimethamine use, is associated with decreased cycloguanil activity [10]. Thus, proguanil efficacy is reduced in areas of pyrimethamine resistance (see Sect. 6.5.1). An attempt was made to combine proguanil with dapsone for malaria treatment [11], and an artemisinin-based combination of proguanil/dapsone/artesunate was also evaluated [12]. However, these combinations have been abandoned because of the reduced activity of cycloguanil (the active metabolite of proguanil). Even if these drugs were developed, dapsone toxicity would have been a limitation (see Sect. 4).

2.2 Proguanil with a Non-Sulpha-Based Drug, Atovaquone

Proguanil has also been combined with atovaquone, an inhibitor of electron-transport to the cytochrome bc_1 complex (coenzyme Q); this combination, known as Malarone®, is primarily used as a prophylactic agent against malaria [13]. Proguanil is converted to cycloguanil, an inhibitor of DHFR, and at the same time, proguanil synergizes with atovaquone (as an inhibitor of electron transport).

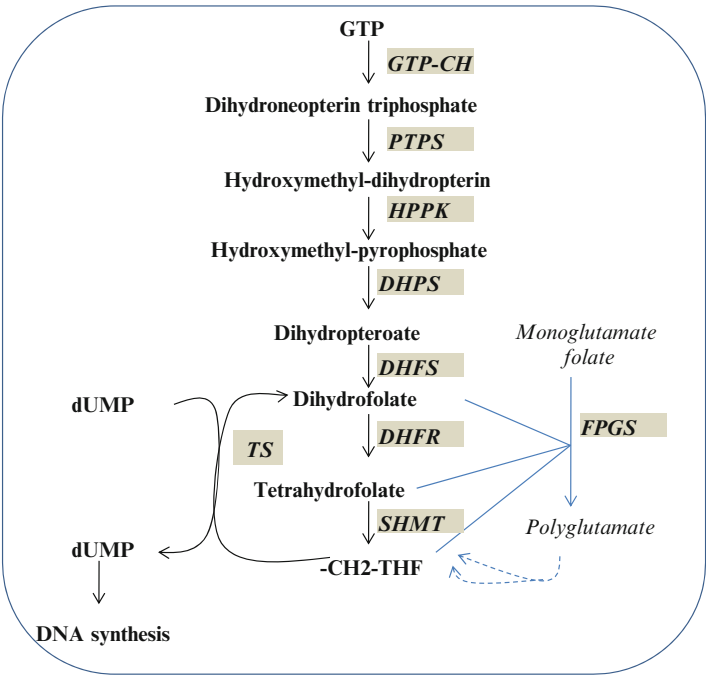


Fig. 1 Folate biochemical pathway in *P. falciparum*. The following abbreviations are used: *GTP-CH* GTP-cyclohydrolase I, *PTPS* pyruvyl tetrahydropterin synthase III, *HPPK* hydroxymethyl dihydropterin pyrophosphokinase, *DHPS* dihydropteroate synthase, *DFHS* dihydrofolate synthase, *FPGS* folylpoly-gamma-glutamate synthase, *DHFR* dihydrofolate reductase, *SHMT* serine hydroxymethyltransferase, *TS* thymidylate synthase. PTPS has recently been characterized in *P. falciparum* [4]

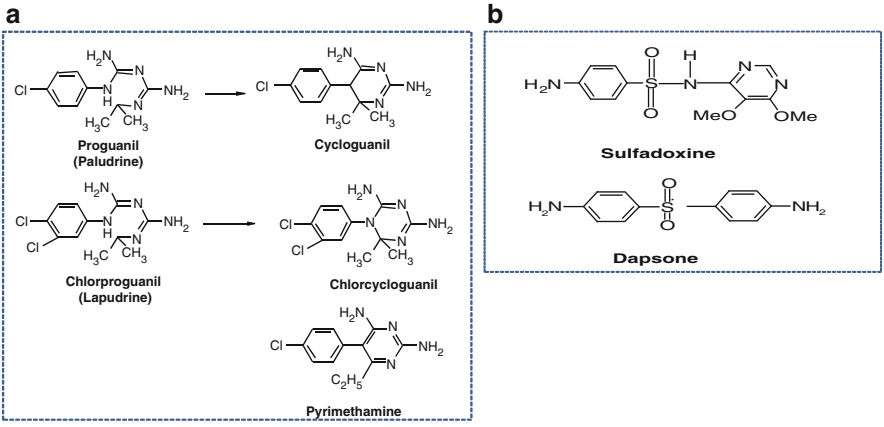


Fig. 2 Chemical structures of inhibitors of dihydrofolate reductase (**a**) and dihydropteroate synthase (**b**) enzymes used in the treatment of malaria

As a result, the sum of both effects account for the in vivo efficacy of Malarone® but in areas of high pyrimethamine resistance (where cycloguanil would be inactive), the potency of Malarone® would primarily be borne by the synergistic effect of proguanil/atovaquone on electron transport (see Sect. 5).

3 Combination with Pyrimethamine

Pyrimethamine, an inhibitor of DFHR, is a derivative of 2,4-diaminopyrimidine that was initially synthesized as an anticancer drug and was identified as an antimalarial based on its structural similarity to proguanil [14]. This drug has been the most widely used antimalarial antifolate agent thus far. It was first used as a monotherapy (known as Daraprim®) [15] and then was combined with sulphadoxine or sulphalene (see below).

3.1 Uncomplicated Malaria Treatment

Pyrimethamine was combined with sulphadoxine (this drug is known as Fansidar®) or sulphalene, under the name of Metakelfin®, for the treatment of uncomplicated malaria. These two sulpha drugs have a long elimination profile, like pyrimethamine, with half-lives >80 h [16]. On the other hand, pyrimethamine has been combined with the short-acting dapsone, under the name of Maloprim®. The half-life of dapsone is around 24 h [17] and, as a result, from the second day of treatment, the synergistic property of the combination is substantially reduced, decreasing the drug efficacy, and explaining the relatively low efficacy of this drug combination [18].

Though the efficacy of Fansidar® and Metakelfin® is comparable [19], Fansidar® has been more widely used than Metakelfin® (partly due to dapsone related toxicity) [19]. However, the efficacy of Fansidar® has been compromised as a result of the emergence of pyrimethamine- and sulphadoxine-resistant parasites, leading to its discontinuation as a drug for mass treatment of malaria in Africa [20, 21]. Combinations of pyrimethamine/sulphadoxine/artesunate and pyrimethamine/sulphadoxine/amodiaquine have been evaluated in several malaria-endemic areas [22]; however, a combination including a failing drug would be of limited benefit.

3.2 Intermittent Preventive Treatment

A body of evidence has shown that the administration of one dose of pyrimethamine/sulphadoxine in the second and third trimester in asymptomatic pregnant women is associated with a reduction of placental parasitaemia, maternal anemia

and low birth weight [23, 24]. This led the World Health Organization to recommend Intermittent Preventive Treatment (IPT) in pregnant women living in malaria-endemic areas, and this concept has now been extended to infants and children [23]. Pyrimethamine/sulphadoxine has been the drug of choice for IPT, even in areas with moderate levels of pyrimethamine/sulphadoxine resistance [23, 25]; however, in the context of high pyrimethamine/sulphadoxine resistance, its efficacy is greatly compromised [26]. Alternative drugs are being evaluated, including the combination of pyrimethamine/sulphadoxine with other antimalarials such as piperaquine and mefloquine [27, 28].

4 Combination with Chlorproguanil

The potency of proguanil led to the search for analogs with higher activity. These studies resulted in the discovery of two important molecules, among others, chlorproguanil and BRL 6331 (WR 99210). Only the information on chlorproguanil is summarized here since BRL 6331 has not reached clinical stages in human trials; however, information on this compound can be found elsewhere [10, 29].

Chlorproguanil is generated by chlorination of proguanil, and is metabolized in vivo to chlorcycloguanil, the active metabolite (Fig. 2a), which is more active than cycloguanil and pyrimethamine [30, 31]. Chlorproguanil was recommended for prophylaxis but was not used as much as proguanil [9]. This antifolate has been combined with dapsone for the treatment of uncomplicated malaria, and a combination of chlorproguanil/dapsone/artesunate was also investigated. However, these drugs have been discontinued because of dapsone toxicity in glucose-6-phosphate-dehydrogenase (G6PD)-deficient patients [32, 33].

5 Antifolate Resistance

5.1 Resistance to the Combination Pyrimethamine/Sulphadoxine

Predominantly, pyrimethamine/sulphadoxine resistance is attributable to parasites that carry point mutations at codons 108 (Ser to Asn), 51 (Asn to Ile) and 59 (Cys to Arg) of *dhfr*. These are triple-mutant parasites. Resistance is augmented by point mutations at codons 437 (Ala to Gly) and/or 540 (Lys to Glu) or 437 and/or 581 (Ala to Lys) of the *dhps* gene [20, 21]. High levels of pyrimethamine/sulphadoxine resistance are associated with the selection of an additional mutation at codon 164 (Ile to Leu) of *dhfr*. Interestingly, the existence of this mutation in Africa has been a matter of debate [34–37] but there is now compelling evidence that it does occur in African isolates [30, 38–40]. While 164-Leu was not found to be associated with in vivo pyrimethamine/sulphadoxine resistance in Kenya [41], recently, Karema

et al. have demonstrated substantially reduced pyrimethamine/sulphadoxine efficacy in an area of Rwanda with high 164-Leu prevalence [38].

Resistance to cycloguanil (the active metabolite of proguanil) is associated with mutation at codons 108 (Ser to Thr) and 16 (Ala to Val). At present, these mutations have been described in South America only, an area where proguanil has been widely as a prophylactic agent [20, 21]. The selection of pyrimethamine resistance has a bearing on the activity of cycloguanil. Indeed, parasites resistant to pyrimethamine are also resistant to cycloguanil, and this cross resistance is the result of the selection of point mutations in DHFR. This explains why the efficacy of proguanil is compromised in areas where resistance to Fansidar is high [20, 21].

The GTP-cyclohydrolase I enzyme (GTP-CH) catalyzes the first step of folate biosynthesis (Fig. 1). Interestingly, investigations have demonstrated that parasites highly resistant to the antifolate pyrimethamine, mainly those with the 164-Leu mutation, have an increased copy number (up to 11) of the *gtp-ch* gene [42]. The presence of mutations in DHFR could be associated with reduced enzyme kinetic properties, leading to a fitness cost [35, 42], though in vitro, these reduced enzyme kinetic properties have so far yielded contradictory results [43, 44]. Thus, the increase in copy number of *gtp-ch*, which is an adaptive phenomenon, could reflect compensatory mechanisms to maintain sufficient folate product (tetrahydrofolate). This increase in copy number in association with the presence of the 164-Leu mutation is a clear indication that GTP-CH modulates antifolate activity [42], along with changes of amino acids in key positions in DHFR and DHPS enzymes.

5.2 *Origin of Antifolate-Resistant Parasites*

Several studies have been dedicated to defining the origin of *dhfr*-mutant parasites. Similar work was undertaken to study chloroquine resistance, and the results indicate that chloroquine-resistant strains originated in South-East Asia – an area known to be the focus for the emergence of the multidrug-resistant parasites – and spread out globally, and into Africa [45]. The origin of new alleles is studied using polymerase chain reaction (PCR)-based assays of microsatellite markers, which consist of many repeated short sequences spread throughout the genome [46]. Reduced microsatellite diversity around the drug-resistant gene indicates selection of resistant strains as a result of drug pressure, and the evolutionary history of this drug resistance can be established by comparing microsatellite haplotypes in regions around the drug-resistant gene. This approach has revealed that African *dhfr* triple-mutant parasites originated mainly from South-East Asia [37, 47–50], though an indigenous origin of some triple mutants, and a few parasites also carrying 164-Leu was suggested [48, 50, 51]. Interestingly, all studies carried out so far indicate an African indigenous origin for *dhfr* double mutants (mutations at codon 108 and 51 or 108 and 59) [40, 48–52]. These observations indicate that highly antifolate-resistant parasites may have originated from outside Africa. Recently, a similar study was carried out to map the origin of *dhps* mutants. The

data show that African *dhps* mutants arose from five geographical foci within Africa, an indication that mutations in this gene may not have been imported from outside Africa [53].

6 Inhibition of Folate Salvage as a Strategy to Increase Antifolate Activity

The malaria parasite can both salvage folate and synthesize it de novo. Both pathways increase the availability of folate in cells. The addition of folate derivatives decreases the activity of antifolate drugs in vitro and in vivo [54, 55]. This clearly shows that folate uptake makes a significant contribution to antifolate drug efficacy. Therefore, inhibition of this salvage pathway could provide a rationale for the development of agents that could potentiate the activity of antifolate drugs acting downstream of the folate uptake processes.

We have demonstrated that probenecid, an inhibitor of anion transporters, at a concentration of <100 μM, which is readily achievable in vivo in humans, substantially increases the activity of inhibitors of DHFR and DHPS [55, 56]. This "chemosensitization" (or increase in antifolate activity) was also associated with a decrease in folate uptake into the parasite. This provides an explanation for how probenecid increases antifolate activity [56, 57]. Interestingly, this "probenecid effect" has been tested in vivo and the results indicate that probenecid significantly increases the efficacy of pyrimethamine/sulphadoxine in African children suffering from *P. falciparum* infection [58–60], although in one in vitro study, probenecid did not potentiate the activity of pyrimethamine [61]. This indicates that inhibitors of the anion transporters (responsible for the uptake of folate derivatives) could be of clinical importance in the treatment of malaria with antifolate drugs, as it has now been proven in cancer treatment [62].

7 Antifolate Anticancer Drugs for the Treatment of Malaria

Our group and others have demonstrated that the anticancer antifolate methotrexate is active against *P. falciparum* pyrimethamine-sensitive and pyrimethamine-resistant laboratory strains and field isolates, including those carrying the 164-Leu *dhfr* mutation [30, 55, 63–65]. In addition, we have shown that the anticancer antifolates aminopterin and trimetrexate are potent inhibitors of *P. falciparum* growth in vitro [30, 55]. $IC_{90/99}$ values (inhibitory concentration that kills 90 and 99% of parasites, respectively) of all these folate-antagonist agents fall between 150 and 350 nM. Thus, if such concentrations can be achieved in vivo with an acceptable toxicity profile, these compounds could potentially be used as antimalarial drugs. However,

anticancer agents in general, and methotrexate in particular, are perceived to be toxic and thus not suitable for malaria treatment.

A law in toxicology, known as "Paracelsus' law" states that "all substances are poisons, and there are none which are not." The right dose differentiates a poison from a remedy; this principle is also known as the "dose–response effect" [66, 67]. Thus, a molecule becomes a drug if the dose required to treat a complication is pharmacologically active with minimal toxicity. This principle has been exploited in the use of anticancer drugs (thus, known to be toxic at high dose) for the treatment of nonneoplastic diseases, at low and relatively safe doses [68]. Methotrexate is one of the examples that vindicate Paracelsus' law.

Methotrexate is used at high dose, up to 5,000–12,000 mg/m^2/week (130–300 mg kg^{-1}) for several weeks for the treatment of cancer, and this dose can yield serum concentrations >1,000 μM, i.e. within the range of concentrations that are associated with methotrexate life-threatening toxicity [69]. On the other hand, a 1,000-fold lower dose of methotrexate (LD-methotrexate) [0.1–0.4 mg kg^{-1}] is used once weekly in the treatment of rheumatoid arthritis, juvenile idiopathic arthritis in children (including infants <1 year old), and psoriasis [70, 71]. This use is associated with a relatively low toxicity, and the drug has become one of the important drugs in the treatment for rheumatoid arthritis.

Interestingly, the proof of concept that methotrexate could be used to treat malaria was established 40 years ago. Two relatively small clinical trials have demonstrated that doses as low as 2.5 mg per day for 3–5 days were safe and effective in treating malaria infection in humans (caused by *P. falciparum* and/or *P. vivax*) [72, 73]. This in vivo efficacy of LD-methotrexate is also supported by its pharmacokinetic behavior. Indeed, a daily dose of 5 mg in adults (0.035–0.1 mg kg^{-1}) could yield serum methotrexate concentrations between 250 and 500 nM [74, 75], concentrations that exceed the $IC_{90/99}$ concentrations required to kill malaria parasites in vitro [30]. Taken together, this information warrants further investigation of the drug as an antimalarial. A Phase I evaluation of LD-methotrexate in 25 volunteers has been carried out (clinicaltrial.gov; NCT 00791531), a step towards its development as an antimalarial. Low dose of the anticancer trimetrexate also has the potential to become an antimalarial [55, 68].

8 Concluding Remarks

In the face of the burgeoning problem of antimalarial drug resistance, new drugs are needed. The disruption of folate metabolism as a strategy to identify new antimalarials, has been exploited for almost 50 years, beginning with the discovery of proguanil and pyrimethamine. Understanding of both the mode of action of, and mechanisms of resistance to, these drugs has permitted the search for and design of new antifolate agents with greater potency, using structure-based drug design, quantitative structure–activity relationships (QSAR), and X-ray crystallography

among others [10, 76–80]. This effort has led to the discovery of new antifolates and some of them have reached advanced preclinical stages [81].

However, while this effort should be pursued, the folate pathway offers other opportunities for drug discovery. The experience gained from using antifolates in cancer has generated useful information that can be exploited to discover new antimalarial strategies. For example, we have provided evidence that the activity of antifolates can be enhanced despite the development of resistance. This concept has been vindicated in vivo. We have also demonstrated that some anticancer antifolates can be used at a low and safe dose to treat malaria. Thus, the opportunity exists to discover new antifolate drugs and extend the therapeutic life of existing ones.

Acknowledgments The author thanks the director of Kenya Medical Research Institute for permission to publish these data. This work was supported by the European Developing Countries Clinical Trials Partnership (EDTCP).

Transparency Declarations None to declare.

References

1. Nzila A, Ward SA, Marsh K, Sims PF, Hyde JE (2005) Comparative folate metabolism in humans and malaria parasites (part II): activities as yet untargeted or specific to *Plasmodium*. Trends Parasitol 21:334–339
2. Nzila A, Ward SA, Marsh K, Sims PF, Hyde JE (2005) Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy. Trends Parasitol 21:292–298
3. Hyde JE (2007) Targeting purine and pyrimidine metabolism in human apicomplexan parasites. Curr Drug Targets 8:31–47
4. Dittrich S, Mitchell SL, Blagborough AM, Wang Q, Wang P, Sims PF, Hyde JE (2008) An atypical orthologue of 6-pyruvoyltetrahydropterin synthase can provide the missing link in the folate biosynthesis pathway of malaria parasites. Mol Microbiol 67:609–618
5. Michel R (1968) Comparative study of the association of sulfalene and pyrimethamine and of sulfalene alone in mass chemoprophylaxis of malaria. Med Trop (Mars) 28:488–494
6. Bushby SR (1969) Combined antibacterial action *in vitro* of trimethoprim and sulphonamides. The *in vitro* nature of synergy. Postgrad Med J 45(Suppl):10–18
7. Curd FHS, Davey DG, Rose FL (1945) Study on synthetic antimalarial drugs. X. Some biguanide derivatives as new types of antimalarial substances with both therapeutic and causal prophylactic activity. Ann Trop Med Parasitol 39:208–216
8. Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL (1951) A metabolite of paludrine with high antimalarial activity. Nature 168:1080
9. Wernsdorfer WH (1990) Chemoprophylaxis of malaria: underlying principles and their realization. Med Trop (Mars) 50:119–124
10. Nzila A (2006) The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. J Antimicrob Chemother 57:1043–1054
11. Mutabingwa TK, Maxwell CA, Sia IG, Msuya FH, Mkongewa S, Vannithone S, Curtis J, Curtis CF (2001) A trial of proguanil-dapsone in comparison with sulfadoxine-pyrimethamine for the clearance of *Plasmodium falciparum* infections in Tanzania. Trans R Soc Trop Med Hyg 95:433–438

12. Krudsood S, Imwong M, Wilairatana P, Pukrittayakamee S, Nonprasert A, Snounou G, White NJ, Looareesuwan S (2005) Artesunate-dapsone-proguanil treatment of falciparum malaria: genotypic determinants of therapeutic response. Trans R Soc Trop Med Hyg 99:142–149
13. Nakato H, Vivancos R, Hunter PR (2007) A systematic review and meta-analysis of the effectiveness and safety of atovaquone proguanil (Malarone) for chemoprophylaxis against malaria. J Antimicrob Chemother 60:929–936
14. Falco EA, Goodwin LG, Hitchings GH, Rollo IM, Russell PB (1951) 2:4-diaminopyrimidines – a new series of antimalarials. Br J Pharmacol Chemother 6:185–200
15. Peters W (1987) Experimental resistance IV: dihydrofolate reductase inhibitors and drugs with related activities. In: Peters W (ed) Chemotherapy and drug resistance in malaria. Academic, London, pp 481–521
16. Watkins WM, Mosobo M (1993) Treatment of *Plasmodium falciparum* malaria with pyrimethamine- sulfadoxine: selective pressure for resistance is a function of long elimination half-life. Trans R Soc Trop Med Hyg 87:75–78
17. Winstanley P, Watkins W, Muhia D, Szwandt S, Amukoye E, Marsh K (1997) Chlorproguanil/dapsone for uncomplicated *Plasmodium falciparum* malaria in young children: pharmacokinetics and therapeutic range. Trans R Soc Trop Med Hyg 91:322–327
18. Segal HE, Chinvanthananond P, Laixuthai B, Pearlman EJ, Hall AP, Phintuyothin P, Na-Nakorn A, Castaneda BF (1975) Comparison of diaminodiphenylsulphonepyrimethamine and sulfadoxine-pyrimethamine combinations in the treatment of falciparum malaria in Thailand. Trans R Soc Trop Med Hyg 69:139–142
19. Peters W (1987) Resistance in human malaria III: dihydrofolate reductase inhibitors. In: Peters W (ed) Chemotherapy and drug resistance in malaria. Academic, London, pp 593–658
20. Gregson A, Plowe CV (2005) Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev 57:117–145
21. Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Nzila AM (2001) Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol 17:582–588
22. Nosten F, White NJ (2007) Artemisinin-based combination treatment of falciparum malaria. Am J Trop Med Hyg 77:181–192
23. Grobusch MP, Egan A, Gosling RD, Newman RD (2007) Intermittent preventive therapy for malaria: progress and future directions. Curr Opin Infect Dis 20:613–620
24. Grobusch MP, Gabor JJ, Aponte JJ, Schwarz NG, Poetschke M, Doernemann J, Schuster K, Koester KB, Profanter K, Borchert LB et al (2009) No rebound of morbidity following intermittent preventive sulfadoxine-pyrimethamine treatment of malaria in infants in Gabon. J Infect Dis 200:1658–1661
25. Aponte JJ, Schellenberg D, Egan A, Breckenridge A, Carneiro I, Critchley J, Danquah I, Dodoo A, Kobbe R, Lell B et al (2009) Efficacy and safety of intermittent preventive treatment with sulfadoxine-pyrimethamine for malaria in African infants: a pooled analysis of six randomised, placebo-controlled trials. Lancet 374:1533–1542
26. Gosling RD, Gesase S, Mosha JF, Carneiro I, Hashim R, Lemnge M, Mosha FW, Greenwood B, Chandramohan D (2009) Protective efficacy and safety of three antimalarial regimens for intermittent preventive treatment for malaria in infants: a randomised, double-blind, placebo-controlled trial. Lancet 374:1521–1532
27. Briand V, Bottero J, Noel H, Masse V, Cordel H, Guerra J, Kossou H, Fayomi B, Ayemonna P, Fievet N et al (2009) Intermittent treatment for the prevention of malaria during pregnancy in Benin: a randomized, open-label equivalence trial comparing sulfadoxine-pyrimethamine with mefloquine. J Infect Dis 200:991–1001
28. Cisse B, Cairns M, Faye E, O ND, Faye B, Cames C, Cheng Y, M ND, Lo AC, Simondon K et al (2009) Randomized trial of piperaquine with sulfadoxine-pyrimethamine or dihydroartemisinin for malaria intermittent preventive treatment in children. PLoS One 4:e7164
29. Diaz DS, Kozar MP, Smith KS, Asher CO, Sousa JC, Schiehser GA, Jacobus DP, Milhous WK, Skillman DR, Shearer TW (2008) Role of specific cytochrome P450 isoforms in the

conversion of phenoxypropoxybiguanide analogs in human liver microsomes to potent antimalarial dihydrotriazines. Drug Metab Dispos 36:380–385
30. Kiara SM, Okombo J, Masseno V, Mwai L, Ochola I, Borrmann S, Nzila A (2009) *In vitro* activity of antifolate and polymorphism in dihydrofolate reductase of *Plasmodium falciparum* isolates from the Kenyan coast: emergence of parasites with Ile-164-Leu mutation. Antimicrob Agents Chemother 53:3793–3798
31. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM (1998) Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and chlorcycloguanil activity *in vitro* and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother 42:164–169
32. Tiono AB, Dicko A, Ndububa DA, Agbenyega T, Pitmang S, Awobusuyi J, Pamba A, Duparc S, Goh LE, Harrell E et al (2009) Chlorproguanil-dapsone-artesunate versus chlorproguanil-dapsone: a randomized, double-blind, phase III trial in African children, adolescents, and adults with uncomplicated *Plasmodium falciparum* malaria. Am J Trop Med Hyg 81:969–978
33. Premji Z, Umeh RE, Owusu-Agyei S, Esamai F, Ezedinachi EU, Oguche S, Borrmann S, Sowunmi A, Duparc S, Kirby PL et al (2009) Chlorproguanil-dapsone-artesunate versus artemether-lumefantrine: a randomized, double-blind phase III trial in African children and adolescents with uncomplicated *Plasmodium falciparum* malaria. PLoS One 4:e6682
34. Hastings MD, Bates SJ, Blackstone EA, Monks SM, Mutabingwa TK, Sibley CH (2002) Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania. Trans R Soc Trop Med Hyg 96:674–676
35. Nzila A, Ochong E, Nduati E, Gilbert K, Winstanley P, Ward S, Marsh K (2005) Why has the dihydrofolate reductase 164 mutation not consistently been found in Africa yet? Trans R Soc Trop Med Hyg 99:341–346
36. Ochong E, Nzila A, Kimani S, Kokwaro G, Mutabingwa T, Watkins W, Marsh K (2003) Molecular monitoring of the Leu-164 mutation of dihydrofolate reductase in a highly sulfadoxine/pyrimethamine-resistant area in Africa. Malar J 2:46
37. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T (2004) Intercontinental spread of pyrimethamine-resistant malaria. Science 305:1124
38. Karema C, Imwong M, Fanello CI, Stepniewska K, Uwimana A, Nakeesathit S, Dondorp A, Day NP, White NJ (2010) Molecular correlates of high-level antifolate resistance in Rwandan children with *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 54:477–483
39. Lynch C, Pearce R, Pota H, Cox J, Abeku TA, Rwakimari J, Naidoo I, Tibenderana J, Roper C (2008) Emergence of a *dhfr* mutation conferring high-level drug resistance in *Plasmodium falciparum* populations from southwest Uganda. J Infect Dis 197:1598–1604
40. McCollum AM, Poe AC, Hamel M, Huber C, Zhou Z, Shi YP, Ouma P, Vulule J, Bloland P, Slutsker L et al (2006) Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel *dhfr* alleles. J Infect Dis 194:189–197
41. Hamel MJ, Poe A, Bloland P, McCollum A, Zhou Z, Shi YP, Ouma P, Otieno K, Vulule J, Escalante A et al (2008) Dihydrofolate reductase I164L mutations in *Plasmodium falciparum* isolates: clinical outcome of 14 Kenyan adults infected with parasites harbouring the I164L mutation. Trans R Soc Trop Med Hyg 102:338–345
42. Nair S, Miller B, Barends M, Jaidee A, Patel J, Mayxay M, Newton P, Nosten F, Ferdig MT, Anderson TJ (2008) Adaptive copy number evolution in malaria parasites. PLoS Genet 4: e1000243
43. Sandefur CI, Wooden JM, Quaye IK, Sirawaraporn W, Sibley CH (2007) Pyrimethamine-resistant dihydrofolate reductase enzymes of *Plasmodium falciparum* are not enzymatically compromised *in vitro*. Mol Biochem Parasitol 154:1–5
44. Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, Santi DV (1997) Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc Natl Acad Sci USA 94:1124–1129

45. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ (2002) Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature 418:320–323
46. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP (1999) Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. Parasitology 119:113–125
47. Maiga O, Djimde AA, Hubert V, Renard E, Aubouy A, Kironde F, Nsimba B, Koram K, Doumbo OK, Le Bras J et al (2007) A shared Asian origin of the triple-mutant *dhfr* allele in *Plasmodium falciparum* from sites across Africa. J Infect Dis 196:165–172
48. McCollum AM, Basco LK, Tahar R, Udhayakumar V, Escalante AA (2008) Hitchhiking and selective sweeps of *Plasmodium falciparum* sulfadoxine and pyrimethamine resistance alleles in a population from central Africa. Antimicrob Agents Chemother 52:4089–4097
49. Mita T (2009) Origins and spread of *pfdhfr* mutant alleles in *Plasmodium falciparum*. Acta Trop 58:201–209
50. Mita T, Tanabe K, Takahashi N, Culleton R, Ndounga M, Dzodzomenyo M, Akhwale WS, Kaneko A, Kobayakawa T (2009) Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa. J Antimicrob Chemother 63:252–255
51. Certain LK, Briceno M, Kiara SM, Nzila AM, Watkins WM, Sibley CH (2008) Characteristics of *Plasmodium falciparum dhfr* haplotypes that confer pyrimethamine resistance, Kilifi, Kenya, 1987–2006. J Infect Dis 197:1743–1751
52. Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, Chandramohan D, Sharp B (2003) Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet 361:1174–1181
53. Pearce RJ, Pota H, Evehe MS, Ba el H, Mombo-Ngoma G, Malisa AL, Ord R, Inojosa W, Matondo A, Diallo DA et al (2009) Multiple origins and regional dispersal of resistant *dhps* in African *Plasmodium falciparum* malaria. PLoS Med 6:e1000055
54. Carter JY, Loolpapit MP, Lema OE, Tome JL, Nagelkerke NJ, Watkins WM (2005) Reduction of the efficacy of antifolate antimalarial therapy by folic acid supplementation. Am J Trop Med Hyg 73:166–170
55. Nduati E, Diriye A, Ommeh S, Mwai L, Kiara S, Masseno V, Kokwaro G, Nzila A (2008) Effect of folate derivatives on the activity of antifolate drugs used against malaria and cancer. Parasitol Res 102:1227–1234
56. Nzila A, Mberu E, Bray P, Kokwaro G, Winstanley P, Marsh K, Ward S (2003) Chemosensitization of *Plasmodium falciparum* by probenecid *in vitro*. Antimicrob Agents Chemother 47:2108–2112
57. Wang P, Wang Q, Sims PF, Hyde JE (2007) Characterisation of exogenous folate transport in *Plasmodium falciparum*. Mol Biochem Parasitol 154:40–51
58. Sowunmi A, Adedeji AA, Fateye BA, Babalola CP (2004) *Plasmodium falciparum* hyperparasitaemia in children. Risk factors, treatment outcomes, and gametocytaemia following treatment. Parasite 11:317–323
59. Sowunmi A, Fehintola FA, Adedeji AA, Gbotosho GO, Falade CO, Tambo E, Fateye BA, Happi TC, Oduola AM (2004) Open randomized study of pyrimethamine-sulphadoxine vs. pyrimethamine-sulphadoxine plus probenecid for the treatment of uncomplicated *Plasmodium falciparum* malaria in children. Trop Med Int Health 9:606–614
60. Sowunmi A, Adedeji AA, Fateye BA, Fehintola FA (2004) Comparative effects of pyrimethamine-sulfadoxine, with and without probenecid, on *Plasmodium falciparum* gametocytes in children with acute, uncomplicated malaria. Ann Trop Med Parasitol 98:873–878
61. Tahar R, Basco LK (2007) Molecular epidemiology of malaria in Cameroon. XXVI. Twelve-year *in vitro* and molecular surveillance of pyrimethamine resistance and experimental studies to modulate pyrimethamine resistance. Am J Trop Med Hyg 77:221–227
62. Fury MG, Krug LM, Azzoli CG, Sharma S, Kemeny N, Wu N, Kris MG, Rizvi NA (2005) A phase I clinical pharmacologic study of pralatrexate in combination with probenecid in adults with advanced solid tumors. Cancer Chemother Pharmacol 57:671–677

63. Dar O, Khan MS, Adagu I (2008) The potential use of methotrexate in the treatment of falciparum malaria: *in vitro* assays against sensitive and multidrug-resistant falciparum strains. Jpn J Infect Dis 61:210–211
64. Fidock DA, Nomura T, Wellems TE (1998) Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. Mol Pharmacol 54:1140–1147
65. Walter RD, Bergmann B, Kansy M, Wiese M, Seydel JK (1991) Pyrimethamin-resistant *Plasmodium falciparum* lack cross-resistance to methotrexate and 2,4-diamino-5-(substituted benzyl) pyrimidines. Parasitol Res 77:346–350
66. Langman LJ, Kapur BM (2006) Toxicology: then and now. Clin Biochem 39:498–510
67. Rozman KK, Doull J (2001) Paracelsus, Haber and Arndt. Toxicology 160:191–196
68. Nzila A, Okombo J, Becker RP, Chilengi R, Lang T, Niehues T (2010) Anticancer agents against malaria: time to revisit? Trends Parasitol 26:125–129
69. Chabner BA, Amrein P, Drucker B, Michealson M, Mitsiades C, Goss P, Ryan D, Ramachandra S, Richardson P, Supko J et al (2006) Antineoplastic agents. In: Brunton L (ed) The pharmacological basis of therapeutics 9/e. McGrwa-Hill, New York, pp 1315–1465
70. Niehues T, Lankisch P (2006) Recommendations for the use of methotrexate in juvenile idiopathic arthritis. Paediatr Drugs 8:347–356
71. Swierkot J, Szechinski J (2006) Methotrexate in rheumatoid arthritis. Pharmacol Rep 58:473–492
72. Sheehy TW, Dempsey H (1970) Methotrexate therapy for *Plasmodium vivax* malaria. JAMA 214:109–114
73. Wildbolz A (1973) Methotrexate in the therapy of malaria. Ther Umsch 30:218–222
74. Chladek J, Grim J, Martinkova J, Simkova M, Vaneckova J (2005) Low-dose methotrexate pharmacokinetics and pharmacodynamics in the therapy of severe psoriasis. Basic Clin Pharmacol Toxicol 96:247–248
75. Chladek J, Grim J, Martinkova J, Simkova M, Vaniekova J, Koudelkova V, Noiekova M (2002) Pharmacokinetics and pharmacodynamics of low-dose methotrexate in the treatment of psoriasis. Br J Clin Pharmacol 54:147–156
76. Chiu TL, So SS (2004) Development of neural network QSPR models for Hansch substituent constants. 2. Applications in QSAR studies of HIV-1 reverse transcriptase and dihydrofolate reductase inhibitors. J Chem Inf Comput Sci 44:154–160
77. Dasgupta T, Chitnumsub P, Kamchonwongpaisan S, Maneeruttanarungroj C, Nichols SE, Lyons TM, Tirado-Rives J, Jorgensen WL, Yuthavong Y, Anderson KS (2009) Exploiting structural analysis, *in silico* screening, and serendipity to identify novel inhibitors of drug-resistant falciparum malaria. ACS Chem Biol 4:29–40
78. Hecht D, Fogel GB (2009) A novel *in silico* approach to drug discovery via computational intelligence. J Chem Inf Model 49:1105–1121
79. Maitarad P, Kamchonwongpaisan S, Vanichtanankul J, Vilaivan T, Yuthavong Y, Hannongbua S (2009) Interactions between cycloguanil derivatives and wild type and resistance-associated mutant *Plasmodium falciparum* dihydrofolate reductases. J Comput Aided Mol Des 23:241–252
80. Thongpanchang C, Taweechai S, Kamchonwongpaisan S, Yuthavong Y, Thebtaranonth Y (2007) Immobilization of malarial (*Plasmodium falciparum*) dihydrofolate reductase for the selection of tight-binding inhibitors from combinatorial library. Anal Chem 79:5006–5012
81. MMV. http://www.mmv.org/IMG/pdf/Global_Malaria_FINALq42009.pdf. Accessed 11 Jan 2011

Naphthoquinones: Atovaquone, and Other Antimalarials Targeting Mitochondrial Functions

Akhil B. Vaidya

Abstract Mitochondria in malaria parasites are highly divergent from their counterparts in mammalian hosts. This degree of divergence underlies the validity of mitochondrial functions as targets for antimalarial drugs. The mitochondrial electron transport chain (mtETC) at the cytochrome bc_1 complex is selectively inhibited in malaria parasites by atovaquone. Proguanil, the synergistic partner of atovaquone, appears to target an alternative pathway that generates electropotential across the inner membrane of parasite mitochondria. However, the rapid emergence of atovaquone-resistance mutations effectively negates the synergistic effect of proguanil. New antimalarials targeting the mtETC with reduced propensity for resistance development could overcome this challenge. A critical function of the mtETC is to serve mitochondrially located dihydroorotate dehydrogensae (DHODH), an enzyme of the pyrimidine biosynthesis pathway. Compounds with selective activity against parasite DHODH are under development as potential new antimalarials. Recent studies on unusual tricarboxylic acid metabolism and ATP synthase structure point to additional opportunities for investigations aimed to identify other selective inhibitors.

1 Introduction

Some of the most potent poisons such as cyanide affect mitochondrial respiration, blocking electron transport and resulting in bioenergetic crisis leading to death. Thus, the idea of using antimalarial drugs that affect the mitochondrial electron transport chain (mtETC) might at first appear to be risky. However, this is precisely what a currently used antimalarial drug, atovaquone, does by selectively inhibiting

A.B. Vaidya (✉)
Department of Microbiology and Immunology Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA
e-mail: Akhil.Vaidya@DrexelMed.edu

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_7,

the parasite mtETC without affecting host mitochondria. The reason for this selectivity lies in structural differences in the cytochrome *b* encoded by the parasite mitochondrial DNA (mtDNA) that distinguishes it from that encoded by the host mtDNA [1]. Clearly, the great evolutionary distance between the parasites and their hosts is reflected in the significant differences observed for their essential physiological processes [2]. Such differences provide opportunities for devising means for selective interference as the basis for developing new antiparasitic drugs. This chapter will focus on some of the recent findings on the unusual mitochondrion of malaria parasites, describing its essential functions and the mechanisms by which some of the antimalarial compounds exert their effects. Some of the insights into mechanisms of action and synergy between atovaquone and proguanil – components of the drug Malarone™ – are important in decisions relating to partner drugs to be used with compounds that target the mtETC.

2 Selective Inhibition of Parasite the mtETC

As with all mitochondria, the main mobile electron carrier for the mtETC in malaria parasites is ubiquinone (also called coenzyme Q, CoQ; see Fig. 1 for the structures of CoQ and other compounds discussed here). Unlike the host mitochondria, however, pyruvate oxidation through the citric acid cycle does not appear to be the source of reducing equivalents in the parasite mitochondria [2, 3]. Also, the parasites do not possess a proton-pumping multisubunit NADH dehydrogenase, but encode a single subunit type II NADH dehydrogenase (NDH2) that reduces CoQ without contributing to the proton gradient [4, 5]. In addition to the NDH2, four other dehydrogenases – dihydroorotate dehydrogenase (DHODH), glycerol-3-phosphate dehydrogenase (GPDH), succinate dehydrogenase (SDH), and malate–quinone oxidoreductase (MQO) – require CoQ as the electron acceptor. The reduced ubihydroquinone ($CoQH_2$) is oxidized by the dimeric cytochrome bc_1 complex (Complex III), which serves an essential step for the continued provision of CoQ to mitochondrial dehydrogenases. As depicted in Fig. 2, $CoQH_2$ oxidation by Complex III is achieved through a process called the Q cycle (see [6, 7] for reviews) in which electrons from $CoQH_2$ are bifurcated at the quinone oxidation (Q_O) site with one electron passed on to the iron–sulfur (2Fe2S) cluster of the Rieske protein on the way to cytochrome c_1 and cytochrome *c*, while the other passes on to the quinone reduction (Q_i) site through the low- and high-potential heme b_L and b_H, reducing a resident quinone to semiquinone. Another round of $CoQH_2$ oxidation results in the reduction of another molecule of cytochrome *c* and full reduction of the semiquinone to $CoQH_2$ at the Q_i site. This process results in the translocation of four protons across the inner mitochondrial membrane and the reduction of two cytochrome *c* molecules. The transfer of electrons by the Rieske iron–sulfur protein requires a large-scale domain movement in which the 2Fe2S cluster moves from a Q_O proximal position to the cytochrome c_1 proximal position. Analysis of limited proteolysis and electron paramagnetic resonance measurements

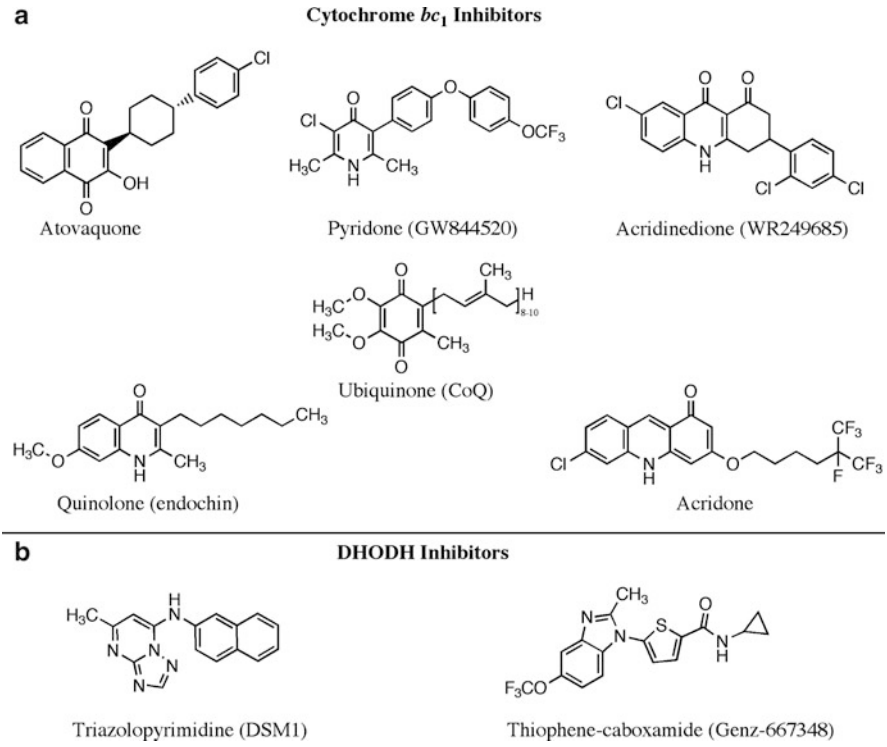


Fig. 1 Structures of compounds discussed in the text. (**a**) Structures of CoQ and five chemical classes of cytochrome bc_1 complex inhibitors. (**b**) Structures of two chemical classes that exhibit selective inhibition of parasite DHODH

of engineered bacterial cytochrome bc_1 complexes showed that atovaquone inhibited the movement of the Rieske protein [8]. Subtle differences in the highly conserved Q_O site residues appear to be responsible for the selectively greater inhibition of the parasite complex compared to the mammalian complex [9]. Molecular modeling studies based on crystal structures of the yeast and bovine Complex III in the presence of known Q_O site inhibitors have postulated an atovaquone binding site, and have proposed the molecular basis for preferential binding of atovaquone to the parasite Complex III [10, 11]. Although there are sufficient caveats in using this surrogate approach, in the absence of authentic crystal structures of the parasite cytochrome bc_1 complex, these studies could provide guidelines for understanding drug action and approaches for further drug design. A critical point is the necessity for identifying compounds that would give the largest therapeutic window so as to minimize the risk of toxicity arising from inhibition of the host Complex III.

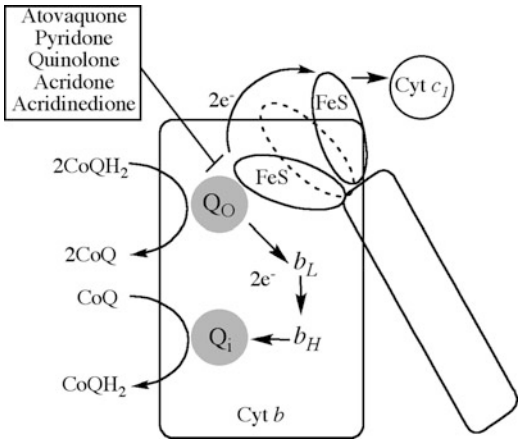


Fig. 2 A schematic representation of the Q cycle within the Complex III for $CoQH_2$ oxidation. With two molecules of $CoQH_2$ being oxidized at the Q_O site of the complex, two electrons are passed on to cytochrome *c*, two electrons to a CoQ molecule resident at the Q_i site, and four protons are translocated across the membrane. Transfer of electrons to cytochrome c_1 involves a large-scale movement of the 2Fe2S domain of the Rieske protein. The five chemical classes of antimalarials shown in the box inhibit this step of electron transport at the Q_O site

3 Atovaquone Resistance

In early clinical trials with atovaquone as a single agent, recrudescent parasites emerged in about 30% of the patients [12]. The recrudescent parasites were >1,000-fold resistant to atovaquone. Even in cultures of *P. falciparum*, atovaquone-resistant parasites could be observed at the frequency as high as 10^{-7} in some strains [13]. Under conditions of suboptimal treatment, several independent lines of the rodent malaria parasite, *P. yoelii,* resistant to atovaquone were readily derived [9]. DNA sequencing of the mitochondrial genome of these resistant parasites revealed mutations centered around the Q_O site of cytochrome *b*, delineating the putative atovaquone-binding region of the parasite Complex III [9]. The subtle differences in the structural features of the Q_O site between the parasite and the host Complex III that form the basis for selective toxicity of atovaquone could explain the propensity of resistance development: mutations could arise in the parasite cytochrome *b* that would make it more like the host protein, thereby gaining resistance to atovaquone.

Could this high frequency of resistance development be due to polymorphisms within the mitochondrial DNA (mtDNA) of malaria parasites in the population? On the contrary, extensive sequence analyses of mtDNA from geographically distant *P. falciparum* have revealed extraordinary sequence conservation [14, 15]. Furthermore, the ease with which atovaquone-resistant parasites could be derived in cultures of cloned parasites argues against the idea that atovaquone-resistant mutants are prevalent in the parasite populations. It is worth noting that no wild-type

mtDNA can be detected once atovaquone resistance has arisen in cultured *P. falciparum*. Given that the parasite mitochondria contain multiple copies of mtDNA [16], this observation is consistent with the idea that under selection, the drug-resistant mutations quickly sweep through the mtDNA population. The suggested mode of parasite mtDNA replication involves extensive gene conversion events [17], which could account for the rapid selective sweeps of atovaquone resistant mutations.

4 Atovaquone–Proguanil Combination

The high frequency of treatment failures in the clinic clearly precluded the use of atovaquone as a single antimalarial agent. A search for a potential partner showed proguanil to have synergistic effect with atovaquone in inhibiting *P. falciparum* growth in vitro [18]. As an antimalarial known since the 1950s [19, 20], proguanil already had an established record of efficacy and safety, favoring its choice as a partner for atovaquone compared to other compounds. A fixed combination of atovaquone and proguanil, registered as Malarone, was extensively tested in clinical trials and found to be highly effective for the prevention and treatment of malaria [21]. No treatment failures due to resistance development were reported in clinical trials of Malarone; however, sporadic cases of prophylaxis and treatment failures due to cytochrome *b* mutations have been reported since its wider use in the field [22–24]. These worrisome reports as well as the high cost of Malarone have restricted its use primarily to a prophylactic drug prescribed for visitors to malaria endemic regions.

By inhibiting electron transport through Complex III, atovaquone also prevents proton translocation, thus decreasing the electropotential across the inner mitochondrial membrane of the parasite. Dose-dependent collapse of mitochondrial membrane potential was demonstrated in a flow-cytometric assay with a cationic fluorescent probe in live intact *P. yoelii* [25]. The same assay also revealed that proguanil by itself did not inhibit mtETC nor did it affect mitochondrial membrane potential [26]. However, when used in combination with atovaquone, proguanil reduced the concentration at which atovaquone collapsed the mitochondrial membrane potential by about six- to eightfold; it also enhanced the magnitude of the collapse [26]. This observation provided an initial indication as to the underlying mechanism for the synergy between atovaquone and proguanil. Importantly, the active metabolite of proguanil, cycloguanil (a selective inhibitor of the parasite dihydrofolate reductase, DHFR), did not demonstrate synergy with atovaquone for neither parasite growth inhibition [18] nor the collapse of the mitochondrial membrane potential [26]. Thus, the synergistic action of proguanil is the property of the prodrug itself; its metabolic activation is not required. More recent studies described later have confirmed and extended these findings.

5 Inhibition of mtETC and the Viability of *P. falciparum*

In metazoan cells, mitochondria play a major role in programmed cell death with mtETC inhibition as well as other insults to the mitochondrial physiology leading to apoptosis. However, apoptotic dismantling of atovaquone-treated parasites has not been observed [27]. Monitoring the viability of parasites exposed to atovaquone and the atovaquone–proguanil combination revealed that ring and schizont-stage *P. falciparum* remained viable for 48 h, and the trophozoite stages remained viable for 24 h under treatment [28]. Thus, an apoptotic program is not initiated by the inhibition of mitochondrial respiration and the collapse of mitochondrial membrane potential. Instead, the parasites seem to enter a "static" state and can survive for up to 48 h in culture. The atovaquone–proguanil combination appears to be a static rather than a cidal drug in vitro. Conversion of proguanil to cycloguanil in vivo, however, could affect the pharmacodynamics of the combination when administered to patients. Nonetheless, it would be prudent to assess the killing rates of antimalarial compounds under development (e.g. pyridones and quinolones; discussed later) that target mitochondrial physiology, so as to optimize their pharmacokinetic and pharmacodynamic properties.

6 Specific Role of mtETC in Blood Stage *P. falciparum*

Among the five mitochondrial dehydrogenases that require CoQ as the electron acceptor, DHODH has been considered the most important as it carries out the fourth step in the pyrimidine biosynthesis pathway, an essential step in parasites unable to salvage pyrimidine. In most eukaryotic organisms, DHODH is localized to mitochondria, but in the yeast *Saccharomyces cerevisiae* the enzyme is localized to the cytosol and uses fumarate as the electron acceptor [29]. Painter et al. [30] generated transgenic *P. falciparum* lines that expressed the yeast DHODH (yDHODH) under the control of a constitutive promoter. Interestingly, the yDHODH-transgenic parasites were completely resistant to atovaquone as well as other mtETC inhibitors. The resistance was not due to mutations affecting mtETC components since mitochondria isolated from the transgenic parasites were fully susceptible to atovaquone. Thus, acquisition of a single metabolic bypass within the pyrimidine biosynthesis pathway was sufficient to render the blood-stage *P. falciparum* independent of the mitochondrial electron transport chain. A critical function of the mtETC in blood-stage *P. falciparum*, therefore, is to provide oxidized CoQ to serve as the electron acceptor for DHODH [30].

These findings further validated the parasite DHODH as a target for antimalarial drug discovery and development, and also raised questions as to the role played by mitochondrial dehydrogenases other than DHODH in blood stage *P. falciparum*. Recent studies have revealed that *P. falciparum* strains of different genetic background have varying reliance on the mtETC. Whereas some of the yDHODH-transgenic

strains could be propagated for long-term growth in the presence of atovaquone, other yDHODH-transgenic strains stopped their growth after ~96 h of exposure to atovaquone. Interestingly, this growth inhibition could be reversed by the inclusion of oxidized decyl-ubiquinone in the culture medium (Ke et al. unpublished results). This suggests that there are strain-specific variations in demands for CoQ under conditions where the mtETC is uncoupled from pyrimidine biosynthesis.

The robust drug resistance imparted by transgenic yDHODH has permitted the construction of transfection vectors with yDHODH as the selectable marker [31]. While atovaquone can be used as the selective agent, strain-specific variations limit its use. Instead, parasite-specific DHODH inhibitors such as triazolopyrimidines [32] (e.g. DSM1; see Fig. 1 for structure) work as better selective agents applicable to all *P. falciparum* strains [31]. Because of the paucity of useful selectable markers for genetic manipulation of *P. falciparum*, the advent of yDHODH as a new selectable marker will likely be of great utility in the field, permitting genetic complementation as well as double- and triple-knockout experiments.

7 The Reversal of mtETC Inhibitor Resistance in yDHODH-Transgenic Parasites by Proguanil

The antimalarial activity of proguanil requires its metabolic conversion to the triazine compound cycloguanil, which is a potent inhibitor of parasite DHFR [19, 20]. However, as mentioned above, synergy with atovaquone is mediated by proguanil, not cycloguanil. Proguanil by itself has minimal intrinsic activity against *P. falciparum* with an EC_{50} of about 50 μM [33], which was also the value observed for the effect of proguanil against the yDHODH-transgenic parasites [30]. Surprisingly, when proguanil was tested in the presence of 100-nM atovaquone, the yDHODH-transgenic parasites had an EC_{50} of about 60 nM for the effect of proguanil, an almost 1,000-fold drop in EC_{50}. Conversely, the inclusion of 1-μM proguanil reduced the atovaquone EC_{50} from >2 μM to 0.7 nM in the transgenic parasites; proguanil completely reversed atovaquone resistance in yDHODH-transgenic *P. falciparum*. Resistance to all mtETC inhibitors in yDHODH-transgenic parasites was similarly reversed by proguanil. The mitochondrial membrane potential was fully collapsed when atovaquone and proguanil were combined [30].

These intriguing observations could be explained by the proposition that maintenance of mitochondrial membrane potential is essential for survival even in yDHODH-transgenic parasites, and that there are two paths operational for maintaining the membrane potential. The dominant path for generating mitochondrial membrane potential is through the mtETC, which, when targeted by atovaquone in the wild-type parasites, results in parasite inhibition due to the block of pyrimidine biosynthesis. Although the yDHODH-transgenic parasites can overcome atovaquone inhibition through the metabolic bypass afforded by the transgene, for survival they still require mitochondrial membrane potential,

which is established through a secondary pathway independent of the mtETC. It is this secondary pathway that is proposed to be inhibited by proguanil, and thus proguanil in the presence of atovaquone results in reversal of the atovaquone resistance of the yDHODH-transgenic parasite. The intrinsic EC_{50} of proguanil appears to be about 60 nM, which becomes apparent only in yDHODH-transgenic parasites in the presence of atovaquone [30]. The dominant mtETC-dependent path for mitochondrial membrane potential would be fully functional in the transgenic parasites in the absence of atovaquone, and thus proguanil by itself would not affect the parasites unless at a 1,000-fold higher concentration, most likely due to its off-target activity. The molecular nature of the proposed secondary pathway targeted by proguanil is unclear at this point.

8 Problems with Atovaquone–Proguanil Combination Therapy

On the face of it, proguanil would appear to be an ideal partner drug for atovaquone as well as other anti-mtETC antimalarials under development. However, a close examination in light of the observations described above raises significant doubts as to the use of the atovaquone–proguanil combination as antimalarials in the field. The atovaquone–proguanil combination could actually be considered as a triple-drug combination (1) atovaquone as the mtETC inhibitor, (2) proguanil with its intrinsic activity against a secondary target that can generate mitochondrial membrane potential, and (3) proguanil as a prodrug for cycloguanil inhibiting the parasite DHFR. Unfortunately, single point mutations within the parasite cytochrome *b* that give rise to atovaquone resistance would render both atovaquone and the intrinsic proguanil activity ineffective. As described in Fig. 3, metabolic conversion of proguanil to cycloguanil requires the host cytochrome P_{450} enzyme CYP2C19 [34]. Genetic polymorphisms resulting in poor metabolism through CYP2C19 are quite prevalent, especially among the populations residing in malaria-endemic areas [35–38]; thus, such individuals will not convert proguanil to cycloguanil in an efficient manner. Furthermore, cycloguanil resistance due to

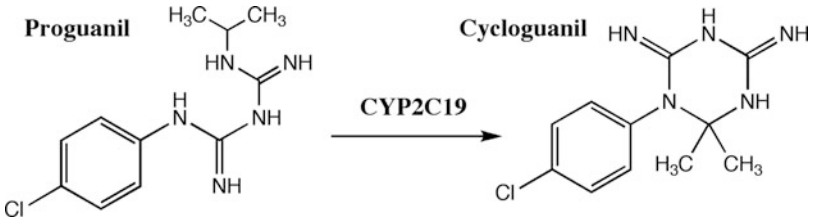


Fig. 3 Structures of proguanil and its metabolite cycloguanil. This conversion is carried out by the cytochrome P_{450} isoform CYP2C19. Genetic polymorphisms involving CYP2C19 with high frequency among certain populations inhibit this metabolic conversion

mutations in the parasite DHFR is quite widespread among the field isolates of *P. falciparum*. Thus, the efficacy of cycloguanil as a stand-alone antimalarial is highly diminished. In light of these points, the atovaquone–proguanil combination in effect turns out to be a monotherapy in two groups of individuals. First, in poor metabolizing individuals, proguanil to cycloguanil conversion would be highly diminished, leaving the parasites unexposed to a DHFR inhibitor. Second, individuals who do metabolize proguanil to cycloguanil, but get infected with cycloguanil-resistant parasites, would not benefit from cycloguanil. Atovaquone resistance could arise very rapidly in such patients due to cytochrome *b* mutations, and, once such parasites arise, proguanil as a prodrug will not provide its synergistic effect, since this effect is apparent only in parasites with atovaquone-sensitive mtETC. Indeed, reports of Malarone prophylaxis and treatment failures [22–24] provide considerable support for this proposition.

The choice of a partner drug for new anti-mtETC inhibitors under development will need to be guided by these insights. A key feature of such compounds has to be a much lower frequency of resistance development compared to atovaquone. Also, the use of Malarone in artemisinin-resistance containment campaigns could become problematic because of emergent resistance to the containment drug combination.

9 Use of yDHODH-Transgenic Parasites to Decipher Mode of Drug Action

The yDHODH-transgenic parasites are resistant to all mtETC inhibitors as well as to the parasite DHODH inhibitors, such as the triazolopyrimidines [32]. Importantly, proguanil is able to reverse the resistance to mtETC inhibitors but not so for the parasite DHODH inhibitors (Morrisey et al. unpublished data). These observations could be used to assess the mode of action for antimalarial compounds identified through high-throughput empirical screens based on parasite growth inhibition. Indeed, recent studies have employed the yDHODH-transgenic parasites in this manner to deconvolute the mode of action for thousands of compounds identified through their antimalarial activities in whole cell-based assays [39, 40]. Several additional mtETC and DHODH inhibitors were identified through this approach.

At least four additional chemical classes of compounds under investigation appear to target the parasite mtETC in a selective manner: pyridones [41], quinolones [42, 43], acridones [44], and acridinediones [45]. Testing representative compounds from each of these classes in yDHODH-transgenic parasites in the presence and the absence of proguanil confirmed that they act through inhibition of the mtETC (Morrisey et al. unpublished data). Furthermore, these assays also revealed that these compounds did not have any off-target activity in *P. falciparum*.

The mode of action for artemisinin and related compounds has been unclear for many years. A few studies have suggested that artemisinin may work through inhibition of the parasite mitochondrial respiration [46, 47]. However, examination of a series of artemisinins for their inhibitory activity against the yDHODH-transgenic parasites failed to demonstrate any reduction in potency (Morrisey et al. unpublished data). Therefore, it appears that artemisinin and derivatives are unlikely to work through parasite mtETC inhibition.

10 DHODH Inhibitors as Antimalarials

Because malaria parasites are completely dependent on de novo pyrimidine biosynthesis, enzymes involved in this pathway are potentially attractive targets for antimalarial drug discovery and development. DHODH carries out the only redox step in this pathway and is a "druggable" enzyme as shown by successful deployment of leflunomide, a human DHODH inhibitor approved for the treatment of rheumatoid arthritis. Two groups have conducted high-throughput screening of large libraries of compounds for their ability to inhibit the parasite DHODH enzymatic activity, and have identified several chemical scaffolds with selective inhibitory activities [32, 48, 49]. Among these compounds, the triazolopyrimidine [32] and substituted thiophene–carboxamide [49] series (see Fig. 1 for structures) have good in vivo activity and are under further development. These are encouraging findings that hold promise for new antimalarials targeting a mitochondrial function.

11 Other Potential Targets in Mitochondrial Functions

Mitochondria in malaria parasites are highly divergent from their hosts' organelles [2]. Indeed, the organelle appears to have much diminished functions compared to the mammalian mitochondria. Many of these functions, however, are critical for parasite survival, and their divergence from their host counterparts presents opportunities for selective antiparasitic agents [50]. Recent studies have revealed that the architecture of the mitochondrial tricarboxylic acid (TCA) metabolism in *P. falciparum* is dramatically different from that of the human mitochondria [51]. Some of the TCA metabolism enzymes, such as malate–quinone oxidoreductase and fumarate hydratase, are significantly different in malaria parasites [50]. Similarly, the ATP synthase complex in *Plasmodium* is also highly divergent in a manner similar to recent observations on a ciliate ATP synthase [52]. These and several other features of *Plasmodium* mitochondria call for detailed studies with the hope of identifying and exploiting their druggable properties.

Acknowledgments I thank colleagues and students in the Center for Molecular Parasitology at Drexel University College of Medicine for discussions and dynamism. I am also grateful for funding from the US National Institutes of Health (Grant number: AI028398) and Medicines for Malaria Venture.

References

1. Vaidya AB, Lashgari MS, Pologe LG, Morrisey J (1993) Structural features of *Plasmodium* cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol Biochem Parasitol 58:33–42
2. Vaidya AB, Mather MW (2009) Mitochondrial evolution and functions in malaria parasites. Annu Rev Microbiol 63:249–267
3. Foth BJ, Stimmler LM, Handman E, Crabb BS, Hodder AN, McFadden GI (2005) The malaria parasite *Plasmodium falciparum* has only one pyruvate dehydrogenase complex, which is located in the apicoplast. Mol Microbiol 55:39–53
4. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S et al (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498–511
5. Fisher N, Bray PG, Ward SA, Biagini GA (2007) The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. Trends Parasitol 23:305–310
6. Crofts AR (2004) The cytochrome bc1 complex: function in the context of structure. Annu Rev Physiol 66:689–733
7. Cooley JW (2010) A structural model for across membrane coupling between the Qo and Qi active sites of cytochrome bc1. Biochim Biophys Acta 1797:1842–1848
8. Mather MW, Darrouzet E, Valkova-Valchanova M, Cooley JW, McIntosh MT, Daldal F, Vaidya AB (2005) Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. J Biol Chem 280:27458–27465
9. Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB (1999) Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Mol Microbiol 33:704–711
10. Kessl JJ, Lange BB, Merbitz-Zahradnik T, Zwicker K, Hill P, Meunier B, Palsdottir H, Hunte C, Meshnick S, Trumpower BL (2003) Molecular basis for atovaquone binding to the cytochrome bc1 complex. J Biol Chem 278:31312–31318
11. Kessl JJ, Meshnick SR, Trumpower BL (2007) Modeling the molecular basis of atovaquone resistance in parasites and pathogenic fungi. Trends Parasitol 23:494–501
12. Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ (1996) Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am J Trop Med Hyg 54:62–66
13. Rathod PK, McErlean T, Lee PC (1997) Variations in frequencies of drug resistance in *Plasmodium falciparum*. Proc Natl Acad Sci USA 94:9389–9393
14. McIntosh MT, Srivastava R, Vaidya AB (1998) Divergent evolutionary constraints on mitochondrial and nuclear genomes of malaria parasites. Mol Biochem Parasitol 95:69–80
15. Joy DA, Feng X, Mu J, Furuya T, Chotivanich K, Krettli AU, Ho M, Wang A, White NJ, Suh E et al (2003) Early origin and recent expansion of *Plasmodium falciparum*. Science 300:318–321
16. Vaidya AB, Arasu P (1987) Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed. Mol Biochem Parasitol 22:249–257
17. Preiser PR, Wilson RJ, Moore PW, McCready S, Hajibagheri MA, Blight KJ, Strath M, Williamson DH (1996) Recombination associated with replication of malarial mitochondrial DNA. EMBO J 15:684–693

18. Canfield CJ, Pudney M, Gutteridge WE (1995) Interactions of atovaquone with other antimalarial drugs against *Plasmodium falciparum in vitro*. Exp Parasitol 80:373–381
19. Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL (1951) A metabolite of "Paludrine" with high antimalarial activity. Nature 168:1080
20. Crowther AF, Levi AA (1953) Proguanil – the isolation of a metabolite with high antimalarial activity. Br J Pharmacol 8:93–97
21. Looareesuwan S, Chulay JD, Canfield CJ, Hutchinson DB (1999) Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Malarone Clinical Trials Study Group. Am J Trop Med Hyg 60:533–541
22. Fivelman QL, Butcher GA, Adagu IS, Warhurst DC, Pasvol G (2002) Malarone treatment failure and *in vitro* confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. Malar J 1:1
23. Kuhn S, Gill MJ, Kain KC (2005) Emergence of atovaquone-proguanil resistance during treatment of *Plasmodium falciparum* malaria acquired by a non-immune north American traveller to west Africa. Am J Trop Med Hyg 72:407–409
24. Krudsood S, Patel SN, Tangpukdee N, Thanachartwet W, Leowattana W, Pornpininworakij K, Boggild AK, Looareesuwan S, Kain KC (2007) Efficacy of atovaquone-proguanil for treatment of acute multidrug-resistant *Plasmodium falciparum* malaria in Thailand. Am J Trop Med Hyg 76:655–658
25. Srivastava IK, Rottenberg H, Vaidya AB (1997) Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. J Biol Chem 272:3961–3966
26. Srivastava IK, Vaidya AB (1999) A mechanism for the synergistic antimalarial action of atovaquone and proguanil. Antimicrob Agents Chemother 43:1334–1339
27. Nyakeriga AM, Perlmann H, Hagstedt M, Berzins K, Troye-Blomberg M, Zhivotovsky B, Perlmann P, Grandien A (2006) Drug-induced death of the asexual blood stages of *Plasmodium falciparum* occurs without typical signs of apoptosis. Microbes Infect 8:1560–1568
28. Painter HJ, Morrisey JM, Vaidya AB (2010) Mitochondrial electron transport inhibition and viability of intraerythrocytic *Plasmodium falciparum*. Antimicrob Agents Chemother 54:5281–5287
29. Nagy M, Lacroute F, Thomas D (1992) Divergent evolution of pyrimidine biosynthesis between anaerobic and aerobic yeasts. Proc Natl Acad Sci USA 89:8966–8970
30. Painter HJ, Morrisey JM, Mather MW, Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 446:88–91
31. Ganesan SM, Morrisey JM, Ke H, Painter HJ, Laroiya K, Phillips MA, Rathod PK, Mather MW, Vaidya AB (2011) Yeast dihydroorotate dehydrogenase as a new selectable marker for *Plasmodium falciparum* transfection. Mol Biochem Parasitol 177:29–34
32. Phillips MA, Gujjar R, Malmquist NA, White J, El Mazouni F, Baldwin J, Rathod PK (2008) Triazolopyrimidine-based dihydroorotate dehydrogenase inhibitors with potent and selective activity against the malaria parasite *Plasmodium falciparum*. J Med Chem 51:3649–3653
33. Fidock DA, Nomura T, Wellems TE (1998) Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. Mol Pharmacol 54:1140–1147
34. Helsby NA, Ward SA, Howells RE, Breckenridge AM (1990) *In vitro* metabolism of the biguanide antimalarials in human liver microsomes: evidence for a role of the mephenytoin hydroxylase (P450 MP) enzyme. Br J Clin Pharmacol 30:287–291
35. Herrlin K, Massele AY, Jande M, Alm C, Tybring G, Abdi YA, Wennerholm A, Johansson I, Dahl ML, Bertilsson L et al (1998) Bantu Tanzanians have a decreased capacity to metabolize omeprazole and mephenytoin in relation to their CYP2C19 genotype. Clin Pharmacol Ther 64:391–401
36. Wanwimolruk S, Bhawan S, Coville PF, Chalcroft SC (1998) Genetic polymorphism of debrisoquine (CYP2D6) and proguanil (CYP2C19) in South Pacific Polynesian populations. Eur J Clin Pharmacol 54:431–435

37. Xie HG, Kim RB, Stein CM, Wilkinson GR, Wood AJ (1999) Genetic polymorphism of (S)-mephenytoin 4′-hydroxylation in populations of African descent. Br J Clin Pharmacol 48:402–408
38. Desta Z, Zhao X, Shin JG, Flockhart DA (2002) Clinical significance of the cytochrome P450 2 C19 genetic polymorphism. Clin Pharmacokinet 41:913–958
39. Guiguemde WA, Shelat AA, Bouck D, Duffy S, Crowther GJ, Davis PH, Smithson DC, Connelly M, Clark J, Zhu F et al (2010) Chemical genetics of *Plasmodium falciparum*. Nature 465:311–315
40. Gamo FJ, Sanz LM, Vidal J, de Cozar C, Alvarez E, Lavandera JL, Vanderwall DE, Green DV, Kumar V, Hasan S et al (2010) Thousands of chemical starting points for antimalarial lead identification. Nature 465:305–310
41. Yeates CL, Batchelor JF, Capon EC, Cheesman NJ, Fry M, Hudson AT, Pudney M, Trimming H, Woolven J, Bueno JM et al (2008) Synthesis and structure-activity relationships of 4-pyridones as potential antimalarials. J Med Chem 51:2845–2852
42. Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Hinrichs D, Riscoe MK (2008) Antimalarial quinolones: synthesis, potency, and mechanistic studies. Exp Parasitol 118:487–497
43. Winter R, Kelly JX, Smilkstein MJ, Hinrichs D, Koop DR, Riscoe MK (2011) Optimization of endochin-like quinolones for antimalarial activity. Exp Parasitol 127(2):545–51
44. Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Bagby GC, Rathbun RK, Levin JI, Hinrichs D, Riscoe MK (2006) Evaluation and lead optimization of anti-malarial acridones. Exp Parasitol 114:47–56
45. Biagini GA, Fisher N, Berry N, Stocks PA, Meunier B, Williams DP, Bonar-Law R, Bray PG, Owen A, O'Neill PM et al (2008) Acridinediones: selective and potent inhibitors of the malaria parasite mitochondrial bc1 complex. Mol Pharmacol 73:1347–1355
46. Li W, Mo W, Shen D, Sun L, Wang J, Lu S, Gitschier JM, Zhou B (2005) Yeast model uncovers dual roles of mitochondria in action of artemisinin. PLoS Genet 1:e36
47. Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, Zhou B (2010) Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One 5:e9582
48. Phillips MA, Rathod PK (2010) *Plasmodium* dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy. Infect Disord Drug Targets 10:226–239
49. Booker ML, Bastos CM, Kramer ML, Barker RH Jr, Skerlj R, Sidhu AB, Deng X, Celatka C, Cortese JF, Guerrero Bravo JE et al (2010) Novel inhibitors of *Plasmodium falciparum* dihydroorotate dehydrogenase with anti-malarial activity in the mouse model. J Biol Chem 285:33054–33064
50. Mather MW, Henry KW, Vaidya AB (2007) Mitochondrial drug targets in apicomplexan parasites. Curr Drug Targets 8:49–60
51. Olszewski KL, Mather MW, Morrisey JM, Garcia BA, Vaidya AB, Rabinowitz JD, Llinas M (2010) Branched tricarboxylic acid metabolism in *Plasmodium falciparum*. Nature 466:774–778
52. Balabaskaran Nina P, Dudkina NV, Kane LA, van Eyk JE, Boekema EJ, Mather MW, Vaidya AB (2010) Highly divergent mitochondrial ATP synthase complexes in Tetrahymena thermophila. PLoS Biol 8:e1000418

Non-Antifolate Antibiotics: Clindamycin, Doxycycline, Azithromycin and Fosmidomycin

Sanjeev Krishna and Henry M. Staines

Abstract A range of antibiotics, in addition to those that target folate metabolism, have demonstrated antimalarial activity. They include those belonging to the lincosamides, tetracyclines and macrolides classes and fosmidomycin, a derivative of phosphonic acid. Predominantly, they target pathways within the apicoplast, a relict plastid found in most apicomplexan parasites including *Plasmodium*. In general, they are not highly active against malarial parasites and are slow acting but are clinically useful when used in combination with other antimalarial drug classes. In addition, some are safe to use in pregnancy and for the treatment of small children. Here, we review the current understanding of their mechanisms of action and clinical use.

1 Introduction

There are many non-antifolate antibiotics that have antimalarial activity including members belonging to the following classes: fluoroquinolones, lincosamides, tetracyclines and macrolides. The fluoroquinolones will not be discussed further here, as they were moderately active against *Plasmodium falciparum* in vitro [1, 2], but this property did not extend to useful antimalarial efficacy when tested in vivo [3, 4]. Fosmidomycin is a phosphonic acid derivative that is also included in discussion, as a potentially interesting new class of antimalarial. The lincosamides, tetracyclines and macrolides have established antimalarial properties and are clinically useful in particular circumstances. None of these antibiotics is highly active and rapidly acting; so, their usage requires combination with other rapidly effective classes of antimalarial, where they contribute to increasing cure rates even with

S. Krishna (✉) • H.M. Staines
Division of Clinical Sciences, Centre for Infection and Immunology, St. George's University of London, Cranmer Terrace, London SW17 0RE, UK
e-mail: sgjf100@sgul.ac.uk; hstaines@sgul.ac.uk

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_8,

shorter courses of treatment. Some antibiotics have the additional advantages of being safe to use in pregnancy or for small children (discussed below).

2 Clindamycin

The lincosamides are named after lincomycin that was extracted from *Streptomyces lincolnensis* in a soil sample in 1962. The structure of lincosamides is unusual, as are their antimicrobial properties. Clindamycin was the only semi-synthetic lincosamide to be developed for clinical use (Fig. 1). Here, it has activity against Gram-positive bacteria and many anaerobes, but not against Gram-negative aerobes. More relevant to its antimalarial properties, clindamycin is active against several apicomplexans including *Plasmodium* spp., *Toxoplasma gondii* and *Babesia microti*.

2.1 Mechanism of Action

Clindamycin acts at the same site on ribosomes as erythromycin and chloramphenicol. It inhibits protein synthesis in bacteria by binding to the 50S ribosomal unit and it can exert concentration-dependent bactericidal activity beyond its accepted bacteriostatic effects [5]. In its actions against apicomplexans, clindamycin targets their apicoplast organelles and interferes with function and survival after one or two rounds of parasite replication have taken place, resulting in a "delayed-death" phenotype. This is well described for *Toxoplasma* [6] as well as for *P. falciparum* with correlations between in vitro and in vivo pharmacodynamics being observed [7–9]. Further evidence for this proposed mechanism of action for clindamycin comes from observations of mutations in the apicoplast genome of parasites obtained from the Peruvian Amazon. A point-mutation in the apicoplast-encoded 23S rRNA gene that confers resistance to lincosamides in other organisms was identified in parasites that were 100-fold more resistant to clindamycin than wild-type parasites [10].

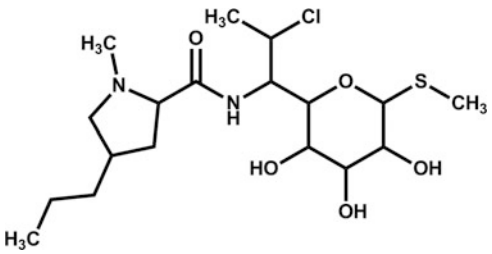


Fig. 1 Structure of clindamycin

2.2 Pharmacokinetics

Clindamycin has good (>90%) bioavailability after oral dosing, is protein bound (>90%) and widely distributed in tissues and has an elimination half-time of approximately 2–3 h. It is eliminated mainly by biliary excretion (with 20% excreted by kidneys) after metabolism to three major derivatives [11].

2.3 Usage in Malaria

The antimalarial properties of clindamycin have been confirmed in many studies, including earlier studies after the first one in 1975 that used clindamycin as a monotherapy before its more successful use in combination treatment regimens [11]. The interesting mechanism of action of clindamycin and its pharmacokinetic properties suggest that a minimum of 5 days treatment with at least twice-daily dosing is needed to achieve adequate cure rates in uncomplicated malaria [11], a regimen that is not currently recommended.

Table 1 summarises the antimalarial properties of clindamycin combined with quinine. Clindamycin dosages ranging from 5 mg/kg twice daily given with quinine, to 8 mg/kg three times a day or 12 mg/kg twice daily in general give acceptable cure rates. One small study [21] found that a 3-day treatment of the combination was as effective as a 7-day monotherapy regimen with quinine alone in travellers returning with uncomplicated malaria. Clindamycin can be used in children and pregnant women and may be combined with artesunate as a partner antimalarial, although short-course regimens may need further study to confirm efficacy [24].

Clindamycin may be less effective in treating *P. vivax* infections [11] even when prolonged courses of monotherapy are used, although a later review suggested that clindamycin is more effective than tetracyclines [25]. It also has no apparent synergism with quinine for *P. vivax* and nor does it have anti-relapse properties [11].

2.4 Tolerance and Safety

Non-specific and relatively common gastro-intestinal side effects of nausea, vomiting, abdominal pain and diarrhoea are associated with clindamycin use. Toxin-producing *Clostridium difficile* colitis can also complicate clindamycin therapy, most commonly in hospitalised patients treated with prolonged courses of clindamycin, and not with the antimalarial regimes that have been most studied in recent years. Rashes may be relatively common side effects, but severe eruptions are not. Transient reversible neutropenia and thrombocytopenia have also occurred.

Table 1 Clinical trials of clindamycin plus quinine against *P. falciparum* malaria

Study details					Regimen							Efficacy (%)	Ref.
Year	Place	Design[a]	Pop.[b]	*N*[c]	Clindamycin		Quinine		Route[d]	Days	Dosing[e]		
					Dosage, form[f]	No. of doses/day	Dosage	No. of doses/day					
1974	United States	WHO	A	5	450 mg, salt	4	540 mg	3	p.o.	3	Yes	100	[12]
1975	United States	WHO	A	5	450 mg, salt	3	560 mg	3	p.o.	3	Yes	60	[13]
1975	United States	WHO	A	2	600 mg, salt	1	560 mg	3	p.o.	3	No	50	[13]
1975	Thailand	WHO	A	4	450 mg, salt	3	540 mg	3	p.o.	3	Yes	100	[14]
1975	Thailand	WHO	A	5	150 mg, salt	3	270 mg	3	p.o.	3	No	60	[14]
1988	Brazil	WHO, RCT	A	40	10 mg/kg, base	2	12 mg/kg	2	p.o.	3	Yes	90	[15]
1994	Gabon	WHO, RCT	C	34	5 mg/kg, base	2	12 mg/kg	2	p.o.	3	Yes	88	[16]
1995	Gabon	WHO, RCT	C[g]	50	5 mg/kg, base	3	8 mg/kg	3	i.v.	4	Yes	96	[17]
1995	Gabon	WHO, RCT	A	40	5 mg/kg, base	2	12 mg/kg	2	p.o.	3[h]	Yes	92	[18]
1997	Gabon	WHO[i]	C	161	8 mg/kg, salt	2	8 mg/kg	2	p.o.	3	Yes	97	[19]
2000	Thailand	WHO, RCT	A	68	5 mg/kg, base	4	8 mg/kg	3	p.o.	7	Yes	100	[20]
2001	France	WHO, RCT	A	53	5 mg/kg, salt	3	8 mg/kg	3	i.v.	3	Yes	100	[21]
2001	Thailand	WHO, RCT	P	65	5 mg/kg, NS[j]	3	8 mg/kg	3	p.o.	7	Yes	100	[22]

[a]WHO, study conducted according to World Health Organisation guidelines [23]; *RCT* randomised controlled trial

[b]Pop., study population; A, adults; C, children; P, pregnant women

[c]*N*, number of subjects

[d]i.v., intravenous; p.o., oral

[e]Adequate dosing (i.e., clindamycin given at least twice daily and more than 3 days)

[f]8 mg of clindamycin hydrochloride salt is equivalent to 5 mg of base

[g]Severe malaria

[h]Quinine was administered for only 1.5 days

[i]Short follow-up (3 weeks)

[j]*NS* not specified. Taken from [11] with kind permission from the the authors and the American Society for Microbiology

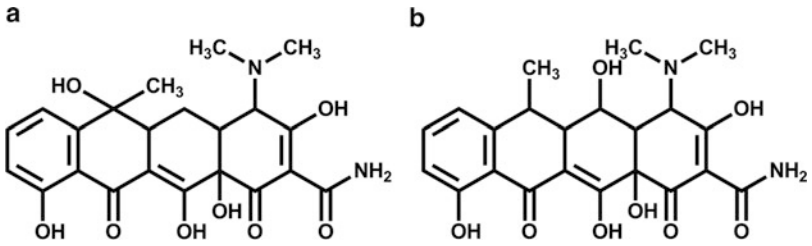


Fig. 2 Structures of tetracycline (**a**) and doxycycline (**b**)

3 Tetracyclines Including Doxycycline

The discovery of chlortetracycline in 1948 preceded that of clindamycin, although it was also from another soil organism *Streptomyces aureofasciens*. Catalytic dehydrogenation of chlortetracycline gave tetracycline in 1953. This class of antibiotic has wide antimicrobial properties, ranging from Gram-positive and Gram-negative bacteria, rickettsiae (where its use can be diagnostic), mycoplasmas, chlamydia and protozoa. Doxycycline is a semi-synthetic derivative of tetracycline first developed in 1967. The structures of tetracycline and doxycycline are given in Fig. 2.

3.1 Mechanism of Action

The tetracyclines including doxycycline act by inhibiting protein synthesis by binding to the 30S ribosomal subunit. They are relatively slow acting as antimalarial agents. Doxycycline is useful as a prophylactic agent on its own, as well as being used in combination treatment regimens to increase cure rates of conventional antimalarial agents [26]. It has limited casual prophylactic activity [26] and does not kill hypnozoites.

3.2 Pharmacokinetics

Doxycyline has ~90% oral bioavailability, which is reduced by food and delayed by alcohol, with peaks without delay occurring at ~2 h after administration [26]. Co-administration with cations such as iron supplements reduces absorption by chelation of the antibiotic, and milk may reduce absorption [26]. Plasma protein binding of doxycycline is 90% and elimination half-time is 18 h, making this one of the most convenient tetracyclines to use in practise. Most elimination of unchanged drug is through the gastrointestinal tract, with about a third being renally excreted.

3.3 Usage in Malaria

Tetracycline requires dosing 4 times as day and is therefore cumbersome to use as an antimalarial. Doxycyline (for adults up to 100 mg base twice daily, with another antimalarial) should only be used in combination with rapidly acting antimalarials such as quinine to increase cure rates, and not on its own. Table 2 summarises antimalarial treatment properties of doxycycline [26]. It can be used as a prophylactic antimalarial (for adults up to 100 mg base once daily starting before travel and continuing for 4 weeks after return), even in areas of multidrug-resistant parasites [26].

3.4 Safety and Toxicity

The tetracyclines are not recommended in pregnancy, and in children whose teeth can be stained (8 years or less). Doxycycline is a photosensitising antibiotic and can also cause oesophageal erosions and heartburn if taken incorrectly. Nausea and abdominal pain are relatively common, but frequency can be reduced by taking doxycycline with food [26].

4 Azithromycin

Azithromycin is a semi-synthetic derivative of erythromycin, whereby a methyl-substituted nitrogen atom is incorporated into the lactone ring system to improve acid stability (Fig. 3). In contrast to doxycycline, azithromycin can be used in younger children and in pregnancy, but its much increased cost compared with doxycycline may limit its applicability for management of malaria. Azithromycin is useful in the management of respiratory tract infections and has activity against *Toxoplasma* and can be used to treat uncomplicated babesiosis when combined with atovaquone in immunocompetent individuals [32].

4.1 Mechanism of Action

Azithromycin acts by binding to the 50S ribosomal sub-unit of susceptible microorganisms and inhibiting protein synthesis. Consistent with the activities of other antimalarial antibiotics, azithromycin is relatively slow acting and should be used in combination therapies for malaria. There may be some cross-resistance between antibiotics as observed for clindamycin-resistant *T. gondii*.

Table 2 Efficacy of doxycycline with or without quinine for treatment of *P. falciparum* malaria

Country (population)	Study type	Sample size/doxycycline dose	Duration of treatment	Cure rate (95% CI)	Ref.
United States (mixture of immune and non-immune)	Challenge, open-label	*P. vivax:*			[27]
		1/100 mg twice a day	4 days	0	
		1/100 mg twice a day	6 days	0	
		P. falciparum:			
		4/100 mg twice a day	5 days	0	
		9/100 mg twice a day	7 days	100%	
West Malaysia (varied, children aged 2 months–8 years)	Challenge, open-label	9/4 mg/kg daily	4 days	44.4%	[28]
		26/4 mg/kg daily	7 days	84.6%	
Irian Jaya, Indonesia (non-immune)	Randomised, open-label, comparator = CQ ($N = 30$) and doxycycline + CQ ($N = 39$)	20/100 mg twice a day	7 days	*P. falciparum*: 64.7% (42.0–87.4%) *P. vivax*: 33.3% (6.6–59.5%)	[29]
Brazil (semi-immune)	Randomised, open-label, comparator = AL ($N = 28$)	31/100 mg twice a day + 500 mg quinine every 8 h	5 days doxycycline 3 days quinine	100%	[30]
Pakistan (unknown immune status)	Challenge, open-label	100/100 mg twice a day + 10 mg/kg quinine every 8 h	7 days doxycycline 3 days quinine	100%	[31]

CQ chloroquine, *AL* artemether-lumefantrine. Taken from [26] with kind permission from the authors and the American Journal of Tropical Medicine and Hygiene

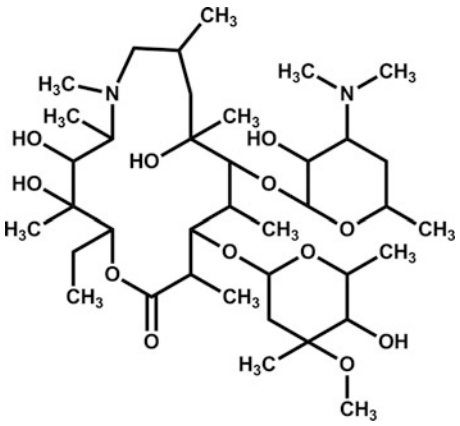


Fig. 3 Structure of azithromycin

4.2 Pharmacokinetics

Azithromycin has moderate oral bioavailability (37% after 500 mg) and this is reduced significantly (by 50%) if given with food; hence, it should be used either 1 h before food or 2 h after. Antacids also reduce availability. The degree of protein binding of azithromycin is dose-dependent, but not usually >50%. The drug is extensively distributed in tissues, which act as a depot with an elimination half-time of ~70 h. Most drugs are probably eliminated unchanged by biliary and gastrointestinal excretion.

4.3 Clinical Studies

Azithromycin by itself is not useful to treat uncomplicated *P. falciparum* or *P. vivax* infections [33] as failure rates can exceed 50% when conventional antibacterial doses for adults of 0.5 g per day for 3 days are used. Cure rates may be increased if the dose of azithromycin is also increased (to for example, 1 g per day) in combination therapies, but comparisons with other antimalarial combinations are not so encouraging (Fig. 4). Current evidence suggests that other combinations should be used to treat malaria unless there is no choice.

4.4 Safety and Toxicity

Azithromycin is relatively well tolerated, with mild gastrointestinal side effects being most commonly reported. Occasional abnormalities in liver function are observed and allergic reactions are rare. Nausea may be more common with higher dose regimens [33].

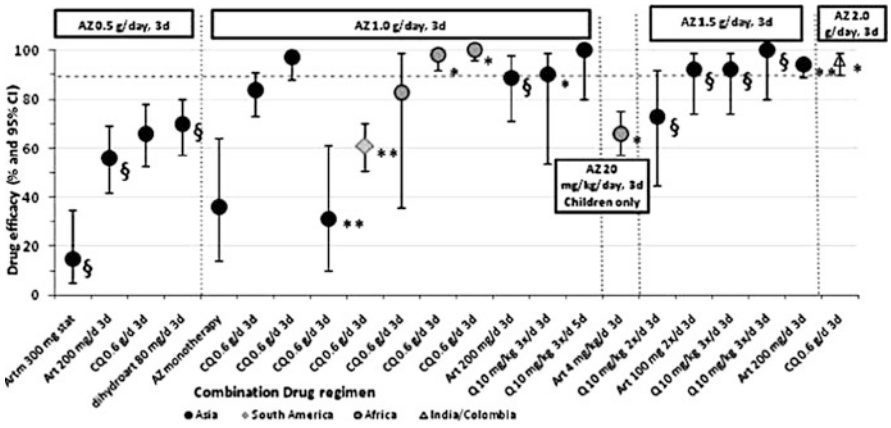


Fig. 4 Efficacy of azithromycin containing treatment regimens for *P. falciparum* (28 day follow-up). *Abbr*: *AZ* azithromycin, *CQ* chloroquine, *Artm* artemether, *Art* artesunate, *dihydroart* dihydroartemisinin, *Q* quinine, *CI* confidence interval, *d* days. *Symbols*: *Asterisk* PCR-corrected, *double asterisk* partially PCR-corrected, *section sign* study conducted in an area without malaria transmission (Bangkok). The AZ dose in the combination with artemisinin (300 mg) was 500 mg at start, followed by 250 mg after 24 h and 48 h. An interrupted line has been drawn at the 90% efficacy level, the minimum level for the 95% confidence interval for a potentially useful drug regimen as recommended by WHO [34]. Taken from [33] with kind permission from the authors and John Wiley & Sons, Ltd on behalf of The Cochrane Collaboration. Copyright Cochrane Collaboration.

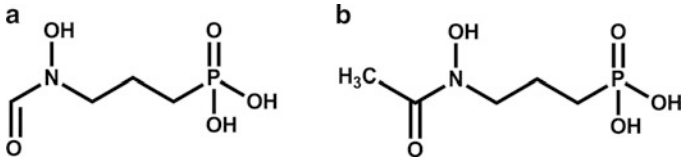


Fig. 5 Structure of fosmidomycin (**a**) and FR-900098 (**b**)

5 Fosmidomycin

Fosmidomycin is a natural antibiotic, originally derived from *Streptomyces lavendulae*. It is currently being investigated as a combination partner in antimalarial chemotherapy regimens, with the idea that it represents a non-artemisinin class of antimalarial with an unusual mode of action. Fosmidomycin's relatively simple chemical structure (Fig. 5) makes it amenable to complete synthesis, which is how it is currently made for investigational studies. Fujisawa Pharmaceutical Company in Osaka, Japan [35], originally developed fosmidomycin as an antibacterial agent [36–39] for treating urinary tract infections approximately three decades ago. It is most effective against enterobacteria and not against Gram-positive organisms or anaerobes. Since its discovery, cephalosporins have emerged as being more effective for recurrent infections and the development of fosmidomycin as an antibacterial

agent has not been taken further forward, until it has been repurposed as an antimalarial.

6 Mechanism of Action

The repurposing of fosmidomycin as an antimalarial depended on several advances in understanding the biology of the malarial parasite. One advance lay in the recognition of the plastid organelle as an excellent target for new drugs, as no similar structure exists in animal cells. Another advance was the identification of an alternative [nonmevalonate or 1-deoxy-D-xylulose 5-phosphate (DOXP)] pathway for isoprenoid synthesis in parasites that was hitherto described in plants and eubacteria. Key enzymes forming part of this synthesis pathway include DOXP reductoisomerase and 2*C*-methyl-D-erythritol-4-(cytidine-5-diphospho) transferase (Fig. 6) and are located in the parasite's apicoplast. This pathway contributes to many functions such as prenylation of membrane-bound proteins and synthesis of carotenoids and terpenoids.

Hassan Jomaa and colleagues [40] tested the idea that inhibiting this pathway would prove lethal to parasites by expressing a recombinant version of DOXP reductoisomerase and showing that it was inhibited by fosmidomycin (with an inhibitory constant of ~30 nM). They also showed that fosmidomycin and compound FR-900098 (Fig. 5), a compound that acts as a pro-drug for fosmidomycin, killed cultured *P. falciparum*, including highly chloroquine-resistant strains (IC_{50} values ranging from 300 to 1,200 nM), as well as being able to cure mice infected with *P. vinckei*. Recently, it has been reported that fosmidomycin also targets a second enzyme in the DOXP pathway, 2*C*-methyl-D-erythritol-4-(cytidine-5-diphospho) transferase (Fig. 6) [41]. Other recent studies have suggested that the uptake of fosmidomycin, which is highly charged, requires specific transport mechanisms [42, 43]. This includes the new permeability pathways (NPP) that are responsible for altered plasma membrane permeability of the host erythrocyte as intracellular plasmodial parasites mature [42, 44]. These new data not only explain the selectively of fosmidomycin for plasmodial parasites over closely related

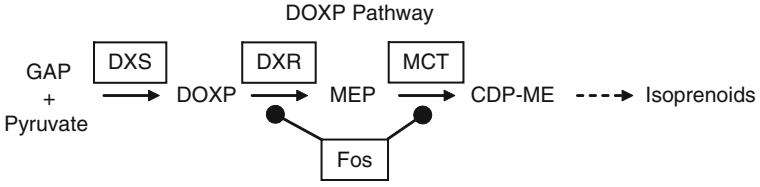


Fig. 6 The 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway for isoprenoid synthesis and the sites of action of fosmidomycin (Fos). *GAP* glyceraldehyde-3-phosphate, *DXS* DOXP synthase, *DXR* DOXP reductoisomerase, *MEP* 2*C*-methyl-D-erythritol-4-phosphate, *MCT* 2*C*-methyl-D-erythritol-4-(cytidine-5-diphospho) transferase, *CDP-ME* 4-(cytidine-5-diphospho)-2*C*-methyl-D-erythritol

Table 3 Fosmidomycin clinical trials data

Region of study	Study design	Regimen	Subjects	Safety and tolerability	Efficacy
Monotherapy					
Gabon/Thailand [48]	Uncontrolled, open-label study	1,200 mg every 8 h for 7 days	26 adults	Well tolerated with mild GI disturbances in 5 patients	At day 28, cure rates were 78% (7/9) in Gabon and 22% (2/9) in Thailand
Gabon [49]	Uncontrolled, open-label study	1,200 mg every 8 h for 5, 4 or 3 days	32 adults	Well tolerated with most frequent AEs being headache, weakness, myalgia, abdominal pain, and loose stools	At day 14, cure rates were 89% (8/9), 88% (7/8) and 60% (6/10) for 5, 4 and 3 day regimens
Combination therapy					
Gabon [50]	Randomised, controlled open-label study	30/5 mg/kg FC, 30 mg/kg F or 5 mg/kg C every 12 h for 5 days	36 children (7–14 years)	Well tolerated with no serious AEs (mostly GI disturbances)	At day 28, cure rates were 100% (12/12), 42% (5/12) and 100% (12/12) for FC, F and C regimens
Gabon [51]	Uncontrolled, dose-reduction study	30/10 mg/kg FC every 12 h for 5, 4, 3, 2, 1 days	52 children (7–14 years)	Well tolerated with most frequent AEs being GI disturbances [mostly loose stools (13 events) and abdominal pain (9 events)]	At day 28, cure rates were 100% (10/10), 100% (10/10), 90% (9/10), 70% (7/10) and 10% (1/10) for 5, 4, 3, 2 and 1 day regimens
Gabon [52]	Uncontrolled, dose-reduction study	30/2 mg/kg F/AS every 12 h for 5, 4, 3, 2, 1 days	50 children (6–12 years)	Well tolerated with most frequent AEs being GI events	At day 28, cure rates were 100% (9/9), 90% (9/10), 100% (10/10), 60% (6/10) and 40% (4/10) for 5, 4, 3, 2 and 1 day regimens
Gabon [53]	Uncontrolled, Phase IIb, single-arm study	30/10 mg/kg FC every 12 h for 3 days	51 children (1–14 years)	Well tolerated with GI disturbances but relatively high rates of treatment-associated	At day 28, cure rates were 89% (42/47) and 62% (5/8) for children 3–14 years and 1–2 years

(continued)

Table 3 (continued)

Region of study	Study design	Regimen	Subjects	Safety and tolerability	Efficacy
				neutropenia (8/51) and falls of haemoglobin concentrations of ≥2 g/dl (7/51)	
Gabon [54]	Randomised study	30/10 mg/kg FC every 12 h for 3 days or 25/1.25 mg/kg SP single dose	105 children (3–14 years)	Both treatments were well tolerated with slightly more AEs occurring in the SP group	At day 28, cure rates were 94% (46/49) and 94% (46/49) for FC and SP regimens
Thailand [46]	Uncontrolled, open-label study	A: 1,200 mg F every 12 h for 7 days or B: 900/600 mg FC every 12 h for 7 days	33 adults	Both treatments were well tolerated with no serious AEs. Mild events included headache and GI disturbance	At day 28, 22% (2/9) and 100% (12/12) for A and B regimens
Thailand [47]	Randomised, uncontrolled open-label study	A: 900/300 mg FC every 6 h for 3 days or B: 1,800/600 mg FC every 12 h for 3 days	114 Adults	Both regimens well tolerated with no serious AEs (GI events were most with abdominal pain developing in 10% of patients, diarrhoea in 5% and vomiting in 4%)	At day 28, 91% (21/23) and 90% (70/78) for A and B regimens

F fosmidomycin, *C* clindamycin, *SP* sulfadoxine-pyrimethamine, *AS* artesunate, *AE* adverse event, *GI* gastro-intestinal

species (e.g. *Toxoplasma*) but can also be used to test potential resistance mechanisms in *Plasmodium* spp.

6.1 Pharmacokinetics

Fosmidomycin has relatively poor oral bioavailability (~25%) and is poorly protein bound (<5%) with an elimination half-time of 1.6–1.8 h [45]. This elimination half time is somewhat prolonged in subjects with malaria to 3.4 h (range 1.4–11.8 h) and not altered importantly after co-administration with clindamycin in one study [46], although particular dosing regimens of this combination (fosmidomycin and clindamycin) may influence pharmacokinetic behaviour of each drug [47].

6.2 Fosmidomycin in Malaria

Table 3 summarises studies of fosmidomycin in children and adults with malaria. Monotherapy studies with fosmidomycin have confirmed its antimalarial activity in patients, and also that, unlike clindamycin, it has relatively rapid antimalarial actions. However, recrudescence rates are rather high, compelling the choice of an appropriate combination partner to achieve adequate clinical and parasitological cure rates. Clindamycin has been the most thoroughly studied partner drug and results of various studies are also included in Table 3. In general, a 3-day treatment regimen with 30 mg/kg fosmidomycin and 10 mg/kg clindamycin given in a 8 or 12 h interval and in the populations reported provided adequate responses in adults and children except for one study in children aged <3 years [53]. This combination was also well tolerated, with mild gastrointestinal side effects being some of the most frequent (Table 3).

Acknowledgments We thank Peter Kremsner and David Hutchinson for useful discussion and the EDTCP (project code: IP.2008.31060.003) for financial support.

References

1. Krishna S, Davis TM, Chan PC, Wells RA, Robson KJ (1988) Ciprofloxacin and malaria. Lancet 1:1231–1232
2. Tripathi KD, Sharma AK, Valecha N, Biswas S (1993) *In vitro* activity of fluoroquinolones against chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. Indian J Malariol 30:67–73
3. Havemann K, Bhibi P, Hellgren U, Rombo L (1992) Norfloxacin is not effective for treatment of *Plasmodium falciparum* infection in Kenya. Trans R Soc Trop Med Hyg 86:586

4. Watt G, Shanks GD, Edstein MD, Pavanand K, Webster HK, Wechgritaya S (1991) Ciprofloxacin treatment of drug-resistant falciparum malaria. J Infect Dis 164:602–604
5. Reusser F (1975) Effect of lincomycin and clindamycin on peptide chain initiation. Antimicrob Agents Chemother 7:32–37
6. Fichera ME, Bhopale MK, Roos DS (1995) *In vitro* assays elucidate peculiar kinetics of clindamycin action against *Toxoplasma gondii*. Antimicrob Agents Chemother 39:1530–1537
7. Burkhardt D, Wiesner J, Stoesser N, Ramharter M, Uhlemann AC, Issifou S, Jomaa H, Krishna S, Kremsner PG, Borrmann S (2007) Delayed parasite elimination in human infections treated with clindamycin parallels 'delayed death' of *Plasmodium falciparum in vitro*. Int J Parasitol 37:777–785
8. Fichera ME, Roos DS (1997) A plastid organelle as a drug target in apicomplexan parasites. Nature 390:407–409
9. Kohler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, Palmer JD, Roos DS (1997) A plastid of probable green algal origin in Apicomplexan parasites. Science 275:1485–1489
10. Dharia NV, Plouffe D, Bopp SE, Gonzalez-Paez GE, Lucas C, Salas C, Soberon V, Bursulaya B, Kochel TJ, Bacon DJ et al (2010) Genome scanning of Amazonian *Plasmodium falciparum* shows subtelomeric instability and clindamycin-resistant parasites. Genome Res 20:1534–1544
11. Lell B, Kremsner PG (2002) Clindamycin as an antimalarial drug: review of clinical trials. Antimicrob Agents Chemother 46:2315–2320
12. Miller LH, Glew RH, Wyler DJ, Howard WA, Collins WE, Contacos PG, Neva FA (1974) Evaluation of clindamycin in combination with quinine against multidrug-resistant strains of *Plasmodium falciparum*. Am J Trop Med Hyg 23:565–569
13. Clyde DF, Gilman RH, McCarthy VC (1975) Antimalarial effects of clindamycin in man. Am J Trop Med Hyg 24:369–370
14. Hall AP, Doberstyn EB, Nanokorn A, Sonkom P (1975) Falciparum malaria semi-resistant to clindamycin. Br Med J 2:12–14
15. Kremsner PG, Zotter GM, Feldmeier H, Graninger W, Rocha RM, Wiedermann G (1988) A comparative trial of three regimens for treating uncomplicated falciparum malaria in Acre, Brazil. J Infect Dis 158:1368–1371
16. Kremsner PG, Winkler S, Brandts C, Neifer S, Bienzle U, Graninger W (1994) Clindamycin in combination with chloroquine or quinine is an effective therapy for uncomplicated *Plasmodium falciparum* malaria in children from Gabon. J Infect Dis 169:467–470
17. Kremsner PG, Radloff P, Metzger W, Wildling E, Mordmuller B, Philipps J, Jenne L, Nkeyi M, Prada J, Bienzle U et al (1995) Quinine plus clindamycin improves chemotherapy of severe malaria in children. Antimicrob Agents Chemother 39:1603–1605
18. Metzger W, Mordmuller B, Graninger W, Bienzle U, Kremsner PG (1995) High efficacy of short-term quinine-antibiotic combinations for treating adult malaria patients in an area in which malaria is hyperendemic. Antimicrob Agents Chemother 39:245–246
19. Vaillant M, Millet P, Luty A, Tshopamba P, Lekoulou F, Mayombo J, Georges AJ, Deloron P (1997) Therapeutic efficacy of clindamycin in combination with quinine for treating uncomplicated malaria in a village dispensary in Gabon. Trop Med Int Health 2:917–919
20. Pukrittayakamee S, Chantra A, Vanijanonta S, Clemens R, Looareesuwan S, White NJ (2000) Therapeutic responses to quinine and clindamycin in multidrug-resistant falciparum malaria. Antimicrob Agents Chemother 44:2395–2398
21. Parola P, Ranque S, Badiaga S, Niang M, Blin O, Charbit JJ, Delmont J, Brouqui P (2001) Controlled trial of 3-day quinine-clindamycin treatment versus 7-day quinine treatment for adult travelers with uncomplicated falciparum malaria imported from the tropics. Antimicrob Agents Chemother 45:932–935
22. McGready R, Cho T, Samuel VL, Brockman A, van Vugt M, Looareesuwan S, White NJ, Nosten F (2001) Randomized comparison of quinine-clindamycin versus artesunate in the treatment of falciparum malaria in pregnancy. Trans R Soc Trop Med Hyg 95:651–656

23. WHO (1996) Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense trassmission. World Health Organisation, Geneva
24. Ramharter M, Oyakhirome S, Klein Klouwenberg P, Adegnika AA, Agnandji ST, Missinou MA, Matsiegui PB, Mordmuller B, Borrmann S, Kun JF et al (2005) Artesunate-clindamycin versus quinine-clindamycin in the treatment of *Plasmodium falciparum* malaria: a randomized controlled trial. Clin Infect Dis 40:1777–1784
25. Pukrittayakamee S, Imwong M, Looareesuwan S, White NJ (2004) Therapeutic responses to antimalarial and antibacterial drugs in vivax malaria. Acta Trop 89:351–356
26. Tan KR, Magill AJ, Parise ME, Arguin PM (2011) Doxycycline for malaria chemoprophylaxis and treatment: report from the CDC expert meeting on malaria chemoprophylaxis. Am J Trop Med Hyg 84:517–531
27. Clyde DF, Miller RM, DuPont HL, Hornick RB (1971) Antimalarial effects of tetracyclines in man. J Trop Med Hyg 74:238–242
28. Ponnampalam JT (1981) Doxycycline in the treatment of falciparum malaria among aborigine children in West Malaysia. Trans R Soc Trop Med Hyg 75:372–377
29. Taylor WR, Widjaja H, Richie TL, Basri H, Ohrt C, Tjitra TE, Jones TR, Kain KC, Hoffman SL (2001) Chloroquine/doxycycline combination versus chloroquine alone, and doxycycline alone for the treatment of *Plasmodium falciparum* and *Plasmodium vivax* malaria in north-eastern Irian Jaya, Indonesia. Am J Trop Med Hyg 64:223–228
30. Alecrim MG, Lacerda MV, Mourao MP, Alecrim WD, Padilha A, Cardoso BS, Boulos M (2006) Successful treatment of *Plasmodium falciparum* malaria with a six-dose regimen of artemether-lumefantrine versus quinine-doxycycline in the Western Amazon region of Brazil. Am J Trop Med Hyg 74:20–25
31. Ejaz A, Haqnawaz K, Hussain Z, Butt R, Awan ZI, Bux H (2007) Treatment of uncomplicated *Plasmodium falciparum* malaria with quinine-doxycycline combination therapy. J Pak Med Assoc 57:502–505
32. Krause PJ, Lepore T, Sikand VK, Gadbaw J Jr, Burke G, Telford SR 3rd, Brassard P, Pearl D, Azlanzadeh J, Christianson D et al (2000) Atovaquone and azithromycin for the treatment of babesiosis. N Engl J Med 343:1454–1458
33. van Eijk AM, Terlouw DJ (2011) Azithromycin for treating uncomplicated malaria. Cochrane Database Syst Rev CD006688
34. WHO (2006) Guidelines for the treatment of malaria. World Health Organisation, Geneva
35. Kamiya T, Hashimoto M, Hemmi K, Takeno H (1980) Hydroxyaminohydrocarbon-phosphonic acids. US Patent 4206156
36. Kanimoto Y, Greenwood D (1987) Activity of fosmidomycin in an *in vitro* model of the treatment of bacterial cystitis. Infection 15:465–468
37. Kuroda Y, Okuhara M, Goto T, Okamoto M, Terano H, Kohsaka M, Aoki H, Imanaka H (1980) Studies on new phosphonic acid antibiotics. IV. Structure determination of FR-33289, FR-31564 and FR-32863. J Antibiot (Tokyo) 33:29–35
38. Mine Y, Kamimura T, Nonoyama S, Nishida M, Goto S, Kuwahara S (1980) *In vitro* and *in vivo* antibacterial activities of FR-31564, a new phosphonic acid antibiotic. J Antibiot (Tokyo) 33:36–43
39. Okuhara M, Kuroda Y, Goto T, Okamoto M, Terano H, Kohsaka M, Aoki H, Imanaka H (1980) Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289. J Antibiot (Tokyo) 33:24–28
40. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK et al (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science 285:1573–1576
41. Zhang B, Watts KM, Hodge D, Kemp LM, Hunstad DA, Hicks LM, Odom AR (2011) A second target of the antimalarial and antibacterial agent fosmidomycin revealed by cellular metabolic profiling. Biochemistry 50:3570–3577
42. Baumeister S, Wiesner J, Reichenberg A, Hintz M, Bietz S, Harb OS, Roos DS, Kordes M, Friesen J, Matuschewski K et al (2011) Fosmidomycin uptake into *Plasmodium* and *Babesia*-

infected erythrocytes is facilitated by parasite-induced new permeability pathways. PLoS One 6:e19334
43. Nair SC, Brooks CF, Goodman CD, Strurm A, McFadden GI, Sundriyal S, Anglin JL, Song Y, Moreno SN, Striepen B (2011) Apicoplast isoprenoid precursor synthesis and the molecular basis of fosmidomycin resistance in *Toxoplasma gondii*. J Exp Med 208:1547–1559
44. Staines HM, Ellory JC, Chibale K (2005) The new permeability pathways: targets and selective routes for the development of new antimalarial agents. Comb Chem High Throughput Screen 8:81–88
45. Kuemmerle HP, Murakawa T, De Santis F (1987) Pharmacokinetic evaluation of fosmidomycin, a new phosphonic acid antibiotic. Chemioterapia 6:113–119
46. Na-Bangchang K, Ruengweerayut R, Karbwang J, Chauemung A, Hutchinson D (2007) Pharmacokinetics and pharmacodynamics of fosmidomycin monotherapy and combination therapy with clindamycin in the treatment of multidrug resistant falciparum malaria. Malar J 6:70
47. Ruangweerayut R, Looareesuwan S, Hutchinson D, Chauemung A, Banmairuroi V, Na-Bangchang K (2008) Assessment of the pharmacokinetics and dynamics of two combination regimens of fosmidomycin-clindamycin in patients with acute uncomplicated falciparum malaria. Malar J 7:225
48. Lell B, Ruangweerayut R, Wiesner J, Missinou MA, Schindler A, Baranek T, Hintz M, Hutchinson D, Jomaa H, Kremsner PG (2003) Fosmidomycin, a novel chemotherapeutic agent for malaria. Antimicrob Agents Chemother 47:735–738
49. Missinou MA, Borrmann S, Schindler A, Issifou S, Adegnika AA, Matsiegui PB, Binder R, Lell B, Wiesner J, Baranek T et al (2002) Fosmidomycin for malaria. Lancet 360:1941–1942
50. Borrmann S, Adegnika AA, Matsiegui PB, Issifou S, Schindler A, Mawili-Mboumba DP, Baranek T, Wiesner J, Jomaa H, Kremsner PG (2004) Fosmidomycin-clindamycin for *Plasmodium falciparum* infections in African children. J Infect Dis 189:901–908
51. Borrmann S, Issifou S, Esser G, Adegnika AA, Ramharter M, Matsiegui PB, Oyakhirome S, Mawili-Mboumba DP, Missinou MA, Kun JF et al (2004) Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. J Infect Dis 190:1534–1540
52. Borrmann S, Adegnika AA, Moussavou F, Oyakhirome S, Esser G, Matsiegui PB, Ramharter M, Lundgren I, Kombila M, Issifou S et al (2005) Short-course regimens of artesunate-fosmidomycin in treatment of uncomplicated *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 49:3749–3754
53. Borrmann S, Lundgren I, Oyakhirome S, Impouma B, Matsiegui PB, Adegnika AA, Issifou S, Kun JF, Hutchinson D, Wiesner J et al (2006) Fosmidomycin plus clindamycin for treatment of pediatric patients aged 1 to 14 years with *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 50:2713–2718
54. Oyakhirome S, Issifou S, Pongratz P, Barondi F, Ramharter M, Kun JF, Missinou MA, Lell B, Kremsner PG (2007) Randomized controlled trial of fosmidomycin-clindamycin versus sulfadoxine-pyrimethamine in the treatment of *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 51:1869–1871

Artemisinins: Artemisinin, Dihydroartemisinin, Artemether and Artesunate

Harin A. Karunajeewa

Abstract Artemisinin comes from the plant, *Artemisia annua*, an ancient Chinese herbal remedy for relapsing fever. Rediscovery of its antimalarial action in China in the 1970s has seen it and its semisynthetic derivatives become the most useful drugs for most malarial illness. Artemisinins have a sesquiterpene lactone structure. Their anti-microbial action relates to a characteristic endoperoxide moiety. The precise mechanism of action remains controversial. Experimental induction of parasite resistance both in vitro and in vivo has been followed by recent initial clinical reports of resistance. Artemisinins are currently preferred as parenteral treatment of severe malaria, pre-referral rectal treatment and, as part of artemisinin combination therapy (ACT) oral treatment of uncomplicated falciparum malaria. Currently, amongst the most widely used drugs in the world, their future will be determined by the rate and extent of development of resistance. Better understanding of mechanisms of action and resistance and policy initiatives to prevent or delay resistance will be crucial.

1 History

The rediscovery of the antimalarial properties of artemisinin by Chinese scientists in the early 1970s has revolutionised the treatment of malaria. Almost all of the world's countries with endemic *P. falciparum* have now enacted policies favouring use of an artemisinin derivative-based combination therapy (ACT) as first-line treatment for uncomplicated malaria and an artemisinin derivative is now the preferred drug for treating severe malaria [1, 2]. When we consider the estimated 400 million annual clinical presentations with malaria [3], in terms of global health

H.A. Karunajeewa (✉)
Harin Karunajeewa, 170 Gladstone Avenue, Northcote, Vic 3070, Australia
e-mail: Harin.Karunajeewa@wh.org.au

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_9,

impact, the development of the artemisinin derivatives represents one of the most important achievements in recent medical history.

Like quinine, artemisinin is one of many plant-derived traditional medicines with antimalarial properties [4]. However, written records of artemisinin's therapeutic use predate that of Cinchona bark in South America by more than 1,000 years. The first known descriptions of its medicinal use are by the revered Chinese physician Ge Hong (284–363 AD) and appear in his text "Emergency prescriptions kept up one's sleeve," dated approximately 340 AD [5]. This recommends use of an extract of the herb, *qinghao* for the treatment of a condition designated by a Chinese medical term best translated as "intermittent fevers." This term, no doubt, included (though by no means exclusively) relapsing fever due to malaria infection. The name, *Qinghao*, means literally "the blue-green hao," referring to the plant's leaves that maintain their green colour into autumn. This description may have been used to distinguish it from a similar plant whose leaves turned yellow, and that has been subsequently described in Chinese medical literature as *huanghuahao* (the "yellow-blossom hao"). The distinction seems to align *Qinghao* with the modern botanical classification of *Artemisia annua* and *huanghuahao* with the species, *Artemisia apiacea*. This is probably important, because *A. annua* yields much higher quantities of artemisinin than other *Artemisia* species. *Artemisia annua*, known in English variously as "sweet wormwood," "sweet Annie," "sweet sagewort" and "annual wormwood" and other species belonging to the genus *Artemisia* are distributed widely throughout the world. In Europe and elsewhere, they have been used as herbal medicine for a myriad of indications, some of which have now been validated by modern medical research (use as a "vermifuge" for the treatment of parasitic worm infections) and some which have not (use as an aphrodisiac) [6]. Perhaps the most famous use of *Artemisia* plants in the Western world has been as a constituent of the drinks, absinthe and vermouth.

The medicinal extract of *qinghao* is known as *quinhaosu*, meaning "essence" of qinghao, and Ge Hong's original description of its preparation is instructive. Rather than the more conventional method of combining dried herbs with hot water to prepare a herbal tea, Ge Hong takes the unusual step of specifically instructing the reader to soak the entire fresh plant in water, to then "wring it out" and ingest the juice. With the benefit of current knowledge of artemisinin's physicochemical properties [7], this unconventional approach may have been crucial in yielding an efficacious preparation, given the poor water solubility of the active constituent, artemisinin. Ge Hong's method is likely to have produced an emulsion with plant oils containing the water-insoluble artemisinin [4] and recent testing of his original technique has yielded concentrations of artemisinin 20-times higher than those of a tea prepared from dried herbs [8].

The modern-day rediscovery of the antimalarial properties of artemisinin was made possible by a unique nexus between traditional Chinese, and conventional Western medicine, that occurred in the unprecedented geo-political context of what is referred to in the West as the Chinese Cultural Revolution (1966–1976). During this time, nationalistic pride saw a rejection of perceived Western influences in all fields, including medicine and science. "Classes for Western medical doctors

learning Chinese medicine" had become mandatory [5]. Mao Tse Tung described China's national heritage as a "treasure house" of knowledge and wisdom that could be used for "serving the people" – regarded as particularly pertinent to the "task of combating malaria" (enumerated as Mao's "task number five hundred and twenty-three") [5]. The importance of malaria to Chinese national interests during this time also, no doubt, lent much to its opposition to American interests in the wars of Indo-China where the burden of malarial disease on combatants had attained crucial strategic importance. It therefore mirrored a similarly massive up-scaling of research into novel antimalarial compounds conducted by the US military that concomitantly led to the development of mefloquine.

The institution credited with the discovery of artemisinin's antimalarial properties is the Academy of Traditional Chinese Medicine. In association with the People's Liberation Army Research Unit, in the late 1960s, they began screening traditional medicines for antimalarial activity. *Qinghao* was included amongst the first ten products screened but initially showed no activity. In retrospect, this was probably because an extraction method suitable to the water insoluble active component was not used. By 1972, however, a group at the Academy led by Professor Tu Youyou had successfully prepared an ether extract described as having 95–100% efficacy when used for treatment in a rodent malaria model. Crucially, Professor Youyou and her team claim to have heeded the original advice of Ge Hong when developing their extraction method, with special attention to avoiding the use of excessive heat. The use of the aprotic solvent, ether, probably also aided the extraction of the oil and water-insoluble active compound and the use of neutral pH would have been important, now that artemisinin is known to be unstable in the presence of both acid and alkali.

Within the same year as the first report of its activity in rodent malaria in 1972, a trial was reported in 21 humans with malaria (both *P. vivax* and *P. falciparum*) in Beijing with a reported efficacy of >90%. The principal investigator in the early human trials of artemisinin derivatives was Professor Li Guoqiao of the Guangzhou University of Traditional Chinese Medicine. The professor and his group subsequently described the efficacy of artemisinin derivatives in severe (including cerebral) malaria as well as demonstrating their extraordinarily rapid in vivo activity on trophozoite blood stages and their potent gametocidal activity [9].

In 1973, Professor Youyou synthesised dihydroartemisinin, the first semi-synthetic artemisinin "derivative." The new compound had higher water solubility, a characteristic later to prove pharmacologically advantageous. Subsequently, further semi-synthetic derivatisation of dihydroartemisinin was employed to produce ethers and esters from dihydroartemisinin. Several hundred of these semi-synthetic derivatives were produced and subjected to preliminary investigation by Chinese scientists in the 1970s. Together with dihydroartemisinin, they are now collectively referred to as the "1st generation endoperoxides" or "artemisinin derivatives" to distinguish them from the subsequent development of other compounds (such as artemisone) and wholly-synthetic endoperoxides. Only a small percentage of these many hundred compounds have been evaluated in human subjects and a smaller number still have achieved widespread therapeutic

use. The most-used therapeutic compounds include artemisinin itself, dihdyroartemisinin, artemether, arteether, and artesunate. These were developed for mass marketing by nationalised pharmaceutical companies in other parts of China (Guilin and Kunming), in regions where China's highest malaria prevalence occurred [10]. The first marketed pharmaceutical preparations were officially available from 1986 onwards. However, it is likely that very widespread use of artemisinin derivatives was employed much earlier in China and also in Cambodia (formerly Kampuchea), as part of Chinese military assistance to the Khmer Rouge regime in the late 1970s.

The first English-language publications regarding clinical efficacy appeared in 1982 [11, 12]. These came at a time of great pessimism and alarm in the face of burgeoning parasite resistance to existing agents, coupled with a paucity of attractive alternative drugs in the existing antimalarial drug development "pipeline," whether in the private (pharmaceutical companies) or public (military programmes) sectors of Western countries [13, 14]. At this time, Thailand and its border areas seemed to face the world's greatest problem of drug-resistant malaria, with the loss of mefloquine efficacy here being viewed as a harbinger of the catastrophic scenario of untreatable *P. falciparum* malaria [14]. In 1991, the first clinical trials demonstrating efficacy against multi-drug resistant strains of *P. falciparum* in Thailand were published [15]. Subsequently, the efficacy of the combination of artesunate and mefloquine for uncomplicated malaria appeared to be excellent, with the introduction of this combination coinciding with a reversal in the trend towards worsening mefloquine resistance in the area [16]. Since this time, susceptibility to mefloquine has not declined further in this area despite use of the artesunate–mefloquine combination for many years [16].

From the early 1990s until the current time, an explosion in interest in the artemisinin derivatives led to development of assays to measure concentrations of drug in biological fluids [17], documentation of the pharmacokinetic properties of the individual derivatives [7], theories regarding their mode of action. [18, 19], development of rectally administered formulations [20] and an extraordinarily large body of clinical trials exploring their efficacy in both uncomplicated and severe malaria [21–24]. At the time of writing well over 12,000 subjects had been enrolled in clinical studies evaluating artemisinin derivatives [23]. At least 50 randomised controlled trials have now been published evaluating the efficacy of ACTs for treatment of uncomplicated malaria [24]. In 2005, the landmark SEAQAMAT study was published [25]. This well-powered multi-centre randomised controlled trial established parenteral artesunate in preference to quinine as the therapy of choice for severe malaria in adults. In doing so, it represented the first therapeutic intervention in recent history to have a demonstrated impact on mortality in severe malaria. More recently, a mortality benefit of artesunate over quinine was also confirmed in a large study of African children with severe malaria [26], effectively ending any controversy as to its preferred status as first-line treatment for severe malaria in most contexts [2]. Until recently, the widespread use of parenteral artesunate had been impeded by its lack of approval by pharmaceutical regulatory bodies. Fortunately, however, the major manufacturer of parenteral artesunate (Guilin Pharmaceuticals, China) has recently achieved prequalification status for

its product by the World Health Organisation (WHO), meaning it is likely to be widely available, including in developed countries.

By the end of the millennium, in the face of burgeoning evidence supporting the use of artemisinin derivatives, opinion leaders, led by Professor Nick White of Oxford and Mahidol Universities, were already calling for a radical re-thinking of malaria treatment policies and guidelines [27]. In particular, the strategy of ACT use was advocated as an alternative to conventional therapies compromised by parasite resistance. In 2006, the WHO officially endorsed ACT as the treatment of choice of uncomplicated *P. falciparum* malaria [28], with emphasis on the theoretical benefits of the strategy of combination therapy to limit the potential for development of parasite resistance [29].

The prototypical ACT was the "loose" combination of separate tablets of artesunate and mefloquine first used in Thailand [30]. Other "loose" ACTs have included sulphadoxine–pyrimethamine, amodiaquine and atovaquone–proguanil as partner drugs. However, "fixed" co-formulations containing both the artemisinin derivative and a non-artemisinin partner drug have been the focus of ACT development in recent years (Table 2). The first co-formulated ACT to achieve regulatory approval was artemether–lumefantrine, endorsed by the WHO in 2006 for use in areas of multi-drug resistance and in Africa, and was shortly followed by endorsement of co-formulated artesunate with amodiaquine [28]. Crucially, manufacturers of these products have provided drug at substantially lower cost for developing countries. Other promising combinations in various stages of development have utilised piperaquine, mefloquine, amodiaquine, fosmidomycin, chloroproguanil–dapsone, pyronaridine and naphthoquine as partner drugs [31].

The effective use of ACTs has been credited as a major factor in very significant reductions in malaria prevalence in many areas of South-East Asia and Sub-Saharan Africa [32, 33]. This has helped reignite interest in the potential for attempts at malaria eradication [34, 35].

The experimental induction of artemisinin resistance in rodent malaria was first reported in 1999 [36]. In 2006, given mounting concerns regarding the potentially disastrous consequences of artemisinin resistance [27, 28, 37–41], the WHO recommended the phasing-out of oral mono-preparations of artemisinin, in order to prevent their use as mono-, rather than combination therapy [42]. Soon after, the first reports indicating impaired responses to artemininin derivatives in vivo were reported from Cambodia in 2008 [43, 44]. At the time of writing, a massive effort is underway to monitor and prevent the spread of parasite resistance in this region [43, 45].

2 Chemistry

Artemisinin ($C_{15}H_{22}O_5$, IUPAC name: (3*R*, 5aS,6*R*,8a*S*,9*R*, 12*S*,12a*R*)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyranol[4,3-*j*]-1,2-benzodioxepin-10(3*H*)-one) is a sesquiterpene lactone, with a distinctive 1,2,4 triaxone ring structure (Fig. 1).

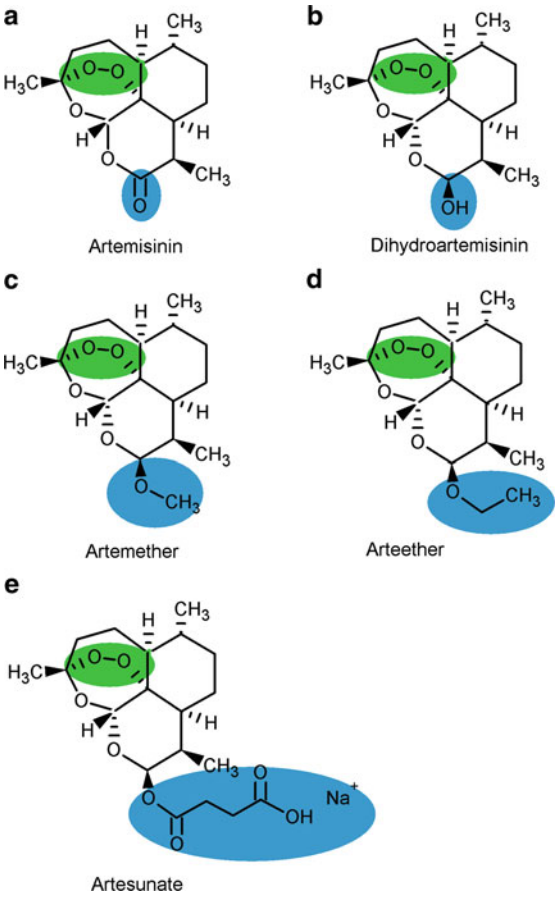


Fig. 1 Chemical structure of artemisinin (**a**) and its derivatives: dihydroartemisinin (**b**), artemether (**c**), arteether (**d**) and artesunate (**e**). The essential endoperoxide pharmacophore common to artemisinin and all its derivatives is highlighted in *green*. The C_{10} position radical that is unique to each individual derivative and that determines its water and lipid solubility (and therefore some of its pharmacokinetic properties) is highlighted in *blue*

Whilst the structure of this "backbone" varies considerably amongst artemisinin's various derivatives and synthetic analogues, all active compounds retain a distinctive endoperoxide moiety that is primarily responsible for the antimalarial activity of this drug class (Fig. 1). Artemisinin itself is poorly soluble in both water and oil, but soluble in many aprotic organic solvents. It has a melting point of 156–157°C and the molecule breaks down at temperatures above 190°C. It's molecular mass is 282.3 Da. It is unstable in the presence of both alkali and acid [46].

In the presence of sodium borohydride, a hydroreduction reaction transforms the lactone group of artemisinin to a lactol, whilst preserving the crucial endoperoxide moiety [46]. This produces dihydroartemisinin. Further semi-synthetic derivatisation produces ethers and esters of the lactol. In addition to artemisinin itself, and dihdyroartemisinin, the most-used derivatives include artemisinin's methyl ether, artemether, β-ethyl ether, arteether, and hemisuccinate ester, artesunate. These all share the same basic chemical structure of artemisinin (Fig. 1) with different chemical groups at the C_{10} (or 2-keto) position [47]. The C_{10} position determines the solubility of each

artemisinin derivative and influences its diffusion across mucosal membranes as well as other pharmacokinetic properties. Artesunate has significantly greater solubility in water than either artemisinin, dihydroartemisinin or artemether [48]. Second generation artemisinins (such as artemisone) are also under investigation.

3 Mechanism of Action

The antimalarial mechanism of action of artemisinin and its derivatives remains unclear. However a number of theories have been proposed that are currently the subject of intense debate. The complex biochemistry underpinning these theories is outlined extensively in a number of recent reviews [46, 49, 50]. Any understanding of the mechanisms of antimalarial activity of the artemisinins must reconcile each of the following considerations.

Firstly, like any good antimicrobial agent that is both safe and effective, the agent produces differential toxicity on pathogen in preference to host cells. Therefore, activity must exploit metabolic pathways or processes, which are either unique to, or preferentially expressed in the parasite. In the case of other antimalarials, this has often related to the food vacuole of the parasite and its role in detoxifying digested haem arising from breakdown of host-cell haemoglobin. The haem pathway has also been extensively investigated as a mode of action of artemisinin and is discussed below. *PfATP6* is an enzyme essential for oxidative metabolism in the parasite, which is absent from mammalian cells. Specific inhibition of this enzyme has been postulated as a mechanism of action and is also discussed below [19]. Much interest has centred on the relationship between artemisinins and free haem and iron that exist in concentrations within the parasite not seen in normal host cells. If these are responsible for "activating" artemisinin, they could explain the differential toxicity of drug in parasitic versus host mammalian cells.

Secondly, the structure–efficacy relationships of artemisinin and other endoperoxides now clearly implicate the endoperoxide moiety as the major determinant of efficacy. Any proposed pathway for the toxicity of artemisinins on plasmodia must incorporate the direct or indirect action of the endoperoxide moiety on a putative molecular target.

Thirdly, mechanistic hypotheses must also reconcile the now well-documented activity of artemisinins on a broad range of organisms and tumour cells [49]. These now include a variety of parasites including helminths (a wide range of trematodes including *Schistosoma*, *Clonorchis*, *Opisthorchis* and *Fasciola* spp.), non-plasmodial *Apicomplexa* (*Toxoplasma gondii*) and protozoa and metazoa (*Trypanosoma* and *Leishmania* spp.). In vitro, and in some cases, in vivo anti-viral activity has been demonstrated against HIV1, Influenza A, hepatitis B and C and human herpes viruses, including CMV where artemisinin therapy has been used with some success in human patients [51]. Some anti-fungal activity has been demonstrated in vitro for *Pneumocystis jirovecii* [49]. A very broad range of tumour cell lines have been shown to be susceptible in vivo and artemisinin is now being

examined as a clinical therapy in a variety of malignancies [49]. It has even shown activity in autoimmune conditions including rheumatoid arthritis and lupus nephritis [49]. All this suggests either multiple or broad and relatively non-specific mechanisms of action. However, it must be said that the sensitivity of other organisms to artemisinin is generally orders of magnitude less than that seen in plasmodia, where IC_{50} values are in the low nanomolar range.

Current concepts under debate include, firstly, the means by which artemisinin becomes biologically activated within the parasite, and secondly, the proposed molecular targets of activated artemisinin [52]. Bioactivation of artemisinins within parasitised erythrocytes is important in explaining the selective toxicity of the artemisinins on the parasite rather than on uninfected host cells and was first proposed by the Meshnick group to be triggered by iron (II) to generate toxic activated oxygen [53]. Subsequently, two opposing models, a "reductive scission model" [54, 55] and an "open peroxide model" [56] have been proposed to further explain this process. These two models differ by their dependency on iron and involvement of carbon-centred radicals [52].

As to the proposed molecular targets of artemisinin, a number of theories have been put forward, four of which are summarised below. These are by no means mutually exclusive and it seems likely that the anti-plasmodial activity of the artemisinins is a multi-faceted process [52].

3.1 Haem Pathway

This theory proposes that the free radicals generated following activation of artemisinin's endoperoxide then alkylate intracellular haem. The alkylated haem is then unable to undergo its usual detoxification by the parasite that ordinarily leads to the formation of the non-toxic crystalline haemozoin pigment. Supporting this theory, haem-drug adducts have clearly been demonstrated by mass spectrometry following exposure of parasites to both artemisinin and synthetic endoperoxides in vitro [57, 58]. However, others [59, 60] have demonstrated a lack of inhibition of haem polymerisation (and therefore of haemozoin formation) under carefully controlled in vitro conditions using stable artemisinin derivatives with good antimalarial activity. They have suggested that previously demonstrated haem polymerisation is an artefact of the instability of artemisinin and dihydroartemisinin in aqueous experimental conditions previously used. They argue that ring-opened products of artemisinin or dihydroartemisinin had been responsible for the haem binding seen previously. Adding to this argument are studies using rodent malaria models that have demonstrated a relative paucity of haem–artemisinin adducts in vitro [60]. In addition, the haem hypothesis fails to explain the toxicity of artemisinins for on other parasite species (such as *Toxoplasma* and *Babesia* spp.) that are not exposed to significant concentrations of haem in vivo.

3.2 Protein Alkylation

Free-radical generation within the parasite may also, through the formation of covalent bonds, alter the function of key parasite proteins involved in a variety of functions [18]. Suggested targets may include membrane transporters, proteases involved in haemoglobin degradation and a variety of other cellular enzymes.

3.3 Inhibition of PfATP6

Recently, specific inhibition of an enzyme, PfATP6, essential for oxidative metabolism in the parasite, was postulated as a mechanism of action [19]. This theory developed following interest in a compound called thapsigargin, which shares artemisinin's sesquiterpnene lactone structure. Thapsigargin is known to be a selective inhibitor of the mammalian sarco/endoplasmic reticulum membrane calcium ATPase (SERCA) that actively concentrates calcium within membrane-bound stores, thereby maintaining low cytosolic free calcium concentrations that are necessary to ensure cell survival. Artemisinin showed similar activity on mammalian SERCA as thapsigargin [19]. Because the PfATP6 of *P. falciparum* is the only enzyme orthologous to that of mammalian SERCA, it was hypothesised that this could be artemisinin's molecular target in the parasite. An elegant series of experiments showed firstly that both artemisinin and thapsigargin did, indeed inhibit PfATP6. Secondly, the mechanism appeared to be highly specific, without effects on non-SERCA Ca^{2+} ATPase or other malarial transporters. Thirdly, incubation of parasitised erythrocytes with the iron chelator, desferrioxamine, abolished the effect of artemisinin (but not thapsigargin), thus supporting the theory that bioactivation of artemisinin by iron was necessary for inhibition of the enzyme, and providing a rationale for the selective toxicity of artemisinin to parasitised cells. Studies of *T. gondii* added weight to the theory by demonstrating a similar effect on this organism's SERCA homologue [61]. A subsequent study suggested that a single amino acid residue could determine the sensitivity of SERCAs to artemisinin, thereby raising the possibility of a single mutation in genes encoding PfATP6 producing artemisinin resistance [62]. This mutation has also been examined by transfection of parasites with interesting results [63].

However, induction of stable parasite artemisinin-resistance in rodent malaria did not show mutations in the parasite's PfATP6 orthologue [39] or suggested alternative resistance pathways [64]. Studies of the synthetic endoperoxide OZ277 have shown that despite its highly potent antimalarial activity, and its competitive inhibition of artemisinin in vitro, it is a very weak inhibitor of PfATP6 [52]. Taken together, these all suggest that if PfATP6 is a molecular target of artemisinin, then it may be one of many through which endoperoxides act and through which parasite resistance could develop [65, 66]. Nevertheless, much more study is required to examine PfATP6 as a target and its mutations in contributing to resistance.

3.4 Mitochondrial Function

Studies of yeast have demonstrated an effect of artemisinin on mitochondrial membrane potential through effects on the electron transport chain [67]. Further studies have shown artemisinins to be distributed to malarial mitochondria, to directly impair mitochondrial function and to induce a strikingly rapid and dramatic production of reactive oxygen species within the mitochondria of both yeast and malaria but not mammalian cells, thereby suggesting a mechanism for selective toxicity [68]. Mitochondrial toxicity demonstrated in these studies appears to be dependent on the peroxide moiety, with derivatives such as deoxyartemisinin (which lacks an endoperoxide bridge) failing to demonstrate production of reactive oxygen species or functional toxicity in mitochondria. Further evidence of the importance of this pathway has been shown by the amelioration of artemisinin's antimalarial toxicity through interference of the mitochondrial electron transport chain, including by use of the iron chelator, desferrioxamine [68]. However, studies in transgenic parasites (see AB Vaiya, Antimalarials targeting mitochondrial functions) do not support the idea that artemisinins kill parasites by a mitochondrial-based mechanism.

4 Resistance

In theory, the artemisinins should be relatively protected from the development of resistance by their extremely short half-lives [7, 69, 70]. This reduces the overall exposure of parasite populations to low or intermediate residual drug concentrations following administration of a conventional treatment course. Historically, this seems to have been important in providing the selection pressure that has driven rapid development of resistance to longer-acting drugs such as mefloquine and sulphadoxine–pyrimethamine [69, 71]. Rapid development of high-level resistance can also occur when a single mutation affects a single enzymatic pathway critical to the drug's activation or mechanism of action. This is the case with the drug atovaquone, which is rendered ineffective by a single base-pair mutation of the gene encoding the *cyt b* enzyme [72]. Therefore, artemisinin resistance has not been ruled out and the implications of widespread artemisinin resistance have been seen as so potentially disastrous that it has long been at the forefront of debate and policy considerations by the WHO and others [28, 37, 38]. It has underpinned calls for the use of artemisinin to be restricted to combination therapies [27], leading to the WHO's recommendation that artemisinin monopreparations for oral treatment be phased out altogether in favour of co-formulated ACTs [42]. Part of the rationale for ACT relies on the supposition that parasite mutants that spontaneously arise with resistance to one drug will still be susceptible to the second, and therefore will not survive to pass on their resistance characteristics to the next generation [71, 73]. A similar approach underlies combination treatment of tuberculosis, HIV, Hepatitis B infections and

most cancers. However, in the case of malaria, the rationale for combination therapy is subject to a number of caveats that mean that ACT should not be regarded as a panacea [29]. In particular, the relatively complex genome of plasmodia (*P. falciparum*'s genome is 2.3×10^7 bp) gives it the advantage of greater number of possible genetic loci that could mediate resistance, when compared with simpler organisms such as *M. tuberculosis* (genome 4.4×10^6 bp) or viruses (3.2×10^3 bp for Hepatitis B). This has also led to the development of more complex drug resistance mechanisms than those seen in bacteria and viruses. For example, mutations affecting drug transport mechanisms can simultaneously lead to resistance to a range of drugs with different molecular targets and modes of action. In *P. falciparum*, the best example is the *P. falciparum* multidrug resistance gene (*Pfmdr1*) that encodes the P-glycoprotein homologue (*Pgh*), involved in drug efflux into the parasite's food vacuole [74, 75]. Resistance can be mediated either by single nucleotide polymorphisms or by increases in gene copy number and *Pfmdr1* has been implicated in resistance to a range of diverse antimalarials including chloroquine, amodiaquine, mefloquine, halofantrine and lumefantrine [76, 77]. The rationale for ACT may also be undermined by the possibility of parasites being "protected" from the activity of one or other drug in a combination either due to sequestration in sites exposed to lower drug concentrations or to drug exposure occurring during non-susceptible phases of the parasite's life cycle [29]. Also, whilst the use of a (conventionally, more longer-acting) partner drug may "protect" the shorter-acting artemisinin component from the development of resistance, it seems less likely that the converse will be true. One study has already demonstrated the unabated spread of sulphadoxine and pyrimethamine resistance despite the introduction of an artesunate plus sulphadoxine–pyrimethamine ACT [78].

A number of groups have been able to select for parasite resistance in rodent malaria in vivo and more recently in *P. falciparum* in vitro [36, 39, 40, 64, 79] adding to concerns regarding the potential for development of resistance. In a study by Peters and Robinson reported in 1999 moderate artemisinin resistance (a 4- to 27-fold difference in sensitivity compared with sensitive strains) was selected in *P. berghei* and *P. yoelli* following sustained selection pressure in a rodent model. However this rapidly resolved with the removal of drug-selection pressure [36]. No specific mechanism for resistance could be identified based on evaluation of putative molecular targets being considered around this time, however resistant strains were shown to accumulate 43% less (radiolabeled) drug in vitro than sensitive strains [40]. The induction of stable artemisinin resistance was not reported until 2006 in a study by Afonso et al. of a *P. chabaudi* rodent model [39]. Strains with 6- to 15-fold increased resistance to artemisinin and artesunate were induced through exposure to increasing drug concentrations during several consecutive passages in mice. Resistance remained stable after cloning, freeze thawing, transmission to mosquitoes and following removal of drug pressure. Nucleotide sequences and gene copy number of putative genetic determinants of resistance (including orthologues of the *P. falciparum* genes, *pfmdr1, pfcg10, pftctp and pfatp6*) were compared between resistant and sensitive strains but failed to demonstrate any differences. Subsequent genetic linkage analysis identified two genetic mutations in a locus encoding a deubiquinating enzyme (UBP-1) [80]. In addition to artesunate, the same mutations

could also be induced independently under drug selection using chloroquine, thereby demonstrating a mechanism for cross-resistance to structurally unrelated antimalarials arising as a single event. However, examination of *P. falciparum* isolates with reduced artemisinin sensitivity failed to identify mutations in the orthologous *P. falciparum* locus. More recently, an artemisinin-tolerant strain of *P. falciparum* has been developed through sustained in vitro drug pressure [64]. Morphologic evaluation revealed a sub-population of ring-form parasites surviving in maturation arrest. Observed transcriptomic modifications included down-regulation of a cell-cycle regulator and DNA biosynthesis protein. Taken together, the authors argued that artemisinin tolerance might be mediated by a quiescence mechanism in *P. falciparum*. A further study by Chavchich et al. induced stable resistance to artemisinin and artelinic acid in three laboratory reference strains of *P. falciparum* [79]. Importantly, this was also associated with decreased susceptibility to mefloquine, quinine, halofantrine and lumefantrine. Again, no mutations were detected in putative genetic determinants of artemisinin resistance (including *pfmdr1*, *pfcrt*, *pfatp6*, *pfctp*, and *pfubcth* genes) or in expression of *pfatp6* or *pftctp*. However, in contrast to the rodent model data from Afonso et al., this study clearly demonstrated increases in *pfmdr1* gene copy number as well as its mRNA and protein expression, suggesting a mechanism for cross-resistance to mefloquine and other drugs. By demonstrating a possible resistance mechanism common to artemisinin and other drug classes, it raises some concerns regarding the underlying rationale for ACT, particularly when drugs such as mefloquine and lumefantrine are used as the partner drug [29]. Together, these studies have provided a proof of principle that induction of stable artemisinin resistance is possible and, even more importantly, that this could arise through more than one mechanism, including some that may simultaneously mediate increased resistance to other structurally unrelated drugs (including ACT partner drugs).

Monitoring of clinical isolates for artemisinin sensitivity using in vitro drug susceptibility assays has methodological challenges due to difficulties in standardisation and, until very recently, the lack of confirmed in vivo resistance to enable clinical correlation. However, a study by Jambou et al. reported in 2005, using a WHO microtest, demonstrated a very wide variability in artemether and artesunate IC_{50} values from 530 clinical isolates from Senegal, French Guiana and Cambodia [41]. Whereas IC_{50} values had previously been reported in a range <30 nmol/l, values as high as 117 nmol/l were reported from French Guiana. Parasites from French Guiana also showed correlation in artemether and mefloquine IC_{50} values. Importantly, polymorphisms of the coding sequences of *pfatp6* were identified and one of these, a S769N mutation, was highly significantly associated with higher IC_{50} values from isolates from French Guiana. Again, analysis of *pftctp, pfmdr1* and *pfcrt* failed to suggest a role for these, although copy number was not assessed. Originally, the S769N mutation was only found in French Guiana (an area of South America with little preceding exposure to artemisinin derivatives) and was not found in a broad range of isolates from Africa, South America and Southeast Asia [81]. Recently, travellers returning to Canada from many African countries with malaria have provided parasites with mutations in *pfatp6* (A623E; S769N). Some of these have significant increases in IC_{50} values

for the effect of artesunate and other derivatives on in vitro parasite growth, and interestingly there may be an interaction between these mutations and mutations in *pfmdr1* [82]. Overall, there was a high level of polymorphic diversity of the *pfatp6* gene throughout these regions though the nature of this could not be explained by genetic drift alone [83]. Given the lack of preceding drug pressure in French Guiana, alternative evolutionary mechanisms are being considered, including host factors, such as endemic haemoglobinopathies that alter intra-erythrocytic calcium homeostasis in a manner that higher cytosolic calcium concentrations might select for *pfatp6* mutants better able to regulate parasite calcium concentrations [81].

The most significant event in the recent history of the artemisinins is the first descriptions of clinical artemisinin resistance, from Western Cambodia from 2008 onwards [43, 44]. An initial report by Noedl et al. prospectively evaluated a 7-day course of artesunate monotherapy in adults with *P. falciparum* in Battambang province of Cambodia. A definition of "resistance" was used that included persistence of parasites 7 days after treatment commencement or re-emergence within 28 days in the presence of acceptable concentrations of dihdyroartemisinin, prolonged time to parasite clearance and reduced in vitro susceptibility to dihydroartemisinin. Of 60 subjects enrolled in the artesunate arm of the study, two met the criteria for artemisinin resistance. IC_{50} values for these two patients were 4 times the mean value for cured patients and 10 times that of a reference strain.

A subsequent study by Dondorp et al. prospectively evaluated the in vivo efficacy of 7-day artesunate monotherapy and of combined artesunate and mefloquine, this time comparing response in 40 patients from each of two locations: a site in Western Cambodia where concerns regarding resistance had emerged and a "control" site in Northeastern Thailand [43]. Significantly longer parasite clearance times were seen at the Cambodian site (84 h vs. 46 h). Although a higher rate of treatment failure (recrudescence) was also seen in the Cambodian patients (total 7/40), this was not statistically significantly higher than at the other site (3/40). When interpreting these data, it is important to consider that parasite clearance rates may reflect not only intrinsic parasite susceptibility to the drug, but numerous host factors, particularly those related to the degree of pre-existing malaria-specific immunity. Because of the study design used, the two groups were not necessarily matched for important factors (such as age, sex, parasite density, disease severity and the malaria transmission intensities that each population was subjected to). These factors could easily have become confounding variables in the analysis. However, the degree of difference was striking and the parasite clearance times documented from the Cambodian site were longer than any described previously worldwide. Moreover, this area has historically seen the early emergence of drug resistance to a number of agents [84]. The emergence of artemisinin resistance here becomes especially plausible when it is considered that artemisinin treatments have probably been used here longer than anywhere else in the world, having been employed since the time of the Khmer Rouge in the late 1970s. Much of this has been poorly regulated monotherapy with, quite possibly, sub-standard drugs [43, 85]. Also, subsequent studies of parasites from the patients studied by Dondorp et al. in Western Cambodia have now suggested that much of the variability in parasite clearance rates can be attributed

to parasite genetic co-lineage, suggesting heritable (and therefore transmissible) parasites, rather than host factors in explaining this phenomenon [86]. However, specific genetic loci associated with delayed parasite clearance are yet to be determined and host genotypes may also need assessment.

Given concerns regarding the apparent development of early artemisinin resistance in Cambodia, a massive effort by the WHO with financial assistance from the Bill & Melinda Gates Foundation is now underway to monitor, control and limit its spread [43, 45]. Strategies will include improved surveillance, enhanced case management (early diagnosis and appropriate treatment), reducing drug pressure (by preventing inappropriate empirical therapy), targeting of mobile populations and optimising vector control and bed-net coverage, with the ultimate aim of effectively eradicating falciparum malaria from this region [43, 87]. A further development of concern in the region has been the development of resistance to commonly used ACT partner drugs, with unacceptably high treatment failure rates emerging for the artesunate–mefloquine combination [88].

5 Basic Clinical Pharmacology of the Artemisinin Derivatives

Artemisinin and its derivatives now exist in a myriad of pharmaceutical products that can be administered either orally (either as mono- or co-formulations), rectally or parenterally. The most widely used in clinical practise include products containing artemisinin itself, dihydroartemisinin, artemether and artesunate. The pharmacology of these four drugs are summarised in Table 1. All derivatives are likely to share a common mechanism of action due to the ubiquitous endoperoxide moiety. There is probably little to separate them in terms of intrinsic efficacy, as IC_{50} values exist in a similar range for each drug [91]. What is more, artesunate and, to a lesser extent artemether, are essentially pro-drugs of dihydroartemisinin. However, each derivative has different physicochemical properties due to the nature of their unique C_{10} (or 2-keto) position radical (Fig. 1). This influences the drug's solubility in oil and water and therefore the molecule's movement across biological membranes. This has important implications for pharmacokinetic properties that may impact on therapeutic response. These are discussed in more detail below.

5.1 Pharmacokinetics

5.1.1 Absorption and Bioavailability

Artemisinin, dihydroartemisinin, artemether and artesunate can all be administered orally or rectally. Artemisinin, artemether and artesunate can also be given by intramuscular injection. However artesunate is the only drug that can be

Table 1 Pharmacokinetic properties of the commonly used artemisinin derivatives

	Elimination half-life	T_{max} – oral administration	T_{max} – intramuscular injection	T_{max} – rectal (suppository) administration
Artemisinin (MW = 282.35)	2.9 h [7]	1–3 h [7]	3.4 h [7]	5.6 h [20]
Dihydroartemisinin (MW = 284.4)	40 min [7]	0.9–1.6 h [7]	NA	4 h [20]
Artemether (MW = 298.4)	3.6 h [7]	1.7–6 h [7]	1.3–8.7 h [7]	3.1 h [20]
Arteether (MW = 312.4)	12.4–30.2 h [89, 90]	NA	4.8–7.0 h [89, 90]	NA
Artesunate (MW = 384.4)	2–5 min [91–93]	15–39 min [93]	12 min [91]	1.6–3 h [20]

T_{max} time to maximal concentration, *MW* molecular weight

administered intravenously [7]. The lack of an intravenous preparation for the other drugs makes it difficult to calculate absolute bioavailability when they are administered by other routes. However oral bioavailability of artesunate has been calculated at between 61 and 88% [94, 95] and is likely to be greater than the less water soluble drugs [91, 96, 97]. Artesunate is clearly the most rapidly absorbed with time to maximal concentration (T_{max}) considerably shorter than those of the other derivatives (Table 1) [7].

Solubility of the different drugs is also likely to influence both bioavailability and absorption profile after rectal administration. All products show a wide degree of inter-individual variability in the rapidity and extent of absorption [20]. However, artesunate is the most rapidly absorbed and achieves the highest maximal drug concentrations following conventional dosing (Table 1) [20].

There are fewer options for parenteral administration of artemisinins. Because of stability issues, artesunate is available as a powder for reconstitution in sodium bicarbonate immediately prior to intravenous injection. However, intravenous administration is more costly and requires equipment and skilled staff often not available in resource-poor settings. Intramuscular injection is likely to be both more practical and have a comparable pharmacokinetic profile, achieving maximal concentrations within 12 min [91]. Artemether has been used widely as a preparation for intramuscular injection [98]. Because of its very poor water solubility it is available in ampoules containing the drug dissolved in peanut oil. It suffers from poor and erratic absorption from the intramuscular injection site. T_{max} values range from 1.3 to 8.7 h [7] and studies from children with severe malaria indicate a wide inter-individual variability in the extent of absorption, such that many children fail to demonstrate detectable concentrations of drug in plasma following intramuscular injection [99]. Its pharmacokinetic profile therefore seems to be inferior to that of artesunate when administered as an intramuscular injection for severe malaria. Arteether and artemisinin have also been administered by intramuscular injection, though much less extensively than artemether. Although data are limited,

pharmacokinetic properties of these preparations are similar to those of artemether (Table 1) [7, 89, 90].

5.1.2 Metabolism and Elimination

All artemisinin derivatives are characterised by their extremely rapid elimination from plasma, with elimination half-lives that are mostly less than a few hours (Table 1) [7]. Both artemether and artesunate are metabolised to dihydroartemisinin by rapid esteratic hydrolysis of artesunate or slower cytochrome P450-mediated demethylation of artemether. Dihydroartemisinin itself and artemisinin are probably metabolised in the liver to inactive metabolites (Table 1) [7].

The pharmacokinetics of artemisinin derivatives do not appear to be influenced significantly by severity of infection [92] and dose adjustment in patients with hepatic or renal impairment is not required. However auto-induction of metabolism of artemisinin has been observed that results in a 5.5-fold increase in oral clearance following 10 days of treatment [100]. This effect appears to be largely attributable to induction of CYP2B6 (a member of the cytochrome P450 enzyme family) and is specific to artemisinin itself rather than to its derivatives.

5.1.3 Pharmacokinetic–Pharmacodynamic Relationships

A clear understanding of the manner by which pharmacokinetic parameters impact on clinical response is lacking for this drug class. Clearly, the rate of absorption (measured by T_{max} or absorption half-life) is important, particularly in severe malaria, where it is important rapidly to achieve parasiticidal drug concentrations. For other antimicrobials, including other classes of antimalarial agents, time-above mean inhibitory concentration (MIC) and area under curve (AUC) relative to pathogen MIC may be the important determinants of efficacy [101, 102]. However, given the excellent efficacy of a very rapidly eliminated drug like artesunate, it seems unlikely that either of these are important determinants of efficacy of artemisinin derivatives. It seems more likely that maximal concentration (C_{max}) is important. Indeed there is some evidence that for artemisinin, C_{max} correlates with parasite clearance time, which is probably the best available surrogate marker for clinical response [103]. Studies of other derivatives, including artesunate have also suggested relationships between absolute dose and parasite clearance time [104]. Whilst maximal concentration may be the important determinant of efficacy, it seems that AUC is likely to be the principal determinant of neurological toxicity (see Sect. 5.3) [105–107]. Therefore, in terms of optimising both efficacy and safety, the ideal pharmacokinetic profile would be one that rapidly achieves high drug concentrations but also sees the rapid elimination of the drug (thereby minimising the AUC). Artesunate appears, therefore, to have a more favourable profile than the other derivatives in this regard, whether administered orally, rectally or by injection.

5.2 Antimalarial Activity

Each of the artemisinin derivatives is active at nanomolar concentrations against asexual blood forms of all the plasmodia that infect humans. The in vitro *P. falciparum* IC_{50} value is similar for all four main artemisinin drugs [7], and is a number of orders of magnitude less than plasma drug concentrations achieved through routine therapeutic administration. The initial reduction in parasitaemia is the most rapid of all available antimalarial drugs, and seems to be a consistent feature, regardless of the derivative or dosing regimen used (Fig. 2) [21, 22, 109]. The parasite reduction ratio (PRR) has been estimated at 4 $\log_{10}$ per 48 h erythrocytic cycle [110], meaning that parasite density characteristically falls by approximately 90% of baseline within the first 12 h (a 1 $\log_{10}$ reduction), by 99% in the first 24 h (2 $\log_{10}$ reduction) and by 99.99% by 48 h [20, 108, 111, 112]. Most clinical infections with *P. falciparum* are thought to represent an initial total body parasite burden of the order of 10^{12} total parasites prior to treatment being commenced [110]. Therefore following artemisinin treatment, a 4 $\log_{10}$ reduction over 48 h will result in a total parasite burden in the region of 10^8 parasites. This is often below the pyrogenic threshold and below the level of detection of peripheral blood parasitaemia using conventional microscopy. Therefore, total fever and parasite clearance times are often less than 48 h following treatment with an artemisinin or derivative-containing regimen [20, 108, 111, 112]. Although the patient may be feeling well and no longer have detectable parasitaemia by this time, if the residual parasite burden is 10^8, assuming an ongoing 4 $\log^{10}$ PRR, another 96 h (at least two 48-h erythrocytic parasite life cycles) will be required before the entire parasite burden has been eradicated and definitive cure ensured. Presumably this is why that, given the very short half-lives of artemisinin and its derivatives in blood, short courses (3–5 days) are associated with recrudescence rates that are typically greater than 25% [22, 109, 113].

Given that artemisinins have very short half-lives, and that parasite clearance persists for many hours after drug is no longer detectable in the circulation, it is likely that the equivalent of a "post-antibiotic effect" exists, where there is persistent suppression of microbial growth following limited exposure to an antimicrobial agent. Artemisinins are also active against some stages of the sexual blood form of the parasites (stage I and II gametocytes) and therefore may have the potential to reduce transmission rates (Fig. 2b) [114]. They do not have significant activity against exoerythrocytic (hepatic) stages, such as the dormant hypnozoite forms in *P. vivax*, and therefore have no ability to prevent *P. vivax* relapse.

5.3 Safety and Tolerability

The artemisinin derivatives appear to have a wide therapeutic margin, being generally well-tolerated at standard therapeutic doses in humans [21, 22, 109, 115].

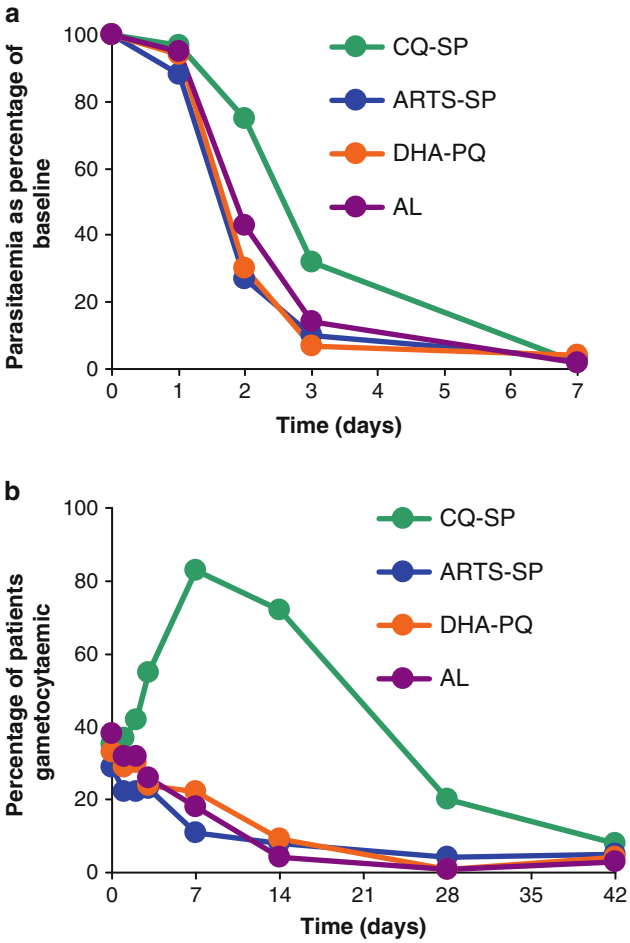


Fig. 2 Comparison of *Plasmodium falciparum* parasite clearance rates in children treated with a non-artemisinin derivative-containing regimen, chloroquine + sulphadoxine/pyrimethamine (CQ: SP) versus three artemisinin-based regimens: artesunate + sulphadoxine–pyrimethamine (ARTS–SP), dihydroartemisinin–piperaquine (DHA–PQ) and artemether–lumefantrine (AL). (**a**) trophozoite (ring forms) and (**b**) gametocytes. Data are from the study by Karunajeewa et al. of Papua New Guinean children with uncomplicated malaria [108]

Reported adverse effects have included nausea, vomiting, bowel disturbance, abdominal pain, headache and dizziness, symptoms that can result from malaria infection itself and are usually mild and self-limiting [115]. Mild and reversible haematological abnormalities, are observed infrequently with routine use at conventional dosages (e.g., artesunate 2–4 mg/kg/day) [115]. However, higher than usual therapeutic doses administered in long courses (artesunate 6 mg/kg/day for 7 days) demonstrated a high (19%) rate of neutropenia, suggesting there is an upper limit to safe dosing [116]. Minor electrocardiographic changes described [115] may reflect malarial disease

itself rather than drug action and are almost certainly not clinically relevant. Animal toxicity studies using these compounds at supra-therapeutic doses in rats and dogs demonstrated a characteristic fatal neurotoxicity affecting predominantly the brainstem of animals receiving high doses over long periods [117, 118]. This toxicity now appears to be dose-related and to be restricted to compounds and dosing regimens that result in sustained high plasma concentrations of the primary drug or its active metabolite dihydroartemisinin [105]. Therefore, it is likely that the depot-like slow release of lipophilic artemisinin derivatives when administered by intra-muscular injection facilitates this toxicity [106]. This theory is supported by data showing that mice fed comparable oral doses of more rapidly cleared water soluble artemisinin derivatives do not develop neurotoxicity [107]. Although somewhat controversial, there is little convincing evidence to suggest neurotoxicity has ever manifested in human subjects. Neurological symptoms of ataxia, slurred speech and hearing loss have been reported in a small numbers of humans treated with artemisinin derivatives [119, 120]. Reports that have attempted to ascribe these to side-effects of artemisinin derivatives (rather than as manifestations of malarial disease itself) have been re-examined in subsequent prospective studies that have failed to substantiate any causal associations [121, 122]. The limited penetration of artemisinin derivatives into the cerebrospinal fluid also makes the possibility of neurotoxicity at therapeutic doses less likely [123]. Nonetheless, some lingering concerns still exist that neurotoxicity may manifest in certain vulnerable individuals, including children, for whom the developing central nervous system may be at greater risk [124]. Ongoing safety monitoring and attention to pharmacokinetic disposition are warranted in order that optimally safe and effective regimens can be developed, particularly if higher dose regimens are to be considered in the face of emerging artemisinin resistance.

A large body of data from animal models (including rats, rabbits and monkeys) now raises significant concerns about the reproductive toxicity of artemisinins, demonstrating embryonic death, foetal resorption, limb dysgenesis and cardiac defects following treatment with therapeutic doses in the first trimester [125]. By contrast, birth outcomes have now been documented in >500 women exposed to artemisinins during their pregnancy, and adverse events have not been reported [126]. Nonetheless, given the strength of the animal data, artemisinin derivatives are considered contraindicated in the first trimester of pregnancy [125].

6 Clinical Uses

The great clinical utility of the artemisinin derivatives owes much to their advantages of extremely rapid parasiticidal activity, overall excellent safety and tolerability, and at the current time, the lack of widespread parasite resistance. However given the reproductive toxicity data from animal studies, their use in pregnancy is limited (see above), at least for the time-being, pending further data. Also, because of their extremely short half-lives, they are not well-suited to use as

prophylaxis (although they have had some use in intermittent presumptive treatment strategies that have utilised ACTs). Their major clinical applications, therefore, are for community/outpatient-based treatment of uncomplicated malaria, parenteral treatment of severe malaria and pre-referral rectal administration of suppositories in presumptive malaria or after confirmation of diagnosis and before definitive treatments can be used. Their use as they apply to each of these three major clinical scenarios is summarised below.

6.1 Treatment of Uncomplicated Malaria

Artemisinin drugs have quickly attained popularity over conventional antimalarials for the treatment of uncomplicated malaria for a number of reasons. Patients and prescribers recognise that they are relatively free from the unpleasant side effects that compromise drugs like quinine, chloroquine and mefloquine. They also appreciate the more rapid relief of symptoms of malaria when compared with other drugs [127]. This is supported by clinical data that consistently demonstrate more rapid fever clearance and parasite clearance rates when compared with conventional therapy (Fig. 2a), with little evidence to separate one derivative from another [22, 108]. However, from very early on in their clinical development it was recognised that although initial cure rates were almost invariably close to 100%, courses of artemisinin-derivative monotherapy $\leq$5 days were associated with recrudescence rates typically greater than 25% [22, 109]. This likely reflects the very short plasma half-lives of artemisinin derivatives coupled with the need for parasiticidal drug concentrations throughout a number of parasite erythrocytic life cycles (each lasting 48 h) to eliminate the entire body parasite burden and thereby produce definitive cure. For long half-life drugs such as chloroquine and mefloquine, this is feasible with short-course therapy because a long "tail" of persisting drug is present for many days after the last dose, and this is sufficient to kill any remaining parasites. This issue can be dealt with by employing courses of monotherapy of at least 5–7 days duration [128]. Prior to the recent development of resistance in Cambodia, 7 day regimens had been described as having cure rates as high as >95% [22]. However, in the developing world, such long courses are likely to be compromised by poor compliance, particularly as defervescence usually occurs within 48 h of artemisinin treatment commencement, diminishing the incentive for the patient to complete the full course.

In addition to concerns regarding the potential for development of parasite resistance, the logistical problems associated with long-course artemisinin monotherapy have added to the rationale for ACT. The artemisinin component provides rapid early reduction in parasite burden and symptom relief for the patient. The longer half-life partner drug achieves parasite killing many days after the last dose has been taken, thereby eliminating the parasite residuum and enabling the feasibility of short-course therapy with high definitive cure rates. Given recent WHO policy directives, it is hoped that in the near future, artemisinin monotherapy

will no longer be used widely and that most artemisinin-based therapy will be in the form of short-course ACT.

A large number of clinical trials have been conducted in recent years evaluating the clinical efficacy of various ACTs [24]. It is likely, however, that rather than the artemisinin component, it is the choice of partner drug that is most crucial in determining the combination's effectiveness [108, 129]. Pre-existing resistance to partner drugs in the area where the combination is used may be especially important [108, 130]. Issues of cost, tolerability/toxicity and dosing convenience must also be considered [31]. The prototypical ACT is the "loose" combination of separate tablets of artesunate and mefloquine first used in Thailand and subsequently adopted as first-line treatment policy for uncomplicated *P. falciparum* infection in much of Southeast Asia [30]. Other "loose" ACTs have included sulphadoxine–-pyrimethamine, amodiaquine and atovaquone–proguanil as partner drugs [31]. However, "fixed" co-formulations containing both the artemisinin derivative and the partner drug have overwhelming operational advantages by preventing inadvertent monotherapy. They have been the focus of ACT development in recent years (Table 2). The most widely used co-formulation, and the first to be endorsed by the WHO, is the combination artemether–lumefantrine. This has consistently demonstrated 28–42 day cure rates >95% when administered as a twice daily regimen for 3 days [131]. However, it has some potential problems that may affect its operational deployment, including the need to be administered with food or drink containing some fat in order to ensure adequate absorption [132] and a relatively complex twice-daily regimen that may compromise compliance. It has now been adopted as first-line treatment policy for laboratory-confirmed uncomplicated *P. falciparum* malaria by at least 46 countries, mostly in Africa and Asia [1]. Importantly, a suspension preparation suitable for administration to infants is also now available [133].

At the time of writing, artesunate–amodiaquine had achieved widespread use in Africa, having been adopted as treatment policy in at least 20 African countries [1]. It is now available as a co-formulation, conveniently administered as three daily doses. In many parts of the world, particularly where amodiaquine has been widely used, the efficacy of this combination is likely to be compromised by pre-existing resistance to amodiaquine. For this reason, it has not achieved widespread use outside Africa [1].

Other promising combinations in various stages of development have utilised piperaquine, mefloquine, amodiaquine, fosmidomycin, chloroproguanil–dapsone, pyronaridine and naphthoquine as partner drugs [31]. A large body of clinical trial data has been accrued to support the dihydroartemisinin–piperaquine combination that is available at an attractive price and has mostly shown excellent efficacy and tolerability, when given as three daily doses. [24, 134] It is used as first-line treatment in Vietnam, China, Myanmar and parts of Indonesia. [1] However, the hurdles to be overcome to achieve regulatory approval have delayed WHO endorsement of this and other preparations manufactured in China or elsewhere in the developing world.

Table 2 Artemisinin derivatives available as co-formulations

	Partner drugs used	Manufacturer(s) and product proprietary-name
Artemisinin	Piperaquine	Artepharm (Artequick®)
	Naphthoquine	Kunming pharmaceuticals (Arco®)
Dihydroartemisinin	Piperaquine	Beijing Holley-Cotec (Duocotecxin®) Sigma-Tau (Eurartekin®) GPC ShenZhen (Combimal®) GVS labs (P-Alaxin®)
Artemether	Lumefantrine	Novartis Pharma (Coartem® and Riamet®)[a] Addis (Artemine®) Ajanta (Artefan®) Medinomics (Fantem®) Cipla (Lumartem® and Lumet®)
Artesunate	Amodiaquine	Sanofi Aventis (Coarsucam®)[a] Guilin Pharmaceuticals (Co-Artusan®)[a] Medinomics (MalmedFD®)
	Mefloquine	Mepha (Artequin®)
	Pyronaridine	Sin Phoong (Pyramax®)

[a]At the time of writing, only these three products (Coartem®, Coarsucam® and Co-artusan®) had achieved WHO pre-qualification

6.2 *Parenteral Treatment of Severe Malaria*

Prior to the availability of the artemisinin derivatives, the only options for parenteral treatment of severe malaria in most of the world had been quinine (or quinidine), administered either by intravenous infusion or intramuscular injection [135], chloroquine (administered by intramuscular or subcutaneous injection) [136] or amodiaquine (given intravenously) [137]. However, whilst clearly highly efficacious in the treatment of chloroquine-sensitive *P. falciparum* malaria, parenteral chloroquine and amodiaquine have been rendered obsolete in most malaria-endemic regions of the world due to the development of widespread 4-aminoquinoline resistance. Therefore, for practical purposes, this leaves quinine as the only viable therapy for severe malaria in most parts of the world.

The possibility of using artemisinin derivatives to treat severe malaria has obvious theoretical advantages over conventional quinine therapy, including faster clearance of trophozoites [21, 22] and a lack of serious toxicity issues (including hypoglycaemia, "blackwater fever," haemodynamic instability, cardiac arrhythmias and tinnitus) that may complicate quinine therapy [115, 138–142]. Artemisinin was first used to treat severe malaria in China as early as 1972, but widespread use for severe malaria would require manufacture of a derivative in a form that was both stable and suitable for injection into patients who were either unconscious or too ill to take medicine orally. Initial interest centred on the oil-based preparations of arteether and artemether that could be administered by intramuscular injection. Numerous trials evaluated their use in severe malaria when compared with parenteral quinine therapy [21]. These consistently demonstrated superiority with regard to the surrogate efficacy endpoints of parasite clearance and fever clearance with artemether.

However, all studies were inadequately powered to demonstrate improvements in the clinically important endpoints of mortality or serious disability. A meta-analysis of data from studies comparing artemether with quinine did show an overall lower mortality in artemether-treated patients but the difference did not reach statistical significance [98]. A number of countries have adopted intramuscular artemether as the standard recommended treatment for severe malaria[1]. However, most early studies investigating the use of artemether for treatment of severe malaria [98] were conducted at a time before the development of robust drug assays and therefore, pharmacokinetic data were limited [97, 99, 143]. It has since become clear that both artemether and arteether have poor and highly variable absorption into the systemic circulation from the intramuscular injection site, raising concerns that a proportion of patients treated might be at risk of sub-optimal therapeutic response [144]. The pharmacokinetic profile of the water-soluble artemisinins appears to be more favourable for treatments of severe malaria. In contrast to artemether, both intravenous and intramuscular injections of artesunate result in consistent and rapid absorption, with peak levels occurring within minutes of administration [92]. The depot effect seen with artemether does not occur so that the drug's apparent plasma elimination half-lives are not distorted by absorption from the injection site. All of these suggested artesunate as a more promising replacement for parenteral quinine.

In 2005, a large multi-centre comparison of parenteral quinine with intravenous artesunate, the South-East Asian Quinine Artesunate (SEAQUAMAT) trial, was published [25]. This showed a clear mortality benefit (relative risk reduction of 35%, absolute risk reduction of 7%, number needed to treat 14) for artesunate over quinine. Because the majority of participants in this study were Southeast Asian non-immune adults, it was not immediately clear whether this mortality benefit could be extrapolated to children living in malaria-endemic areas of the world such as Africa. However, a more recent large multi-centre study of African children with severe malaria, [26] has also demonstrated a clear, although somewhat smaller mortality benefit (relative risk reduction 22.5%, absolute risk reduction 1.4%, number needed to treat 71) Parenteral artesunate is now, therefore, widely considered as the treatment of choice for severe malaria, in both children and adults regardless of context, with the only possible exception being women in the first trimester of pregnancy [2].

At the present time, it is not clear whether or to what extent early reports of delayed parasite clearance following artemisinin treatment in Cambodia will affect the efficacy of artemisinins for treatment of severe malaria. However, for the time being parenteral administration of artesunate should be considered the gold standard by which other treatments for severe malaria will be judged.

6.3 Rectal Administration

Because the majority of deaths from *P. falciparum* infection occur in children living in remote rural areas with limited access to medical care, a rapidly acting

antimalarial drug in suppository form has great potential value [145, 146]. In contrast to oral therapy, rectal administration is not precluded by vomiting, prostration or impaired consciousness, all common features of severe malaria. Although parenteral therapy can also be used when oral dosing is problematic, equipment and trained personnel necessary to safely administer injections are often unavailable in these areas. An effective rectally administered treatment could mean that those too sick to take medication orally could be treated in the community, either as emergency "pre-referral" treatment (before transportation to a health facility for higher-level treatment) or, if referral is not possible, as part of a complete treatment course given at home [28, 38]. It may be feasible for suppository administration and subsequent monitoring for extrusion to be performed by a village health worker, or even by a sick child's mother, with minimal training. Because the artemisinin drugs have an excellent safety profile and a reasonably wide therapeutic margin, rectal administration could be safely and effectively performed by individuals with little or no medical training.

Artemisinin derivatives that have been formulated for rectal administration have included artemether, artemisinin, dihydroartemisinin and artesunate (Table 1). At present, most data accrued regarding rectally administered artemisinin derivatives comes from studies of artesunate suppositories, which of all the rectally administered artemisinins, appear to have the most favourable pharmacokinetic properties based on existing data [20]. These have been produced by two manufacturers and are usually formulated as 50 mg or 200 mg suppositories (Plasmotrim Rectocaps®, Mepha Pharmaceuticals, Aesch-Basel, Switzerland; Scanpharma A/E Denmark).

Rectally administered artemisinins are likely to have their greatest value in critically ill patients residing in geographically remote settings in whom definitive therapy may be many hours or days away. Therefore, the primary imperative is for early and rapid parasite clearance. This should prevent or delay the progression of microvascular parasite sequestration and the consequent metabolic derangements that can lead to death,[147] and buy time while the patient is being transported to a health facility. For this reason, the most useful indicators of clinical efficacy are those that describe the rapidity of parasite clearance, particularly in the first 12–24 h [20, 147]. These have been shown to be equivalent to or superior to those of both artemisinin derivative or quinine-based parenteral treatments for severe malaria [20, 112, 148]. Nevertheless, the high degree of inter-individual variability in absorption raises the possibility of sub-optimal response in a subset of children [20, 149]. Therefore, parenteral administration (intramuscular or intravenous artesunate) should be considered more reliable when facilities for safe injection are available. Definitive clinical and parasitological cure is determined largely by either the duration of therapy or the efficacy of the longer acting partner drug used as part of an ACT once the patient is able to swallow tablets and is therefore of secondary interest in evaluation of rectal artemisinin drugs.

A recent large multi-centre placebo-controlled trial has shown that rectal artesunate given as pre-referral therapy to children with suspected severe malaria results in a significant reduction in overall death and disability, especially when

arrival at the health clinic is delayed by >6 h from the time of administration [145]. In common with oral and parenteral formulations of artemisinin derivatives, their introduction into clinical use has not followed classical pathways of rational drug development, and dosage regimens have largely been derived empirically. With the exception of artesunate, pharmacokinetic data describing the adequacy and consistency of absorption of other rectally administered derivatives is lacking. The situation is complicated by the number of rectal artemisinin derivatives on the market with the potential for multiple manufacturers of single formulations. Because pharmaceutical factors may also influence pharmaceutical disposition, preparations from different manufacturers cannot necessarily be assumed to be bioequivalent.

7 Future Potential

The future of the artemisinin-derivative drug class will be dependent on the extent to which significant parasite resistance can be avoided. The principles underlying ACT as a means of preventing artemisinin resistance are now widely accepted and implemented as health policy throughout most of the malaria-endemic world. However, the theory that ACT will prevent resistance developing is one that is difficult to test and to prove. The real effects of this strategy on the population genetics of antimalarial drug resistance remain to be seen. Its success will rely on availability of a suite of suitably effective partner drugs and on operational factors that limit the use of inappropriate artemisinin monotherapy. The development of apparent artemisinin resistance in Cambodia is of particular concern. However, this phenomenon will become doubly alarming if resistance mechanisms evolve that affect a pathway common to both drugs within ACTs, through, for example, drug efflux mechanisms mediated by *pfmdr1* copy number [76, 77, 79].

Improved understanding is required of the mechanisms of artemisinin resistance, including their underlying parasite genetics. This may enable molecular tools and PCR-based diagnostics for monitoring the spread and the development of resistance. Elucidation of the metabolic pathways involved may also shed much-needed light on the mechanisms of action of the artemisinin derivative drug class. This will be useful for the development of subsequent generations of endoperoxide drugs that must aim to subvert these resistance mechanisms.

For the most part, the introduction of the artemisinin derivatives into clinical use has not followed classical pathways of rational drug development, and dosage regimens have largely been derived empirically. However, when dealing with sensitive parasites, the therapeutic margin appears to have been wide enough that therapeutic outcomes have been universally good, regardless of the dosages administered up until now. However, this may change with the advent of reduced susceptibility to artemisinin derivatives. As the therapeutic margin becomes narrower, improved understanding of pharmacokinetic–pharmacodynamic

relationships will become more important for optimally effective and safe dosing regimens to be devised.

With recent evidence that community-based pre-referral rectal administration of artesunate suppositories may have a population mortality benefit [145], artemisinin derivatives formulated for rectal administration have the potential to be amongst the most widely used of all drugs in tropical countries. Because currently available suppository formulations are all mono-preparations of an artemisinin, and because use in this context will be difficult to regulate, this raises concerns about the potential for this strategy to contribute to driving selection pressure for artemisinin resistance, and suggests a need for development of co-formulated (ACT) suppositories [38]. A wide range of preparations of different artemisinin derivatives formulated for rectal administration is now available. However, robust pharmacokinetic data are lacking for many of these and it is not clear if these can be considered therapeutically equivalent to rectal preparations of artesunate that have been the subject of most clinical studies [20]. In addition, optimal strategies for deploying intrarectal artemisinin drugs at community level are yet to be determined. In particular, it is not clear to what extent this strategy is socially or culturally acceptable and feasible across the range of societies in which this intervention could be deployed. Information on existing attitudes to rectal administration in specific cultural contexts should help to inform the development of the most appropriate deployment strategies [127].

Because *A. annua* can be easily grown in a wide range of climates and environments, it has been suggested that it could be grown as a crop at the village-level to enable preparation of a "home-grown" local malaria remedy, similar to its original use in ancient China [5, 150]. This could be useful in remote areas with poor access to conventional health-care and in some societies, the mode of delivery might be more culturally acceptable than modern pharmaceutical administration. However it grows less well and yields less active drug in tropical climates, where the world's greatest malaria burden occurs. In addtion, administration in this way would effectively constitute monotherapy with raw artemisinin, a compound with lesser activity than its pharmaceutical derivatives (such as artesunate). In particular, because it would be difficult to regulate dosage, it seems unlikely that such a strategy would receive official endorsement from the WHO or others, given current existing concerns about the development of resistance.

Although the recent advent of resistance in a small focus in Southeast Asia is concerning, it should be remembered this has emerged up to 30 years after artemisinin drugs were first used in this area. Artemisinin derivatives have now been used heavily for many years in many other parts of the world, without such problems emerging. They represent the most important class of antimalarial drug at the current time and are likely to remain so for the foreseeable future. Following recent WHO policy directives, in the near future, a substantial proportion (if not most) of the estimated 400 million annual clinical presentations with suspected malarial illness will be treated with an artemisinin-containing regimen [1, 28]. Artemisinin derivatives could therefore become one of the most-used classes of drug in the world, raising significant issues regarding global production capacity.

An estimated 10,000 ha of *A. annua* will be required to produce the equivalent of 100 million adult treatment courses [151]. To address these massive global demands, higher yielding varieties of *A. annua* are being investigated [152] and alternatives to agricultural manufacture of raw artemisinin, including recombinant microbial production and wholly synthetic methods of producing artemisinin and other synthetic endoperoxides, may also emerge as feasible means of ensuring a consistent supply of affordable drug .

Acknowledgements Tim Davis and Ken Ilett have been an invaluable source of information and ideas used in the writing of this chapter. Figure 2 is reproduced from Karunajeewa et al. New England Journal of Medicine, 2008, 359 (24) pp. 2545–57 [108] with the permission of the authors.

References

1. WHO. Malaria Treatment Policies (by region). http://www.who.int/malaria/publications/treatment-policies/en/. Accessed 24 Apr 2010
2. WHO (2010) Guidelines for the treatment of malaria, 2nd edn. World Health Organization, Geneva
3. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 434:214–217
4. Willcox M, Bodeker G, Bourdy G, Dhingra V, Falquet J, Ferreira JFS, Graz B, Hirt H-M (2004) *Artemisia annua* as a traditional herbal antimalarial. In: Willcox M, Bodeker G, Raoanaivo P (eds) Traditional medicinal plants and malaria. CRC, Boca Raton, pp 43–59
5. Hsu E (2006) Reflections on the 'discovery' of the antimalarial qinghao. Br J Clin Pharmacol 61:666–670
6. Keiser J, Utzinger J (2007) Artemisinins and synthetic trioxolanes in the treatment of helminth infections. Curr Opin Infect Dis 20:605–612
7. Ilett K, Batty K (2005) Artemisinin and its derivatives. In: Yu VL, Edwards G, McKinnon PS, Peloquin CA (eds) Antimicrobial therapy and vaccines. ESun Technologies, Pitsburg, PA, pp 981–1002
8. Wright CW, Linley PA, Brun R, Wittlin S, Hsu E (2010) Ancient Chinese methods are remarkably effective for the preparation of artemisinin-rich extracts of Qing Hao with potent antimalarial activity. Molecules 15:804–812
9. China Cooperative Research Group on qinghaosu and its derivatives as antimalarials (1982) Clinical studies on the treatment of malaria with qinghaosu and its derivatives. J Tradit Chin Med 2:45–50
10. Schlitzer M (2007) Malaria chemotherapeutics. Part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. ChemMedChem 2:944–986
11. Bruce-Chwatt LJ (1982) Qinghaosu: a new antimalarial. Br Med J (Clin Res Ed) 284:767–768
12. Jiang JB, Li GQ, Guo XB, Kong YC, Arnold K (1982) Antimalarial activity of mefloquine and qinghaosu. Lancet 2:285–288
13. Bruce-Chwatt LJ (1985) Recent trends of chemotherapy and vaccination against malaria: new lamps for old. Br Med J (Clin Res Ed) 291:1072–1076
14. Nosten F, ter Kuile F, Chongsuphajaisiddhi T, Luxemburger C, Webster HK, Edstein M, Phaipun L, Thew KL, White NJ (1991) Mefloquine-resistant falciparum malaria on the Thai-Burmese border. Lancet 337:1140–1143

15. Bunnag D, Viravan C, Looareesuwan S, Karbwang J, Harinasuta T (1991) Clinical trial of artesunate and artemether on multidrug resistant falciparum malaria in Thailand. A preliminary report. SE Asian J Trop Med Publ Health 22:380–385
16. Nosten F, van Vugt M, Price R, Luxemburger C, Thway KL, Brockman A, McGready R, ter Kuile F, Looareesuwan S, White NJ (2000) Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study. Lancet 356:297–302
17. Edwards G (1994) Measurement of artemisinin and its derivatives in biological fluids. Trans R Soc Trop Med Hyg 88:37–39
18. Meshnick SR (2002) Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 32:1655–1660
19. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 424:957–961
20. Karunajeewa HA, Manning L, Mueller I, Ilett KF, Davis TM (2007) Rectal administration of artemisinin derivatives for the treatment of malaria. JAMA 297:2381–2390
21. McIntosh HM, Olliaro P (2000) Artemisinin derivatives for treating severe malaria. Cochrane Database Syst Rev CD000527
22. McIntosh HM, Olliaro P (2000) Artemisinin derivatives for treating uncomplicated malaria. Cochrane Database Syst Rev CD000256
23. Myint HY, Tipmanee P, Nosten F, Day NPJ, Pukrittayakamee S, Looareesuwan S, White NJ (2004) A systematic overview of published antimalarial drug trials. Trans R Soc Trop Med Hyg 98:73–81
24. Sinclair D, Zani B, Donegan S, Olliaro P, Garner P (2009) Artemisinin-based combination therapy for treating uncomplicated malaria. Cochrane Database Syst Rev CD007483
25. Dondorp A, Nosten F, Stepniewska K, Day N, White N (2005) Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. Lancet 366:717–725
26. Dondorp AM, Fanello CI, Hendriksen IC, Gomes E, Seni A, Chhaganlal KD, Bojang K, Olaosebikan R, Anunobi N, Maitland K et al (2010) Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet 376:1647–1657
27. White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, Kokwaro G, Ouma J, Hien TT, Molyneux ME et al (1999) Averting a malaria disaster. Lancet 353:1965–1967
28. WHO (2006) Guidelines for the treatment of malaria. WHO, Geneva
29. Kremsner PG, Krishna S (2004) Antimalarial combinations. Lancet 364:285–294
30. Nosten F, Luxemburger C, ter Kuile FO, Woodrow C, Eh JP, Chongsuphajaisiddhi T, White NJ (1994) Treatment of multidrug-resistant *Plasmodium falciparum* malaria with 3-day artesunate-mefloquine combination. J Infect Dis 170:971–977
31. Davis A (2004) Clinical trials in parasitic diseases. Trans R Soc Trop Med Hyg 98:139–141
32. Barnes KI, Chanda P, Ab Barnabas G (2009) Impact of the large-scale deployment of artemether/lumefantrine on the malaria disease burden in Africa: case studies of South Africa, Zambia and Ethiopia. Malar J 8:S8
33. Barnes KI, Durrheim DN, Little F, Jackson A, Mehta U, Allen E, Dlamini SS, Tsoka J, Bredenkamp B, Mthembu DJ et al (2005) Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa. PLoS Med 2: e330
34. Greenwood B (2009) Can malaria be eliminated? Trans R Soc Trop Med Hyg 103:S2–S5
35. White NJ (2008) The role of anti-malarial drugs in eliminating malaria. Malar J 7:S8
36. Peters W, Robinson BL (1999) The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. Ann Trop Med Parasitol 93:325–329
37. WHO (2001) Antimalarial drug combination therapy. Report of a technical consultation. WHO, Geneva

38. WHO (2007) Use of rectal artesmisinin based suppositories in the management of severe malaria. Report of a WHO Informal Consultation, 27–28 Mar 2006
39. Afonso A, Hunt P, Cheesman S, Alves AC, Cunha CV, do Rosario V, Cravo P (2006) Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes *atp6* (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), *tctp*, *mdr1*, and *cg10*. Antimicrob Agents Chemother 50:480–489
40. Walker DJ, Pitsch JL, Peng MM, Robinson BL, Peters W, Bhisutthibhan J, Meshnick SR (2000) Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii*. Antimicrob Agents Chemother 44:344–347
41. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T et al (2005) Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 366:1960–1963
42. Rehwagen C (2006) WHO ultimatum on artemisinin monotherapy is showing results. BMJ 332:1176
43. Dondorp AM, Yeung S, White L, Nguon C, Day NP, Socheat D, von Seidlein L (2010) Artemisinin resistance: current status and scenarios for containment. Nat Rev Microbiol 8:272–280
44. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620
45. Samarasekera U (2009) Countries race to contain resistance to key antimalarial. Lancet 374:277–280
46. Li J, Zhou B (2010) Biological actions of artemisinin: insights from medicinal chemistry studies. Molecules 15:1378–1397
47. Schlitzer M (2008) Antimalarial drugs – what is in use and what is in the pipeline. Arch Pharm (Weinheim) 341:149–163
48. Chemical Abstracts Services SciFinder Scholar database. American Chemical Society, Washington DC, USA
49. Krishna S, Bustamante L, Haynes RK, Staines HM (2008) Artemisinins: their growing importance in medicine. Trends Pharmacol Sci 29:520–527
50. O'Neill PM, Barton VE, Ward SA (2010) The molecular mechanism of action of artemisinin–the debate continues. Molecules 15:1705–1721
51. Efferth T, Romero MR, Wolf DG, Stamminger T, Marin JJ, Marschall M (2008) The antiviral activities of artemisinin and artesunate. Clin Infect Dis 47:804–811
52. O'Neil MT, Korsinczky ML, Gresty KJ, Auliff A, Cheng Q (2007) A novel *Plasmodium falciparum* expression system for assessing antifolate resistance caused by mutant *P. vivax* dihydrofolate reductase-thymidylate synthase. J Infect Dis 196:467–474
53. Meshnick SR (1994) The mode of action of antimalarial endoperoxides. Trans R Soc Trop Med Hyg 88:31–32
54. Jefford CW (2001) Why artemisinin and certain synthetic peroxides are potent antimalarials. Implications for the mode of action. Curr Med Chem 8:1803–1826
55. Posner GH, Wang D, Cumming JN, Oh CH, French AN, Bodley AL, Shapiro TA (1995) Further evidence supporting the importance of and the restrictions on a carbon-centered radical for high antimalarial activity of 1,2,4-trioxanes like artemisinin. J Med Chem 38:2273–2275
56. Haynes RK, Chan WC, Lung CM, Uhlemann AC, Eckstein U, Taramelli D, Parapini S, Monti D, Krishna S (2007) The Fe^{2+}-mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other artemisinins: the unlikelihood of C-centred radicals as bioactive intermediates. ChemMedChem 2:1480–1497
57. Creek DJ, Charman WN, Chiu FC, Prankerd RJ, Dong Y, Vennerstrom JL, Charman SA (2008) Relationship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. Antimicrob Agents Chemother 52:1291–1296
58. Meshnick SR, Haynes RK, Monti D, Taramelli D, Basilico N, Parapini S, Olliaro P (2003) Artemisinin and heme. Antimicrob Agents Chemother 47:2712–2713

59. Haynes RK, Monti D, Taramelli D, Basilico N, Parapini S, Olliaro P (2003) Artemisinin antimalarials do not inhibit hemozoin formation. Antimicrob Agents Chemother 47:1175
60. Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O (2006) Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. Trends Mol Med 12:200–205
61. Nagamune K, Beatty WL, Sibley LD (2007) Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. Eukaryot Cell 6:2147–2156
62. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK et al (2005) A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 12:628–629
63. Valderramos SG, Scanfeld D, Uhlemann AC, Fidock DA, Krishna S (2010) Investigations into the role of the *Plasmodium falciparum* SERCA (PfATP6) L263E mutation in artemisinin action and resistance. Antimicrob Agents Chemother 54:3842–3852
64. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F (2010) Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob Agents Chemother 54:1872–1877
65. Krishna S, Pulcini S, Fatih F, Staines H (2010) Artemisinins and the biological basis for the PfATP6/SERCA hypothesis. Trends Parasitol 26:517–523
66. Woodrow CJ, Bustamante LY (2011) Mechanisms of artemisinin action and resistance: wider focus is needed. Trends Parasitol 27:2–3; author reply 3–4
67. Li W, Mo W, Shen D, Sun L, Wang J, Lu S, Gitschier JM, Zhou B (2005) Yeast model uncovers dual roles of mitochondria in action of artemisinin. PLoS Genet 1:e36
68. Wang J, Huang L, Li J, Fan Q, Long Y, Li Y, Zhou B (2010) Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. PLoS One 5:e9582
69. Hastings IM, Watkins WM, White NJ (2002) The evolution of drug-resistant malaria: the role of drug elimination half-life. Philos Trans R Soc Lond B Biol Sci 357:505–519
70. Stepniewska K, White NJ (2008) The pharmacokinetic determinants of the window of selection for antimalarial drug resistance. Antimicrob Agents Chemother 52(5):1589–96
71. White NJ (2004) Antimalarial drug resistance. J Clin Invest 113:1084–1092
72. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q (2000) Mutations in *Plasmodium falciparum* cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother 44:2100–2108
73. White N (1999) Antimalarial drug resistance and combination chemotherapy. Philos Trans R Soc Lond B Biol Sci 354:739–749
74. Cowman AF, Galatis D, Thompson JK (1994) Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. Proc Natl Acad Sci USA 91:1143–1147
75. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403:906–909
76. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ et al (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 364:438–447
77. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S et al (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin Infect Dis 42:1570–1577
78. Raman J, Little F, Roper C, Kleinschmidt I, Cassam Y, Maharaj R, Barnes KI (2010) Five years of large-scale *dhfr* and *dhps* mutation surveillance following the phased implementation of artesunate plus sulfadoxine-pyrimethamine in Maputo Province, Southern Mozambique. Am J Trop Med Hyg 82:788–794
79. Chavchich M, Gerena L, Peters J, Chen N, Cheng Q, Kyle DE (2010) Induction of resistance to artemisinin derivatives in *Plasmodium falciparum*: role of *Pfmdr1* amplification and expression. Antimicrob Agents Chemother 54(6):2455–64

80. Hunt P, Afonso A, Creasey A, Culleton R, Sidhu AB, Logan J, Valderramos SG, McNae I, Cheesman S, do Rosario V et al (2007) Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. Mol Microbiol 65:27–40
81. Jambou R, Martinelli A, Pinto J, Gribaldo S, Legrand E, Niang M, Kim N, Pharath L, Volnay B, Ekala MT et al (2010) Geographic structuring of the *Plasmodium falciparum* sarco(endo) plasmic reticulum Ca^{2+} ATPase (PfSERCA) gene diversity. PLoS One 5:e9424
82. Shahinas D, Lau R, Khairnar K, Hancock D, Pillai DR (2011) Artesunate misuse and *Plasmodium falciparum* malaria in traveler returning from Africa. Emerg Infect Dis 16:1608–1610
83. Tanabe K, Zakeri S, Palacpac NM, Afsharpad M, Randrianarivelojosia M, Kaneko A, Marma AS, Horii T, Mita T (2011) Spontaneous mutations in the *Plasmodium falciparum* sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (*PfATP6*) gene among geographically widespread parasite populations unexposed to artemisinin-based combination therapies. Antimicrob Agents Chemother 55:94–100
84. Vinayak S, Alam MT, Mixson-Hayden T, McCollum AM, Sem R, Shah NK, Lim P, Muth S, Rogers WO, Fandeur T et al (2010) Origin and evolution of sulfadoxine resistant *Plasmodium falciparum*. PLoS Pathog 6:e1000830
85. Newton P, Proux S, Green M, Smithuis F, Rozendaal J, Prakongpan S, Chotivanich K, Mayxay M, Looareesuwan S, Farrar J et al (2001) Fake artesunate in southeast Asia. Lancet 357:1948–1950
86. Anderson TJ, Nair S, Nkhoma S, Williams JT, Imwong M, Yi P, Socheat D, Das D, Chotivanich K, Day NP et al (2010) High heritability of malaria parasite clearance rate indicates a genetic basis for artemisinin resistance in western Cambodia. J Infect Dis 201:1326–1330
87. Maude RJ, Pontavornpinyo W, Saralamba S, Aguas R, Yeung S, Dondorp AM, Day NP, White NJ, White LJ (2009) The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. Malar J 8:31
88. Rogers WO, Sem R, Tero T, Chim P, Lim P, Muth S, Socheat D, Ariey F, Wongsrichanalai C (2009) Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. Malar J 8:10
89. Li Q, Lugt CB, Looareesuwan S, Krudsood S, Wilairatana P, Vannaphan S, Chalearmrult K, Milhous WK (2004) Pharmacokinetic investigation on the therapeutic potential of artemotil (beta-arteether) in Thai patients with severe *Plasmodium falciparum* malaria. Am J Trop Med Hyg 71:723–731
90. Sabarinath SN, Asthana OP, Puri SK, Srivastava K, Madhusudanan KP, Gupta RC (2005) Clinical pharmacokinetics of the diastereomers of arteether in healthy volunteers. Clin Pharmacokinet 44:1191–1203
91. Ilett KF, Batty KT, Powell SM, Binh TQ, le Thu TA, Phuong HL, Hung NC, Davis TM (2002) The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated falciparum malaria. Br J Clin Pharmacol 53:23–30
92. Davis TM, Phuong HL, Ilett KF, Hung NC, Batty KT, Phuong VD, Powell SM, Thien HV, Binh TQ (2001) Pharmacokinetics and pharmacodynamics of intravenous artesunate in severe falciparum malaria. Antimicrob Agents Chemother 45:181–186
93. Batty KT, Thu LT, Davis TM, Ilett KF, Mai TX, Hung NC, Tien NP, Powell SM, Thien HV, Binh TQ et al (1998) A pharmacokinetic and pharmacodynamic study of intravenous vs oral artesunate in uncomplicated falciparum malaria. Br J Clin Pharmacol 45:123–129
94. Binh TQ, Ilett KF, Batty KT, Davis TM, Hung NC, Powell SM, Thu LT, Thien HV, Phuong HL, Phuong VD (2001) Oral bioavailability of dihydroartemisinin in Vietnamese volunteers and in patients with falciparum malaria. Br J Clin Pharmacol 51:541–546
95. Newton P, Suputtamongkol Y, Teja-Isavadharm P, Pukrittayakamee S, Navaratnam V, Bates I, White N (2000) Antimalarial bioavailability and disposition of artesunate in acute falciparum malaria. Antimicrob Agents Chemother 44:972–977

96. Titulaer HA, Zuidema J, Kager PA, Wetsteyn JC, Lugt CB, Merkus FW (1990) The pharmacokinetics of artemisinin after oral, intramuscular and rectal administration to volunteers. J Pharm Pharmacol 42:810–813
97. Karbwang J, Na-Bangchang K, Congpuong K, Molunto P, Thanavibul A (1997) Pharmacokinetics and bioavailability of oral and intramuscular artemether. Eur J Clin Pharmacol 52:307–310
98. Artemether-Quinine Meta-analysis Study Group (2001) A meta-analysis using individual patient data of trials comparing artemether with quinine in the treatment of severe falciparum malaria. Trans R Soc Trop Med Hyg 95:637–650
99. Murphy SA, Mberu E, Muhia D, English M, Crawley J, Waruiru C, Lowe B, Newton CR, Winstanley P, Marsh K et al (1997) The disposition of intramuscular artemether in children with cerebral malaria; a preliminary study. Trans R Soc Trop Med Hyg 91:331–334
100. Simonsson US, Jansson B, Hai TN, Huong DX, Tybring G, Ashton M (2003) Artemisinin autoinduction is caused by involvement of cytochrome P450 2B6 but not 2 C9. Clin Pharmacol Ther 74:32–43
101. Czock D, Markert C, Hartman B, Keller F (2009) Pharmacokinetics and pharmacodynamics of antimicrobial drugs. Expert Opin Drug Metab Toxicol 5:475–487
102. Gautam A, Ahmed T, Batra V, Paliwal J (2009) Pharmacokinetics and pharmacodynamics of endoperoxide antimalarials. Curr Drug Metab 10:289–306
103. Alin MH, Ashton M, Kihamia CM, Mtey GJ, Bjorkman A (1996) Clinical efficacy and pharmacokinetics of artemisinin monotherapy and in combination with mefloquine in patients with falciparum malaria. Br J Clin Pharmacol 41:587–592
104. Angus BJ, Thaiaporn I, Chanthapadith K, Suputtamongkol Y, White NJ (2002) Oral artesunate dose-response relationship in acute falciparum malaria. Antimicrob Agents Chemother 46:778–782
105. Li QG, Mog SR, Si YZ, Kyle DE, Gettayacamin M, Milhous WK (2002) Neurotoxicity and efficacy of arteether related to its exposure times and exposure levels in rodents. Am J Trop Med Hyg 66:516–525
106. Gordi T, Lepist EI (2004) Artemisinin derivatives: toxic for laboratory animals, safe for humans? Toxicol Lett 147:99–107
107. Genovese RF, Newman DB, Brewer TG (2000) Behavioral and neural toxicity of the artemisinin antimalarial, arteether, but not artesunate and artelinate, in rats. Pharmacol Biochem Behav 67:37–44
108. Karunajeewa HA, Mueller I, Senn M, Lin E, Law I, Gomorrai PS, Oa O, Griffin S, Kotab K, Suano P et al (2008) A trial of combination antimalarial therapies in children from Papua New Guinea. N Engl J Med 359:2545–2557
109. Hien TT, White NJ (1993) Qinghaosu. Lancet 341:603–608
110. White NJ (2003) Malaria. In: Cook G, Zumla A (eds) Manson's tropical diseases. Elsevier, Amsterdam, pp 1205–1296
111. Karunajeewa HA, Kemiki A, Alpers MP, Lorry K, Batty KT, Ilett KF, Davis TM (2003) Safety and therapeutic efficacy of artesunate suppositories for treatment of malaria in children in Papua New Guinea. Pediatr Infect Dis J 22:251–256
112. Karunajeewa HA, Reeder J, Lorry K, Dabod E, Hamzah J, Page-Sharp M, Chiswell GM, Ilett KF, Davis TM (2006) Artesunate suppositories versus intramuscular artemether for treatment of severe malaria in children in Papua New Guinea. Antimicrob Agents Chemother 50:968–974
113. Borrmann S, Adegnika AA, Moussavou F, Oyakhirome S, Esser G, Matsiegui PB, Ramharter M, Lundgren I, Kombila M, Issifou S et al (2005) Short-course regimens of artesunate-fosmidomycin in treatment of uncomplicated *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 49:3749–3754
114. Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, White NJ (1996) Effects of artemisinin derivatives on malaria transmissibility. Lancet 347:1654–1658

115. Price R, van Vugt M, Phaipun L, Luxemburger C, Simpson J, McGready R, ter Kuile F, Kham A, Chongsuphajaisiddhi T, White NJ et al (1999) Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. Am J Trop Med Hyg 60:547–555
116. Bethell D, Se Y, Lon C, Socheat D, Saunders D, Teja-Isavadharm P, Khemawoot P, Darapiseth S, Lin J, Sriwichai S et al (2010) Dose-dependent risk of neutropenia after 7-day courses of artesunate monotherapy in Cambodian patients with acute *Plasmodium falciparum* malaria. Clin Infect Dis 51:e105–e114
117. Brewer TG, Peggins JO, Grate SJ, Petras JM, Levine BS, Weina PJ, Swearengen J, Heiffer MH, Schuster BG (1994) Neurotoxicity in animals due to arteether and artemether. Trans R Soc Trop Med Hyg 88:33–36
118. Brewer TG, Grate SJ, Peggins JO, Weina PJ, Petras JM, Levine BS, Heiffer MH, Schuster BG (1994) Fatal neurotoxicity of arteether and artemether. Am J Trop Med Hyg 51:251–259
119. Miller LG, Panosian CB (1997) Ataxia and slurred speech after artesunate treatment for falciparum malaria. N Engl J Med 336:1328
120. Toovey S, Jamieson A (2004) Audiometric changes associated with the treatment of uncomplicated falciparum malaria with co-artemether. Trans R Soc Trop Med Hyg 98:261–267
121. Kissinger E, Hien TT, Hung NT, Nam ND, Tuyen NL, Dinh BV, Mann C, Phu NH, Loc PP, Simpson JA et al (2000) Clinical and neurophysiological study of the effects of multiple doses of artemisinin on brain-stem function in Vietnamese patients. Am J Trop Med Hyg 63:48–55
122. Davis TM, Edwards GO, McCarthy JS (1997) Artesunate and cerebellar dysfunction in falciparum malaria. N Engl J Med 337:792; author reply 793
123. Davis TM, Binh TQ, Ilett KF, Batty KT, Phuong HL, Chiswell GM, Phuong VD, Agus C (2003) Penetration of dihydroartemisinin into cerebrospinal fluid after administration of intravenous artesunate in severe falciparum malaria. Antimicrob Agents Chemother 47:368–370
124. Johann-Liang R, Albrecht R (2003) Safety evaluations of drugs containing artemisinin derivatives for the treatment of malaria. Clin Infect Dis 36:1626–1627; author reply 1627–1628
125. Clark RL (2009) Embryotoxicity of the artemisinin antimalarials and potential consequences for use in women in the first trimester. Reprod Toxicol 28(3):285–96
126. McGready R, Cho T, Keo NK, Thwai KL, Villegas L, Looareesuwan S, White NJ, Nosten F (2001) Artemisinin antimalarials in pregnancy: a prospective treatment study of 539 episodes of multidrug-resistant *Plasmodium falciparum*. Clin Infect Dis 33:2009–2016
127. Hinton RL, Auwun A, Pongua G, Oa O, Davis TM, Karunajeewa HA, Reeder JC (2007) Caregivers' acceptance of using artesunate suppositories for treating childhood malaria in papua new Guinea. Am J Trop Med Hyg 76:634–640
128. Giao PT, Binh TQ, Kager PA, Long HP, Van Thang N, Van Nam N, de Vries PJ (2001) Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy? Am J Trop Med Hyg 65:690–695
129. Davis TM, Karunajeewa HA, Ilett KF (2005) Artemisinin-based combination therapies for uncomplicated malaria. Med J Aust 182:181–185
130. Adjuik M, Babiker A, Garner P, Olliaro P, Taylor W, White N (2004) Artesunate combinations for treatment of malaria: meta-analysis. Lancet 363:9–17
131. Omari AAA, Preston C, Garner PA (2005) Artemether-lumefantrine (six-dose regimen) for treating uncomplicated falciparum malaria. Cochrane Database Syst Rev CD005564
132. Ashley EA, Stepniewska K, Lindegardh N, Annerberg A, Kham A, Brockman A, Singhasivanon P, White NJ, Nosten F (2007) How much fat is necessary to optimize lumefantrine oral bioavailability? Trop Med Int Health 12:195–200
133. Juma EA, Obonyo CO, Akhwale WS, Ogutu BR (2008) A randomized, open-label, comparative efficacy trial of artemether-lumefantrine suspension versus artemether-lumefantrine tablets for treatment of uncomplicated *Plasmodium falciparum* malaria in children in western Kenya. Malar J 7:262

134. Karunajeewa H (2010) Piperaquine. In: Grayson ML (eds): Kucers' the use of antibiotics. Hodder Education, London
135. Winstanley P, Newton C, Watkins W, Mberu E, Ward S, Warn P, Mwangi I, Waruiru C, Pasvol G, Warrell D et al (1993) Towards optimal regimens of parenteral quinine for young African children with cerebral malaria: the importance of unbound quinine concentration. Trans R Soc Trop Med Hyg 87:201–206
136. White NJ, Miller KD, Churchill FC, Berry C, Brown J, Williams SB, Greenwood BM (1988) Chloroquine treatment of severe malaria in children. Pharmacokinetics, toxicity, and new dosage recommendations. N Engl J Med 319:1493–1500
137. Looareesuwan S, Phillips RE, White NJ, Karbwang J, Benjasurat Y, Attanath P, Warrell DA (1985) Intravenous amodiaquine and oral amodiaquine/erythromycin in the treatment of chloroquine-resistant falciparum malaria. Lancet 2:805–808
138. Roche RJ, Silamut K, Pukrittayakamee S, Looareesuwan S, Molunto P, Boonamrung S, White NJ (1990) Quinine induces reversible high-tone hearing loss. Br J Clin Pharmacol 29:780–782
139. Krishna S, White NJ (1996) Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. Clin Pharmacokinet 30:263–299
140. Touze JE, Heno P, Fourcade L, Deharo JC, Thomas G, Bohan S, Paule P, Riviere P, Kouassi E, Buguet A (2002) The effects of antimalarial drugs on ventricular repolarization. Am J Trop Med Hyg 67:54–60
141. Taylor WR, White NJ (2004) Antimalarial drug toxicity: a review. Drug Saf 27:25–61
142. White NJ (1994) Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives. Trans R Soc Trop Med Hyg 88:S41–S43
143. Karbwang J, Na-Bangchang K, Tin T, Sukontason K, Rimchala W, Harinasuta T (1998) Pharmacokinetics of intramuscular artemether in patients with severe falciparum malaria with or without acute renal failure. Br J Clin Pharmacol 45:597–600
144. Mithwani S, Aarons L, Kokwaro GO, Majid O, Muchohi S, Edwards G, Mohamed S, Marsh K, Watkins W (2004) Population pharmacokinetics of artemether and dihydroartemisinin following single intramuscular dosing of artemether in African children with severe falciparum malaria. Br J Clin Pharmacol 57:146–152
145. Gomes MF, Faiz MA, Gyapong JO, Warsame M, Agbenyega T, Babiker A, Baiden F, Yunus EB, Binka F, Clerk C et al (2009) Pre-referral rectal artesunate to prevent death and disability in severe malaria: a placebo-controlled trial. Lancet 373:557–566
146. TDR/GEN/01.5 Special Programme for Research and Training in Tropical Diseases. 15th Programme Report. Progress 1999–2000
147. White NJ (1997) Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. Antimicrob Agents Chemother 41:1413–1422
148. Gomes M, Ribeiro I, Warsame M, Karunajeewa H, Petzold M (2008) Rectal artemisinins for malaria: a review of efficacy and safety from individual patient data in clinical studies. BMC Infect Dis 8:39
149. Karunajeewa HA, Ilett KF, Dufall K, Kemiki A, Bockarie M, Alpers MP, Barrett PH, Vicini P, Davis TM (2004) Disposition of artesunate and dihydroartemisinin after administration of artesunate suppositories in children from Papua New Guinea with uncomplicated malaria. Antimicrob Agents Chemother 48:2966–2972
150. de Ridder S, van der Kooy F, Verpoorte R (2008) Artemisia annua as a self-reliant treatment for malaria in developing countries. J Ethnopharmacol 120:302–314
151. Hommel M (2008) The future of artemisinins: natural, synthetic or recombinant? J Biol 7:38
152. Centre for Novel Agricultural Products DoB, University of York. The CNAP Artemisia Research Project: Project Update Number 9, May 2011. http://www.york.ac.uk/cnap/artemisaproject/pdfs/update-009.pdf. Accessed 15 May 2011

Second-Generation Peroxides: The OZs and Artemisone

Dejan M. Opsenica and Bogdan A. Šolaja

Abstract The emergence of multi-drug resistant strains of *Plasmodium falciparum* has rendered many affordable antimalarials, such as chloroquine, much less effective in addressing the severe health issues in sub-Saharan Africa, Southeast Asia and the Amazon region. In order to overcome the neurotoxicity of an initial series of artemisinin-derived drugs and their relatively high production costs, an intensive and all-inclusive research programme to develop new derivatives has been undertaken. Two efficient antimalarial drug candidates of different chemotype have been devised, the artemisinin derivative artemisone and 1,2,4-troxolane **OZ277**. Both are nontoxic, more potent than artemisinin and should be affordable to people of endemic regions. The same may hold for the backup candidates artemiside and **OZ439**.

1 Introduction

Great efforts have been expended over the last 30 years to discover new and efficacious endoperoxide antimalarials based on a natural product isolated from sweet wormwood – artemisinin, and related derivatives (collectively known as artemisinins) - primarily because, until very recently [1, 2], resistance to this type of drug had not been observed (Fig. 1) [3].

The main drawback of the initial series of artemisinin derivatives (Fig. 1) is their relative metabolic instability, i.e., susceptibility to hydrolysis (esters), or

D.M. Opsenica
Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 16, Belgrade 11158, Serbia
e-mail: dopsen@chem.bg.ac.rs

B.A. Šolaja (✉)
Faculty of Chemistry, University of Belgrade, Studentski trg 16, P.O. Box 51, Belgrade 11158, Serbia
e-mail: bsolaja@chem.bg.ac.rs

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_10,

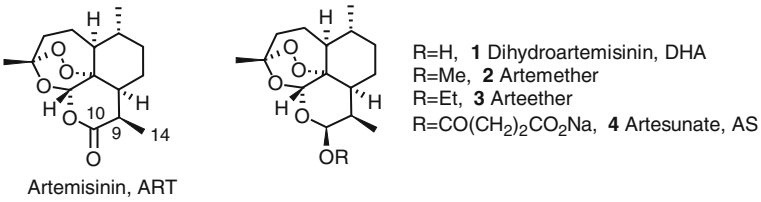


Fig. 1 Artemisinin and its early derivatives

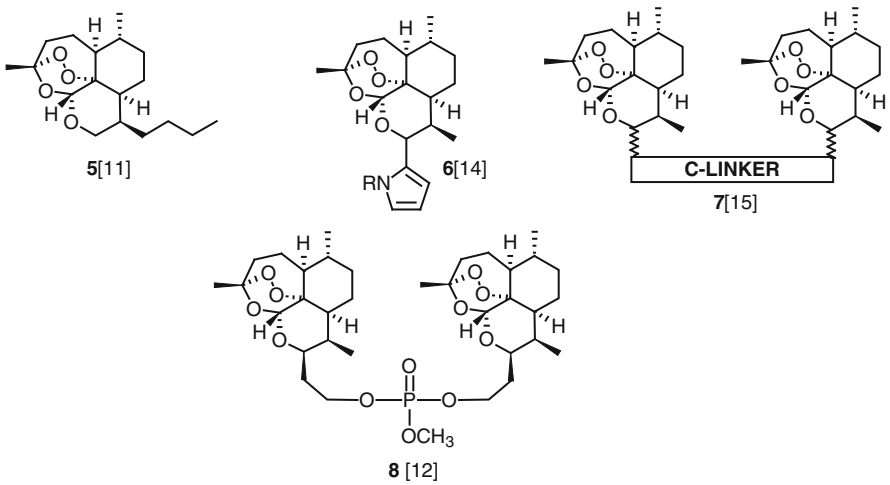
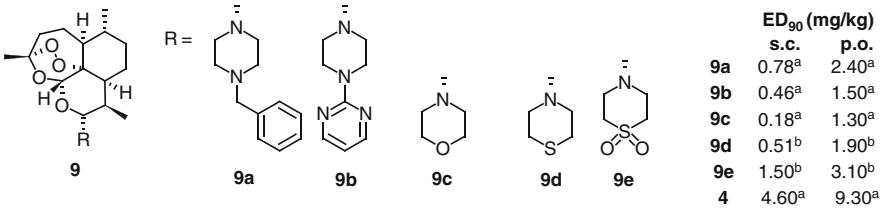


Fig. 2 Semi-synthetic artemisinins: C(14) and C(10) carba derivatives

oxidative-dealkylation (ethers) to dihydroartemisinin [4]. Dihydroartemisinin, apart from being a more potent antimalarial than artemisinin [5], was found to be neurotoxic in vitro [6, 7] and in mice [8]. However, similar neurotoxicity has not been observed in humans [9]. Although embryotoxicity has been detected within a narrow window of embryogenesis, this has not been convincingly observed in clinical trials from 1,837 pregnant women [10].

These potential toxicity issues provoked an extensive search for metabolically stable artemisinin derivatives and two major semi-synthetic artemisinin sub-classes were developed: C(10) deoxyartemisinin derivatives usually functionalized at C(9) or at C(14) (**5**) [11], and derivatives possessing a new C(10)-NR, or C(10)-C bond (**6**–**8**) [12–15]. The second sub-class is more diversified with artemisinin-derived dimers and artemisone (**9e**) as the most prominent members (Figs. 2 and 3).

The quest for efficacious and cheap antimalarial drugs that can be administered as a single dose, preferably orally, is not limited to natural product-derived semi-synthetic artemisinins, like artemisone, but also to other classes of peroxide antimalarials. Of these, the most prominent peroxide classes are the fully synthetic 1,2,4-trioxanes[16, 17], 1,2,4-trioxolanes (ozonides, OZs) and mixed 1,2,4,5-



	ED_{90} (mg/kg) s.c.	p.o.
9a	0.78[a]	2.40[a]
9b	0.46[a]	1.50[a]
9c	0.18[a]	1.30[a]
9d	0.51[b]	1.90[b]
9e	1.50[b]	3.10[b]
4	4.60[a]	9.30[a]

[a] Data taken from ref. 22; [b] Data taken from ref. 21.

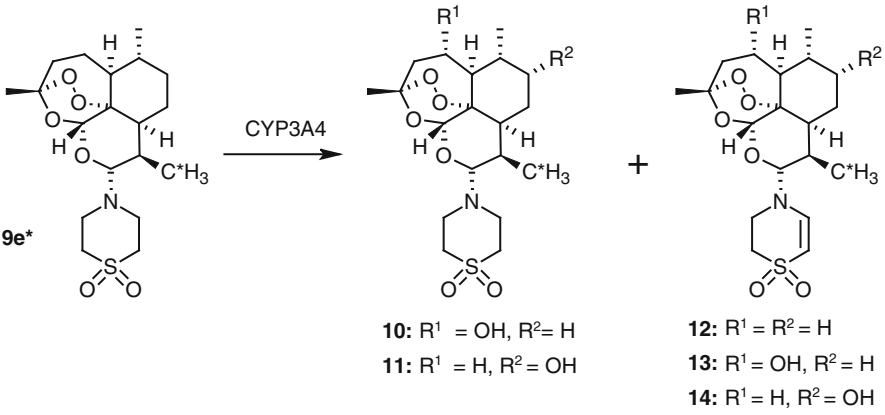
Fig. 3 Structures and antimalarial activities of derivatives **9**

tetraoxanes [18–20]. Two representatives of this class that have entered studies in humans are discussed below in detail.

2 Artemisone (Artemifon)

With the aim of preventing metabolic transformation of artemisinins to dihydroartemisinin (**1**, Fig. 1) and the drive to improve pharmacokinetic characteristics, several 10-(alkylamino)artemisinins **9** were designed (Fig. 3) [21, 22]. All the tested compounds showed excellent in vivo activities against *P. berghei* (**9c** was the most active derivative, almost 25 times (given *s.c.*) and 7 times (given *p.o.*) more active than artesunate **4**). Unfortunately, **9c** suffers from serious neurotoxicity issues even at low doses, thus indicating, that more lipophilic artemisinins are more toxic [21, 23].

A detailed antimalarial efficacy and drug–drug interaction study of artemisone (a drug examined recently in clinical Phase II trials [24]) was performed (**9e**, Fig. 3) [25]. It was shown that the in vitro antimalarial activities of artemisone against 12 different *P. falciparum* strains were comparable (exhibiting a mean IC_{50} value of 0.83 nM), independent of their drug-susceptibility profile to other antimalarial drug classes [21, 25]. During examination of the in vitro drug–drug interaction against drug sensitive 3D7 and multi-drug resistant K1 strains, it was noticed that artemisone showed slight antagonistic effects with chloroquine, amodiaquine, tafenoquine, atovaquone or pyrimethamine, and slight synergism with mefloquine. In vivo screening using the 4-day Peters test against drug-susceptible (NY), primaquine-resistant (P) and sulphadoxine/pyrimethamine-resistant (KFY) lines of *P. berghei*, chloroquine-resistant (NS) and artemisinin-resistant lines of *P. yoelii* NS and drug-susceptible *P. chabaudi* (AS) showed artemisone has superior ED_{50} and ED_{90} activity in comparison with artesunate (**4**, Fig. 1). Artemisone exhibited a 7-times greater activity (lower dosage) than artesunate (artemisone $ED_{90} = 12.13$ mg/kg vs. artesunate $ED_{90} = 87.50$ mg/kg) against the *P. yoelii* artemisinin-resistant line. The above results appear quite important in light of the recent in vitro isolation of artemether-resistant *P. falciparum* strains from humans [26] and



Scheme 1 Metabolic transformations of artemisone

emerging evidence for resistance in vivo [1, 2], suggesting more potent derivatives may be efficacious where first generation compounds are failing.

During in vivo drug–drug interaction examinations against the drug-susceptible *P. berghei* NY and the mefloquine-resistant *P. berghei* N1100 lines, artemisone showed synergism with mefloquine against both parasite lines. In combination with chloroquine, no interaction against drug-susceptible *P. berghei* NY parasites was detected; however, a synergistic effect against the chloroquine-resistant line, *P. yoelii* NS, was observed.

No dihydroartemisinin had been produced after 30 min, when isotopically labelled artemisone **9e*** (Scheme 1) was incubated with human liver microsomes. Only dehydrogenated **12** and mono-hydroxylated metabolites **10** and **11** and **13** and **14**, with *syn*-hydroxyl and peroxide groups were observed (Scheme 1) [21], clearly distinguishing artemisone and similar compounds from the first-generation artemisinins.[1] Incubation with microsomes and 14 recombinant CYP isoforms, together with selective inhibitors of CYP, showed that artemisone was primarily metabolised by recombinant CYP3A4. Interestingly, artemisinin induces its own metabolism [27] and is metabolised principally by CYP2B6 [28]. These results indicate that artemisone and artemisinin, in spite of being structurally similar, have a different metabolic profile in *P. falciparum;* therefore, it is possible that they can exert their antimalarial activity through similar but not identical mechanisms (vide infra). Isolated artemisone metabolites were tested against the *P. falciparum* K1 strain and were also found to be potent antimalarials, with **11** and **12** being the most active (**11**: $IC_{50} = 5.51$ nM and **12**: $IC_{50} = 4.26$ nM, artemisone: $IC_{50} = 1.99$ nM) [21, 29].

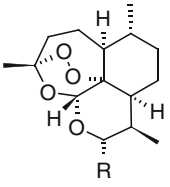
[1] However, dihydroartemisinin was detected in plasma during assessment of the safety of artemisone [27]. The concentrations were low, with geometric mean C_{max} values of 10 ng/ml after an 80 mg dose.

In preclinical studies, artemisone showed enhanced efficacy and improved pharmacokinetics in comparison with artesunate and did not demonstrate neurotoxicity in vitro and in vivo [21, 29], which is a characteristic of the current artemisinin derivatives used in clinical treatment [30]. Administration of artemisone as a single dose (10–80 mg) or multiple doses (40 mg or 80 mg given once daily for 3 days) was well tolerated. It appears that artemisone is devoid of time-dependent pharmacokinetics (unlike artemisinin and artemether), with comparable C_{max}, AUC and $t_{½}$ values after the first and third doses following the 3-day courses of artemisone. Although not being so active in vitro as the parent drug, the relatively high concentrations of metabolites obtained after artemisone administration probably add to the overall parasiticidal effect of artemisone [29]. In vivo testing on non-immune *Aotus* monkeys infected with *P. falciparum* showed that a single dose of artemisone (10 mg/kg) in combination with single doses of mefloquine (12.5 mg/kg or 5 mg/kg) cleared parasitaemia by day 1, with complete cure for all four monkeys tested [31]. With a single dose of mefloquine (2.5 mg/kg) parasitaemia was cleared by day 1 but without cure. For 3 days of treatment with a combination of artemisone (10 mg/kg/day) and amodiaquine (20 mg/kg/day), all three monkeys tested were cured, in contrast to those that were administered with the individual drugs for 3 days. From this study, it is clear that various total dosages of artemisone (20–90 mg/kg) alone, administered over 1–3 days, were unable to cure non-immune *Aotus* monkeys infected with *P. falciparum*. However, cure can be achieved when artemisone is combined with a single, sub-curative dose of mefloquine, or with a 3-day treatment course of amodiaquine (or clindamycin).

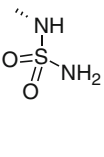
Efficacy of several artemisinin derivatives was examined for the treatment of murine cerebral malaria, CM, ECM [32]. It was shown that artemisone and artemiside (**9d**, Fig. 3) were more effective in the treatment of ICR and C57BL mice in comparison with dihydroartemisinin or artesunate. In all experiments performed on *P. berghei*-infected mice, treatment with artemisone led to complete cure (at least parasitaemia was reduced to an undetectable level). The observed recrudescent parasites were successfully eradicated by repeating the treatment with artemisone without selecting for parasite resistance. In addition, it was shown that complete cure of infected mice could be achieved even when treatment with artemisone was commenced at late stages of pathogenesis, 6 days post-infection. These results opened a considerable time window for adequate treatment, since human malaria is diagnosed after clinical symptoms are apparent. Following WHO's instructions for artemisinin combination therapy (ACT), the efficacy of artemisone–CQ combination was examined and it was shown that this combination was more successful than single therapy of both drugs individually. Combination of these two drugs prevented recrudescence and cured all mice (2×5 mg/kg/day artemisone + 15 mg/kg CQ). In this study, artemiside appeared to be even more successful then artemisone, but this derivative has to be submitted to more detailed preclinical toxicological evaluation. However, the preliminary results suggest that artemiside may represent a new option for antimalarial therapy based on artemisinin derivatives [32].

2.1 Possible Mechanisms: Fe(II) Interaction and the Peroxide Bond

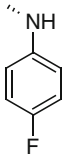
In an effort to define more clearly a reasonable mechanism for the action of artemisone and related derivatives, experiments with various Fe(II) salts were performed [33]. It was found that artemisone, unlike other examined aminoartemisinin derivatives **9a**, **9b** (Fig. 3) and **15**–**17** (Fig. 4), reacts with Fe (II) salts under aqueous conditions to afford the products **18**–**20**, which are structurally similar to the ones obtained from artemisinin and dihydroartemisinin. The relative ratio of products **18**–**20** (Scheme 2) depends on the employed salts and solvent mixtures. A reasonable mechanism was proposed for Fe(II)-induced generation of the products, including artemisone → **18**, **19** formation via iminium cations. Evidence that a primary C-radical is generated during Fe(II) peroxide scission came from the isolation of adduct **22** (Scheme 2), arising from reaction in the presence of the radical trapper 4-oxo-TEMPO. However, the yield of this



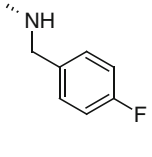
R =



15



16

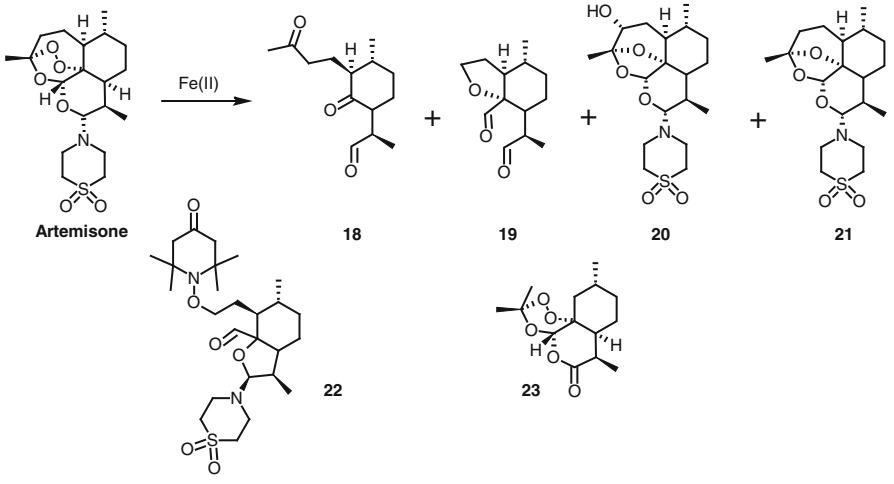


17

	IC_{50} (nM)[a]	
	W2[b]	**D10**[b]
9a	0.66	1.17
9b	1.53	2.04
9e	0.60	1.17
15	3.03	7.89
16	0.82	2.07
ART	4.82	7.33

[a] Data taken from ref. 33; [b] W2 is *P. f.* CQR strain, D10 is *P. f.* CQS strain.

Fig. 4 Semi-synthetic artemisinins: C(10) aza derivatives



Scheme 2 Products **18**–**21** obtained after reaction of artemisone with various Fe(II) salts

product is low (10%) and the authors argued that the role of Fe(II) is during decomposition rather than activation of artemisone [33].

Others have shown that artemisone readily reacts with haem in vitro producing alkylated products [34] very similar to artemisinins [35]. The ability of artemisinin itself to alkylate haem has been confirmed in vitro [36, 37] and in vivo [38]. These data indicate that Fe(II) species are able to activate artemisinins, including artemisone, in their antimalarial action. However, it should be noted that the conditions used to determine alkylation by artemisinins have been questioned, in particular the use of dimethyl sulphoxide that produces a reducing environment [39, 40], and, currently, there is no evidence that artemisone alkylates haem in vivo. In addition, the theory of Fe(II)-initiated activation of artemisinins could not explain the pronounced antimalarial activity of 5-nor-4,5-seco-artemisinin **23** [41–43], which is clearly less capable of forming C radicals upon peroxide bridge cleavage by Fe(II). Another inconsistency in applying the general Fe(II) mechanism (vide supra) to artemisinin and artemisone, and their derivatives, arises from their activities in the presence of desferrioxamine B (DFO). DFO acts as a free radical scavenger (for hydroxyl and peroxyl radicals) and as a Fe(III) chelator and reduces artemisinin inhibition of PfATP6 (a proposed site of action; see below) [44]. In contrast, artemisone, which exhibits approximately the same reactivity with aqueous Fe(II) as artemisinin, retains inhibition potency towards PfATP6 in the presence of DFO [33]. This detail strongly indicates that although the compounds are of similar structure, they might not exert their antimalarial activity by sharing the same mechanism of action.

In order to understand better the manner in which artemisinins exert their antimalarial activity, extensive research was launched bearing in mind that cleavage of the peroxide bridge via an iron-dependent mechanism (as the sole mechanism) does not provide complete answers to observed peroxide drug behaviour. Based on the finding that the in vitro antimalarial activity of artemisinin was significantly increased under a 2% carbon monoxide atmosphere (by 40–50%) and under a 20% oxygen atmosphere (by 20–30%) [45], a detailed study of the interaction of artemisone and related derivatives with various forms of haemoglobin (Hb), haem, as well as an analysis of their antimalarial activity was performed [39]. In contrast to artemisinin, artemisone does not react with Hb–Fe(II) and oxyHb–Fe(II) to produce metHb–Fe(III); in addition, it was shown that both artemisinin and artemisone (and **9a**, **9b**, **16**) are inactive towards Hb–Fe(II) in a 2% carbon monoxide atmosphere. On the other hand, both compounds induced the oxidation of the haemoglobin catabolic product, haem–Fe(II), to produce haem–Fe(III). In addition, on exposure of haem–Fe(II) and Hb–Fe(II) to carbon monoxide, stable complexes were formed, which are also inactive for reactions with peroxide antimalarials. However, in a biologically relevant experiment, both compounds showed increased antimalarial activity against *P. falciparum* W2 strain under a 2% carbon monoxide atmosphere, while the activity of chloroquine remained unchanged. The authors suggested [39] that peroxide antimalarials behave as reactive oxygen species (ROS), or that they produce ROS via Haber–Weiss chemistry. Furthermore, it was concluded that passivation of haem–Fe(II) by its conversion

into the CO complex results in decreased decomposition of artemisinins (artemisone included), thus making them more available for reaction with their actual target. The authors proposed that Fe(II), regardless of its origin (Hb–Fe(II) or haem–Fe(II)), is not the sole initiator of O–O scission, and suggested that another mechanism be sought [39].

2.2 *Possible Mechanisms: Primary Targets, Accumulation and Co-Factors*

The discovery that artemisinins target SERCA orthologues in *P. falciparum* (PfATP6) and *P. vivax* (PvSERCA) [44] shed new light and encouraged research towards the same and other novel targets for peroxide antimalarials. Thus, it was found that artemisone is more potent against plasmodial SERCA orthologues [$K_i = 1.7 \pm 0.6$ nM (PfATP6) and $K_i = 0.072 \pm 0.012$ nM (PvSERCA)], as compared with values for artemisinin [$K_i = 169 \pm 31$ nM (PfATP6) and $K_i = 7.7 \pm 4.9$ nM (PvSERCA)] [46].

In order to better understand the mechanism of the accumulation into infected and uninfected erythrocytes, radiolabelled artemisone **9e*** was submitted to uptake assays [47]. It was found that artemisone was accumulated in infected erythrocytes at significantly higher levels than in uninfected ones, by a saturable, competitive, time- and temperature-dependent mechanism. Most radioactivity was detected in pellet fractions which predominantly contain proteins suggesting that artemisone is probably associated with proteins [48]. The distribution of artemisone is dependent on the stage of parasite maturation: after incubation with immature parasites, which are highly susceptible to action of artemisinins in vitro, as well as in vivo, most artemisone was found in a Triton-soluble fraction, which contains proteins, DNA and RNA. In mature parasites most artemisone was found in the Triton-insoluble fraction, which predominantly contains haemozoin. This finding suggests that mature infected erythrocytes can act by removing the artemisinins. This was further supported by the observation that mature infected erythrocytes take up artemisone much faster than do the more sensitive ring-stage-infected erythrocytes.

Methylene blue (MB) was the first synthetic drug ever used in humans, and it was Paul Erlich who cured two patients from malaria using MB in 1891 [49]. The discovery that artemisinin and artemether exhibit synergic effects with methylene blue [50], unlike chloroaminoquinolines, initiated systematic examination of reactivity relationships between the drugs with the aim to possibly correlate their mechanism of antimalarial action. Subsequent research by the same group [51] revealed MB as a redox-cycling agent that produces H_2O_2 at the expense of O_2 and of NADPH in each cycle (Fig. 5). Thus, MB consumes NADPH and O_2 needed for the pathogen's metabolism, with probably serious consequences to the NADPH/NADP ratio.

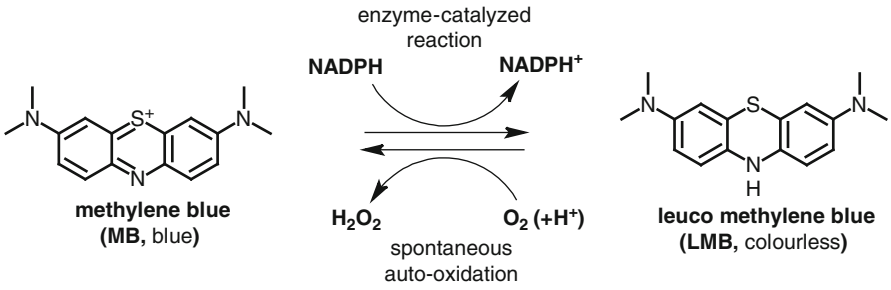
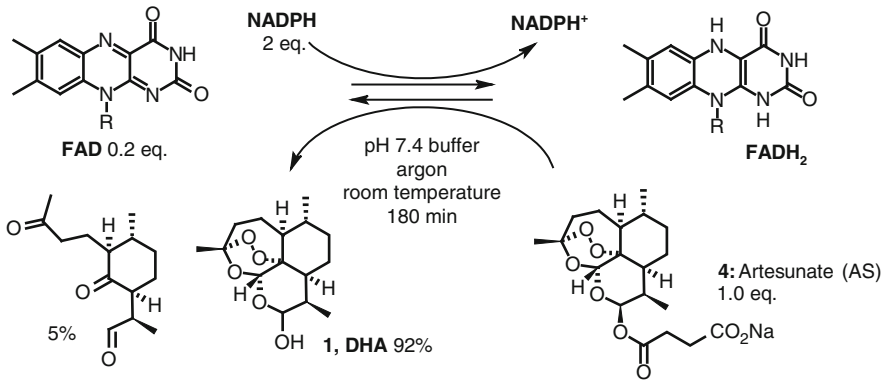


Fig. 5 Redox-cycling interconversion between methylene blue (MB) and leuco methylene blue (LMB)



Scheme 3 Biomimetic catalytic system under physiological conditions: works with other flavins, all examined artemisinins, tetraoxane and trioxolane antimalarials

The results of subsequent research revealed that artemisone, other artemisinins [52] and other peroxide antimalarials like ozonides and tetraoxanes [53] are active in the presence of MB-ascorbic acid, MB–*N*-benzyl-1,4-dihydronicotinamide (BNAH), riboflavin–BNAH and riboflavin–NADPH systems and yield identical products to those that were isolated from the reaction of the same antimalarials with Fe(II). According to this proposal, antimalarial peroxides act as oxidants re-oxidising LMB and $FADH_2$, so contributing to depletion of NADPH (Scheme 3).

The observed results suggest that peroxide antimalarials disrupt a highly sensitive redox balance established in the parasite and thereby cause its death. The results also correlate well with the observed SAR of artemisinins, i.e., artemisinins that exhibited low antimalarial activity, like 9-epiartemisinin [54, 55], also show low reactivity under the applied reaction conditions. In addition, high antimalarial activity of 5-nor-4,5-seco-artemisinin **23** can easily be comprehended using the new mechanism proposal. The proposed explanation involves iron-free reactions; however, Fe(III) rapidly oxidises $FADH_2$, and thus contributes to redox cycling without interfering with artemisinins.

3 1,2,4-Trioxolanes (Ozonides)

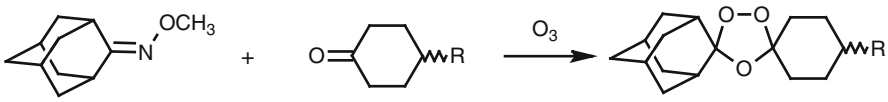
1,2,4-Trioxolanes are a very well-known class of organic compounds. They are intermediates in the transformation of olefins into carbonyls during ozonolysis. It was an unexpected and surprising discovery [56] that ozonides are relatively stable and that many of them express excellent activity against malaria parasites, as do the structurally similar 1,2,4-trioxanes.

3.1 Development of 1,2,4-Trioxolanes: OZ209, OZ277 and OZ339

Preparation of 1,2,4-trioxolanes is relatively simple and relies on the Griesbaum co-ozonolysis of suitable methyl oximes and ketones (Scheme 4) [57–59]. This method provides a highly applicable synthetic approach to tetrasubstituted 1,2,4-trioxolanes (ozonides), otherwise accessible only with great difficulty by ozonolysis of the corresponding alkenes, or by other means.

The synthesis of the final OZ antimalarial drug significantly benefits from the stability of the ozonide peroxide bridge to reduction and alkylation conditions, as well as to other standard reaction conditions. This enables transformation of the initial co-ozonolysis products into a vast array of unsymmetrically substituted ozonides ($n > 500$!) [60]. Usually, the final 1,2,4-trioxolane antimalarials are prepared in 1–4 steps, depending on the modifications required [57–59, 61–63], affording the final products in yields up to 75%. The vast majority of these antimalarials are achiral, which greatly facilitates production in the developmental step. A substantial improvement in drug design in the antimalarial field was the development of the 4-substituted cyclohexyl-adamantyl ozonide (adamantane-2-spiro-3′-1′,2′,4′-trioxaspiro[4.5]decane) chemotype. The advantage of OZ antimalarials is the use of the adamantane moiety that has lipophilic functionality, allowing the opposite part of the molecule to be fine-tuned using a number of polar functional groups, preferably basic in nature [56, 58–62]. Many of the OZ compounds obtained in this way were active in all stages of development of the malaria parasite and are more active than artemether and artesunate, both in vivo and in vitro. All these findings, gathered during much experimentation, contributed to the discovery of amines **OZ209** (**24** as mesylate, Fig. 6) and **OZ277** (**25** as tosylate, Fig. 6) as the best drug candidates.

In comparison with other OZ compounds, trioxolanes **OZ209** and **OZ277** showed superior pharmacokinetic results, such as prolonged half-life and enhanced



Scheme 4 Griesbaum co-ozonolysis: Ozonides

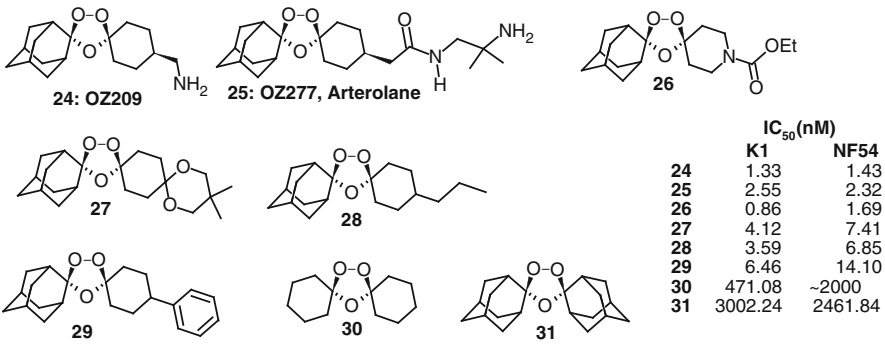


Fig. 6 1,2,4-Troxolanes **OZ209** and **OZ277**, and selected congeners. Compound **OZ277** has been advanced to Phase III clinical trials (vide infra)

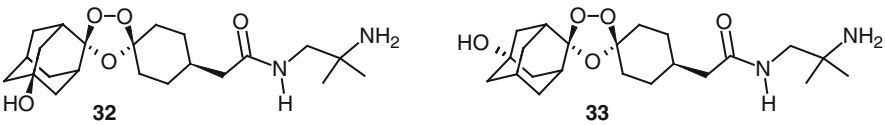


Fig. 7 Microsomal metabolites of ozonide **OZ277**

bioavailability after a single oral dose. Compound **OZ209** had somewhat better antimalarial results and a lower recrudescence level. However, **OZ277** was chosen as the development candidate, primarily because of its improved toxicological profile and reduced concentrations in brain tissue after oral dosing [56]. For example, 2 h after dosing, both **OZ209** and **OZ277** were distributed throughout the liver, kidney, lung and heart, while after 18 h, **OZ277** was detected only in the lungs and in several-fold lower concentrations than **OZ209**.

Unlike **OZ209**, which was quantified in brain tissue after both 2 h and 18 h, trioxolane **OZ277** could not be quantified in this organ at all. In view of potential neurotoxicity issues, these findings were taken as a considerable advantage of **OZ277** over **OZ209**. Trioxolane **OZ277** appeared quite stable to metabolic transformation ($t_{½}$ = 17 h, *p.o.* in healthy rats) [56]. The metabolic profile of **OZ277** was studied with human liver microsomes and only two, monohydroxylated derivatives at the adamantane angular positions (**32** and **33**, Fig. 7), were identified as major metabolites, thus confirming the stability of the trioxolane moiety to metabolic transformation. Interestingly, both metabolites were inactive against the *P. falciparum* K1 strain (IC_{50} > 100 ng/ml), thus demonstrating the indispensability of the unsubstituted spiro-adamantane moiety to the antimalarial activity of **OZ277** (IC_{50} (K1) = 1.0 ng/ml) [64]. Unlike the artemisone products (Scheme 1), OZ metabolic derivatives **32** and **33** very probably lower the overall **OZ277** antimalarial activity. The other derivatives **26–29** (Fig. 5) afforded further insight into SAR in the context of the physico-chemical, biopharmaceutical and toxicological profiles of trioxolanes [61].

Recently, the same authors revealed data for a series of OZ compounds with weak base functional groups, which were responsible for a high antimalarial efficacy in *P. berghei*-infected mice [65]. Their antimalarial efficacy and ADME profiles are equal or superior to **OZ277**. One of the most promising is **OZ339** (as tosylate salt). The two trioxolanes, **OZ339** and **OZ277** are evaluated in Table 1, with artesunate added for comparison. Despite the obvious difference in in vitro activity, both ozonides eradicate parasitaemia below the detectable level 1 day after administration (99.9%, 1 ×10 mg/kg, and 3 × 3 mg/kg). The drug candidate **OZ277** is a powerful fast-acting antimalarial with a 67% cure record at a 3 × 10 mg/kg dosage (mice) [56]. However, at a 3 × 3 mg/kg dosage, the same compound cured no mice, while trioxolane **OZ339** cured 3/5 mice with an excellent survival time of 27 days (**OZ277** had a 2.4 times lower survival time). These good pharmacokinetic characteristics are additionally enhanced by the favourable bioavailability data for **OZ339** (78%, Table 1). In all experiments, artesunate showed inferior activity. Inhibition assays revealed that **OZ339**, like **OZ277**, did not inhibit CYP3A4, CYP2C9 and 2D6CYP450 at concentrations up to 50 μM. Finally, preliminary toxicological experiments indicated that **OZ339** was minimally toxic (liver) and, similar to **OZ277**, demonstrated no detectable signs of neurotoxicity.

As mentioned above, the tolerance of the 1,2,4-trioxolane moiety to diverse reaction conditions [57] and resistance to metabolic transformation [64] enabled the synthesis of a significant number of derivatives and many of them showed very good antimalarial activity, e.g., derivatives **34**–**38** (Fig. 8) [66], and derivatives which contain aliphatic and aromatic amino functional groups or azole heterocycles as substituents (**39**–**45**) (Fig. 8) [62].

The lack of activity of trioxolane **46** [62], and the isolation of inactive hydroxylated **OZ277** metabolites [64], point to the essential contribution of an unsubstituted spiro-adamantane system to the antimalarial properties of this class of compounds.

Many of the examined derivatives exhibited excellent in vitro results, but failed during in vivo tests, toxicity trials or metabolic stability and bioavailability tests. More lipophilic trioxolanes tend to have better oral activities and are metabolically less stable than their more polar counterparts. Such behaviour is consistent with results obtained for other classes of synthetic peroxides. Trioxolanes with a wide range of neutral and basic groups had good antimalarial profiles, unlike derivatives with acidic groups. Based on the collected extensive screening results, the authors concluded that in vitro activities of 1,2,4-trioxolanes are not (always) a reliable predictor of in vivo potency [66]. Rather, their experiments in *P. berghei*-infected mice confirmed that in vivo results were essential for compound differentiation and selection for further metabolic and pharmacokinetic profiling [65].

Trioxolane **OZ277** alkylates haem (Fig. 9) [67], and its in vitro activity against *P. falciparum* is antagonised by DFO [68]. In vitro, artesunate and **OZ277** act antagonistically against *P. falciparum*. These findings, together with only weak interaction with the proposed artemisinin target PfATP6 [44], unlike artemisone [33], suggest that interaction with food vacuole-generated haem is probably how trioxolanes are activated. Further support can be found in the fact that the

Table 1 Comparative data for fast-acting antimalarials: **33** (**OZ339**), **24** (**OZ277**) and sodium **4** (**AS**)

	IC_{50} (ng/mL)		In vivo activity (3 × 3 mg/kg, *p.o.*)[a]							
Compound	K1	NF54	Activity (1 × 10 mg/kg, *p.o.* %)[b]	ER[c]	Activity (%)	Survival (days)	Cure[d] (%)	*t*½ (*i.v.*, min)	V_d (*i.v.*, L/kg)	Bioavailability (*p.o.*, %)
34 (**OZ339**)[e,f]	0.35	0.39	>99.9	0.45	>99.9	27.0	60	83	19	78
25 (**OZ277**)[e,f]	1.0	0.91	99.9	0.32	>99.9	11.4	0	76	16	26
4 (**AS**)[f]	1.3	1.6	67	0.43	70	9.2	0	40 (DHA)[g]	3.0 (DHA)	N.D.

N.D. not dosed

[a]Groups of five *P. berghei*-infected NMRI mice were treated orally on days +1, +2, and + 3 (3 × 3 mg/kg). Activity measured on day +4

[b]Groups of five *P. berghei*-infected NMRI mice were treated orally on day +1 (1 × 10 mg/kg). Activity measured on day +2

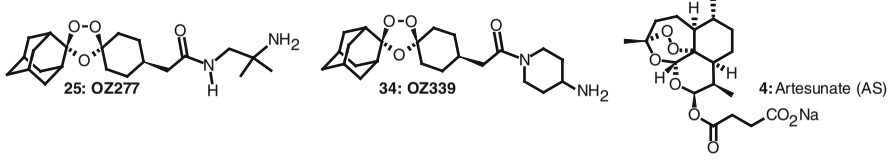
[c]Predicted hepatic extraction ratios (ERs) using human microsomes

[d]Percentage of mice alive on day 30 with no evidence of blood parasites

[e]Tosylate salt

[f]Taken from [61]

[g]Taken from [58, 59]



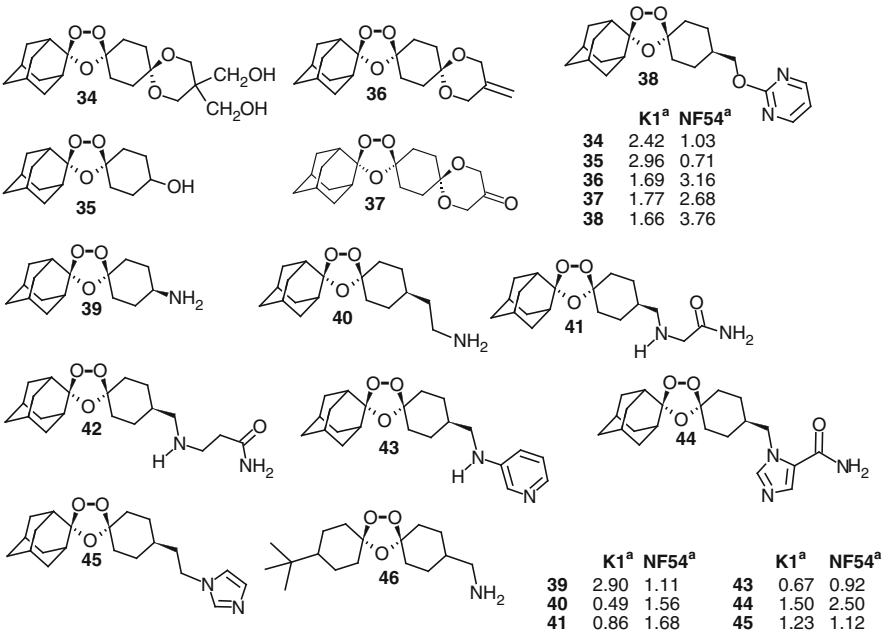


Fig. 8 Structures of ozonides **33**–**45** with their in vitro antimalarial activity

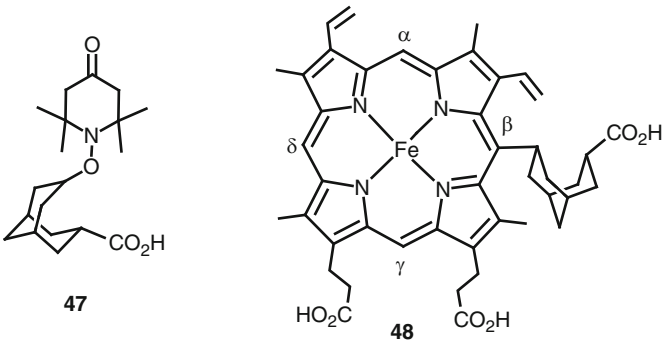
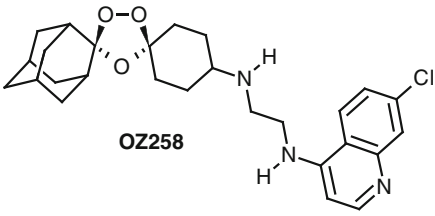


Fig. 9 Structures of adducts of the secondary radical derived from **OZ277** with 4-oxo-TEMPO and haem

stereochemistry of a given compound has little effect on the in vitro potency of trioxolane antimalarials, thereby strongly pointing to the interaction of an antimalarial peroxide (chiral or achiral) with an achiral target (haem). The selectivity of trioxolanes towards infected and not-healthy erythrocytes may be explained based on their reactivity towards free haem and stability in the presence of oxy- and deoxyHb [69].



IC_{50} (nM)		% Activity (10 mg/kg)	
K1	NF54	p.o.	s.c.
11.98	10.74	75	99.95

Fig. 10 Chimaeric trioxolane **OZ258**

Based on the concept that compounds with two integrated pharmacophores might have enhanced activity [70], the chimaeric trioxolane **OZ258** was prepared (Fig. 10) [58, 59]. Although it is very active in vitro against the K1 and NF54 strains of *P. falciparum* and in vivo against the ANKA strain of *P. berghei*, **OZ258** did not achieve the synergic effect of two pharmacophores, especially when compared with trioxolanes **39** and **43**. The same holds for other chloroaminoquinoline and acridine chimaeras [71].

Very often, promising peroxide drugs eradicate parasitaemia quickly, which is crucial for rapid treatment of life-threatening cerebral malaria, and this property is inherently protective against the development of resistance. Since the drugs are typically administered for only a few days and they have short half-lives, the recrudescence of malaria parasites occurs frequently (artemisone cf. [24, 32]; **OZ277** cf. [58]). In an attempt to overcome this problem, artemisinin-based combination therapies (ACTs) are recommended (as indicated for artemisone, see above) by the WHO. The WHO currently distributes, under a no-loss and no-profit agreement with Novartis, the fixed-dose ACT drug Coartem® (artemether 20 mg/lumefantrine 120 mg) [72]. The drug has been recently approved by the FDA for the treatment of acute, uncomplicated malaria infections [73]. Although each ACT is specific [73, 74], the following concept can be applied to all: antimalarial peroxides eliminate most of the infection and the remaining parasites are then exposed to high concentrations of the slow-acting partner drugs; because of the rapid reduction in parasites, the selective pressure for the emergence of mutant parasites is greatly reduced. In accord, **OZ277** (RBX-11160) entered Phase III clinical trials in combination with piperaquine (arterolane maleate + piperaquine phosphate) [75].

3.2 The Second Generation of 1,2,4-Trioxolane Drug Candidates: OZ439

In Phase I clinical trials, the half-life of **OZ277** in healthy volunteers was only about two- to threefold longer than that of dihydroartemisinin. OZ277's possible first-generation ozonide alternative, **OZ339**, only has a slightly higher $t½$ value

Table 2 Comparative data for first and second generation of ozonide antimalarials: **25** (**OZ277**) vs. **49** (**OZ439**)

	IC_{50} (ng/mL)		Postinfection in vivo activity[a]		In vivo prophylactic activity (1 × 30 mg/kg, *p.o.*)[b]					
Compound	K1	NF54	1 × 30 mg/kg, *p.o.* %	Cure[c]	Activity (%)	Survival (days)	Cure[c] (%)	*t*½ (*p.o.*, min)	V_d (*i.v.*, L/kg)	Bioavailability (*p.o.*, %)
25 (**OZ277**)[d,e]	0.71	0.63	99.9	0	0	7	0	55	4	13
49 (**OZ439**) [e]	1.6	1.9	>99.9	100	99.8	>30	100	1380	15	76
4 (**AS**)[e]	1.2	1.5	92	0	21	7	0	40 (DHA)[f]	3.0 (DHA)	N.D.

N.D. not dosed

[a]Groups of *P. berghei*-infected ANKA mice ($n = 5$) were treated orally on day +1 (1 × 30 mg/kg). Activity measured on day +3

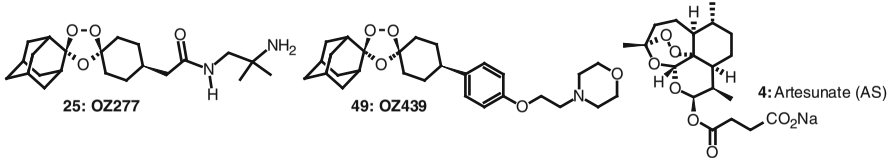
[b]Groups of *P. berghei*-infected ANKA mice ($n = 5$) were treated orally on day −1 (1 × 30 mg/kg). Activity measured on day +3

[c]Percentage of mice alive on day 30 with no evidence of blood parasites

[d]Tosylate salt

[e]Taken from [76]

[f]Intravenous, taken from [58, 59]



(Table 1); so the search for an ozonide with significantly increased half-life continued.

As a result, screening of the second generation of ozonide antimalarials has been completed, recently [76]. Of the several very active OZ compounds of undisclosed structure it appears that the most promising antimalarial candidate is **OZ439** (Table 2) [76]. Initial results indicate that this compound provides single-dose oral cure in a murine malaria model at 20 mg/kg, a situation not known for any of the peroxide antimalarials except for artelininc acid at >7 times higher dose [76]. The second-generation ozonide **OZ439** completed Phase I studies and is currently undergoing Phase IIa clinical trials. In accord with other ozonide antimalarials, **OZ439** is considered to be an Fe(II)-initiated pro-drug. However, it is >50-fold more stable to Fe(II)-mediated degradation compared with **OZ277** [76]. Consistent with proposed Fe(II) degradation of ozonides [67] is the significantly enhanced stability (15–20 times) of **OZ439** over **OZ277** in healthy and infected human and rat blood. This prolonged blood stability and improved pharmacokinetic characteristics (Table 2) led to the positioning of **OZ439** as the current major OZ drug candidate – with respect to post-infection cure (3 × 5 mg/kg/day and 20 mg/kg single dose), and exclusive prophylactic characteristics (Table 2). The absence of metabolic products significantly contributes to overall activity (prophylactic and post-infection) of **OZ439** relative to other peroxide antimalarial drugs [76].

To conclude, as a consequence of intensive and comprehensive research, efficient antimalarial drug candidates of different chemotype have been devised: artemisone and 1,2,4-trioxolane **OZ277**. They are nontoxic, effective at small doses and very probably inexpensive to produce[2] [77]. The same may hold for a prospective backup candidate artemiside and the newest breakthrough drug candidate **OZ439**. It would be of benefit if their combination partner would cure malaria through different mechanisms, since resistance is then less likely to occur.

References

1. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
2. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620
3. Jefford CW (2007) New developments in synthetic peroxidic drugs as artemisinin mimics. Drug Discov Today 12:487–495

[2] The annual demand for artemisinin as a starting material for transformation into semi-synthetic products amounts to ca. 114 tonnes for Coartem® production only. Since synthesis of artemisinin is uneconomical currently, Novartis initiated an increase of the agricultural cultivation of *Artemisia annua* in Kenya, Tanzania and Uganda and extraction of artemisinin therefrom, in addition to Chinese supplies of artemisinin.

4. Lin AJ, Miller RE (1995) Antimalarial activity of new dihydroartemisinin derivatives. 6. alpha-Alkylbenzylic ethers. J Med Chem 38:764–770
5. Lin AJ, Lee M, Klayman DL (1989) Antimalarial activity of new water-soluble dihydroartemisinin derivatives. 2. Stereospecificity of the ether side chain. J Med Chem 32:1249–1252
6. Fishwick J, McLean WG, Edwards G, Ward SA (1995) The toxicity of artemisinin and related compounds on neuronal and glial cells in culture. Chem Biol Interact 96:263–271
7. Wesche DL, DeCoster MA, Tortella FC, Brewer TG (1994) Neurotoxicity of artemisinin analogs *in vitro*. Antimicrob Agents Chemother 38:1813–1819
8. Nontprasert A, Pukrittayakamee S, Prakongpan S, Supanaranond W, Looareesuwan S, White NJ (2002) Assessment of the neurotoxicity of oral dihydroartemisinin in mice. Trans R Soc Trop Med Hyg 96:99–101
9. Davis TM, Binh TQ, Ilett KF, Batty KT, Phuong HL, Chiswell GM, Phuong VD, Agus C (2003) Penetration of dihydroartemisinin into cerebrospinal fluid after administration of intravenous artesunate in severe falciparum malaria. Antimicrob Agents Chemother 47:368–370
10. Li Q, Weina PJ (2010) Severe embryotoxicity of artemisinin derivatives in experimental animals, but possibly safe in pregnant women. Molecules 15:40–57
11. Avery MA, Alvim-Gaston M, Vroman JA, Wu B, Ager A, Peters W, Robinson BL, Charman W (2002) Structure-activity relationships of the antimalarial agent artemisinin. 7. Direct modification of (+)-artemisinin and *in vivo* antimalarial screening of new, potential preclinical antimalarial candidates. J Med Chem 45:4321–4335
12. Chadwick J, Mercer AE, Park BK, Cosstick R, O'Neill PM (2009) Synthesis and biological evaluation of extraordinarily potent C-10 carba artemisinin dimers against *P. falciparum* malaria parasites and HL-60 cancer cells. Bioorg Med Chem 17:1325–1338
13. Pacorel B, Leung SC, Stachulski AV, Davies J, Vivas L, Lander H, Ward SA, Kaiser M, Brun R, O'Neill PM (2010) Modular synthesis and *in vitro* and *in vivo* antimalarial assessment of C-10 pyrrole mannich base derivatives of artemisinin. J Med Chem 53:633–640
14. Posner GH, Parker MH, Northrop J, Elias JS, Ploypradith P, Xie S, Shapiro TA (1999) Orally active, hydrolytically stable, semisynthetic, antimalarial trioxanes in the artemisinin family. J Med Chem 42:300–304
15. Posner GH, Ploypradith P, Parker MH, O'Dowd H, Woo SH, Northrop J, Krasavin M, Dolan P, Kensler TW, Xie S et al (1999) Antimalarial, antiproliferative, and antitumor activities of artemisinin-derived, chemically robust, trioxane dimers. J Med Chem 42:4275–4280
16. Jefford CW (2001) Why artemisinin and certain synthetic peroxides are potent antimalarials. Implications for the mode of action. Curr Med Chem 8:1803–1826
17. McCullough KJ, Nojima M (2001) Recent advances in the chemistry of cyclic peroxides. Curr Org Chem 5:601–636
18. O'Neill PM, Amewu RK, Nixon GL, Bousejra ElGarah F, Mungthin M, Chadwick J, Shone AE, Vivas L, Lander H, Barton V et al (2010) Identification of a 1,2,4,5-tetraoxane antimalarial drug-development candidate (RKA 182) with superior properties to the semisynthetic artemisinins. Angew Chem Int Ed Engl 49:5693–5697
19. Opsenica I, Opsenica D, Smith KS, Milhous WK, Šolaja BA (2008) Chemical stability of the peroxide bond enables diversified synthesis of potent tetraoxane antimalarials. J Med Chem 51:2261–2266
20. Šolaja B, Opsenica DM, Pocsfalvi G, Milhous WK, Kyle DE (2005) Mixed steroidal 1,2,4,5-tetraoxane compounds and methods of making and using thereof. US Patent 6906098 B2
21. Haynes RK, Fugmann B, Stetter J, Rieckmann K, Heilmann HD, Chan HW, Cheung MK, Lam WL, Wong HN, Croft SL et al (2006) Artemisone–a highly active antimalarial drug of the artemisinin class. Angew Chem Int Ed Engl 45:2082–2088
22. Haynes RK, Ho WY, Chan HW, Fugmann B, Stetter J, Croft SL, Vivas L, Peters W, Robinson BL (2004) Highly antimalaria-active artemisinin derivatives: biological activity does not correlate with chemical reactivity. Angew Chem Int Ed Engl 43:1381–1385

23. Bhattacharjee AK, Karle JM (1999) Stereoelectronic properties of antimalarial artemisinin analogues in relation to neurotoxicity. Chem Res Toxicol 12:422–428
24. http://www.public-health.tu-dresden.de/dotnetnuke3/Portals/0/VKliPhaDresden%202005.pdf. Accessed 04 Jan 2011
25. Vivas L, Rattray L, Stewart LB, Robinson BL, Fugmann B, Haynes RK, Peters W, Croft SL (2007) Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone *in vitro* and *in vivo*. J Antimicrob Chemother 59:658–665
26. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T et al (2005) Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 366:1960–1963
27. Ashton M, Hai TN, Sy ND, Huong DX, Van Huong N, Nieu NT, Cong LD (1998) Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. Drug Metab Dispos 26:25–27
28. Svensson US, Ashton M (1999) Identification of the human cytochrome P450 enzymes involved in the *in vitro* metabolism of artemisinin. Br J Clin Pharmacol 48:528–535
29. Nagelschmitz J, Voith B, Wensing G, Roemer A, Fugmann B, Haynes RK, Kotecka BM, Rieckmann KH, Edstein MD (2008) First assessment in humans of the safety, tolerability, pharmacokinetics, and *ex vivo* pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. Antimicrob Agents Chemother 52:3085–3091
30. Brewer TG, Grate SJ, Peggins JO, Weina PJ, Petras JM, Levine BS, Heiffer MH, Schuster BG (1994) Fatal neurotoxicity of arteether and artemether. Am J Trop Med Hyg 51:251–259
31. Obaldia N 3rd, Kotecka BM, Edstein MD, Haynes RK, Fugmann B, Kyle DE, Rieckmann KH (2009) Evaluation of artemisone combinations in *Aotus* monkeys infected with *Plasmodium falciparum*. Antimicrob Agents Chemother 53:3592–3594
32. Waknine-Grinberg JH, Hunt N, Bentura-Marciano A, McQuillan JA, Chan HW, Chan WC, Barenholz Y, Haynes RK, Golenser J (2010) Artemisone effective against murine cerebral malaria. Malar J 9:227
33. Haynes RK, Chan WC, Lung CM, Uhlemann AC, Eckstein U, Taramelli D, Parapini S, Monti D, Krishna S (2007) The Fe^{2+}-mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other artemisinins: the unlikelihood of C-centered radicals as bioactive intermediates. ChemMedChem 2:1480–1497
34. Bousejra-El Garah F, Meunier B, Robert A (2008) The antimalarial artemisone is an efficient heme alkylating agent. Eur J Inorg Chem 2008:2133–2135
35. Laurent SA, Loup C, Mourgues S, Robert A, Meunier B (2005) Heme alkylation by artesunic acid and trioxaquine DU1301, two antimalarial trioxanes. ChemBioChem 6:653–658
36. Robert A, Cazelles J, Meunier B (2001) Characterization of the alkylation product of heme by the antimalarial drug artemisinin. Angew Chem Int Ed Engl 40:1954–1957
37. Robert A, Coppel Y, Meunier B (2002) Alkylation of heme by the antimalarial drug artemisinin. Chem Commun 2002:414–415
38. Robert A, Benoit-Vical F, Claparols C, Meunier B (2005) The antimalarial drug artemisinin alkylates heme in infected mice. Proc Natl Acad Sci USA 102:13676–13680
39. Coghi P, Basilico N, Taramelli D, Chan WC, Haynes RK, Monti D (2009) Interaction of artemisinins with oxyhemoglobin Hb-FeII, Hb-FeII, carboxyHb-FeII, heme-FeII, and carboxyheme FeII: significance for mode of action and implications for therapy of cerebral malaria. ChemMedChem 4:2045–2053
40. Haynes RK (2005) Reply to comments on “Highly antimalaria-active artemisinin derivatives: biological activity does not correlate with chemical reactivity”. Angew Chem Int Ed Engl 44:2064–2065
41. Avery MA, Chong WKM, Bupp JE (1990) Tricyclic analogues of artemisinin: synthesis and antimalarial activity of (+)-4,5=secoartemisinin and (−)-5-nor-4,5-secoartemisinin. J Chem Soc Chem Commun 1487–1489

42. Avery MA, Gao F, Chong WKM, Hendrickson TF, Inman WD, Crews P (1994) Synthesis, conformational analysis, and antimalarial activity of tricyclic analogs of artemisinin. Tetrahedron 50:952–957
43. Haynes RK, Vonwiller SC (1996) The behaviour of qinghaosu (artemisinin) in the presence of non-heme iron(II) and (III). Tetrahedron Lett 37:257–260
44. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 424:957–961
45. Parapini S, Basilico N, Mondani M, Olliaro P, Taramelli D, Monti D (2004) Evidence that haem iron in the malaria parasite is not needed for the antimalarial effects of artemisinin. FEBS Lett 575:91–94
46. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK et al (2005) A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 12:628–629
47. Pooley S, Fatih FA, Krishna S, Gerisch M, Haynes RK, Wong HN, Staines HM (2011) Artemisone uptake in *Plasmodium falciparum*-infected erythrocytes. Antimicrob Agents Chemother 55:550–556
48. Yang YZ, Little B, Meshnick SR (1994) Alkylation of proteins by artemisinin. Effects of heme, pH, and drug structure. Biochem Pharmacol 48:569–573
49. Rosenthal PJ (2001) Antimalarial chemotherapy: mechanisms of action, resistance, and new directions in drug discovery. Humana, Totowa, NJ
50. Akoachere M, Buchholz K, Fischer E, Burhenne J, Haefeli WE, Schirmer RH, Becker K (2005) *In vitro* assessment of methylene blue on chloroquine-sensitive and -resistant *Plasmodium falciparum* strains reveals synergistic action with artemisinins. Antimicrob Agents Chemother 49:4592–4597
51. Buchholz K, Schirmer RH, Eubel JK, Akoachere MB, Dandekar T, Becker K, Gromer S (2008) Interactions of methylene blue with human disulfide reductases and their orthologues from *Plasmodium falciparum*. Antimicrob Agents Chemother 52:183–191
52. Haynes RK, Chan WC, Wong HN, Li KY, Wu WK, Fan KM, Sung HH, Williams ID, Prosperi D, Melato S et al (2010) Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and antimalarial mechanism of action. ChemMedChem 5:1282–1299
53. Haynes RK, Cheu KW, Tang MM, Chen MJ, Guo ZF, Guo ZH, Coghi P, Monti D (2011) Reactions of antimalarial peroxides with each of leucomethylene blue and dihydroflavins: flavin reductase and the cofactor model exemplified. ChemMedChem 6:279–291
54. Avery MA, Gao F, Chong WK, Mehrotra S, Milhous WK (1993) Structure-activity relationships of the antimalarial agent artemisinin. 1. Synthesis and comparative molecular field analysis of C-9 analogs of artemisinin and 10-deoxoartemisinin. J Med Chem 36:4264–4275
55. Jefford CW, Burger U, Millasson-Schmidt P, Bernardinelli G, Robinson BL, Peters W (2000) Epiartemisinin, a remarkably poor antimalarial: implications for the mode of action. Helv Chim Acta 83:1239–1246
56. Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, Dong Y, Dorn A, Hunziker D, Matile H et al (2004) Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430:900–904
57. Tang Y, Dong Y, Karle JM, DiTusa CA, Vennerstrom JL (2004) Synthesis of tetrasubstituted ozonides by the Griesbaum coozonolysis reaction: diastereoselectivity and functional group transformations by post-ozonolysis reactions. J Org Chem 69:6470–6473
58. Vennerstrom JL, Dong Y, Chollet J, Matile H (2002) Spiro and dispiro 1,2,4-trioxolane antimalarials. US Patent 6486199
59. Vennerstrom JL, Tang Y, Dong Y, Chollet J, Matile H, Padmanilayam M, Charman WN (2004) Spiro and dispiro 1,2,4-trioxolane antimalarials. US Patent 6825230

60. Vennerstrom JL, Dong Y, Charman SA, Wittlin S, Chollet J, Wang X, Sriraghavan K, Zhou L, Matile H, Charman WN (2008) Spiro and dispiro 1,2,4-trioxolane antimalarials. US Patent 2008/0125441 A1
61. Dong Y, Chollet J, Matile H, Charman SA, Chiu FC, Charman WN, Scorneaux B, Urwyler H, Santo Tomas J, Scheurer C et al (2005) Spiro and dispiro-1,2,4-trioxolanes as antimalarial peroxides: charting a workable structure-activity relationship using simple prototypes. J Med Chem 48:4953–4961
62. Tang Y, Dong Y, Wittlin S, Charman SA, Chollet J, Chiu FC, Charman WN, Matile H, Urwyler H, Dorn A et al (2007) Weak base dispiro-1,2,4-trioxolanes: potent antimalarial ozonides. Bioorg Med Chem Lett 17:1260–1265
63. Vennerstrom JL, Dong Y, Chollet J, Matile H, Wang X, Sriraghavan K, Charman WN (2008) Spiro and dispiro 1,2,4-trioxolane antimalarials. US Patent 7371778
64. Zhou L, Alker A, Ruf A, Wang X, Chiu FC, Morizzi J, Charman SA, Charman WN, Scheurer C, Wittlin S et al (2008) Characterization of the two major CYP450 metabolites of ozonide (1,2,4-trioxolane) OZ277. Bioorg Med Chem Lett 18:1555–1558
65. Dong Y, Wittlin S, Sriraghavan K, Chollet J, Charman SA, Charman WN, Scheurer C, Urwyler H, Santo Tomas J, Snyder C et al (2010) The structure-activity relationship of the antimalarial ozonide arterolane (OZ277). J Med Chem 53:481–491
66. Dong Y, Tang Y, Chollet J, Matile H, Wittlin S, Charman SA, Charman WN, Tomas JS, Scheurer C, Snyder C et al (2006) Effect of functional group polarity on the antimalarial activity of spiro and dispiro-1,2,4-trioxolanes. Bioorg Med Chem 14:6368–6382
67. Creek DJ, Charman WN, Chiu FC, Prankerd RJ, Dong Y, Vennerstrom JL, Charman SA (2008) Relationship between antimalarial activity and heme alkylation for spiro- and dispiro-1,2,4-trioxolane antimalarials. Antimicrob Agents Chemother 52:1291–1296
68. Uhlemann AC, Wittlin S, Matile H, Bustamante LY, Krishna S (2007) Mechanism of antimalarial action of the synthetic trioxolane RBX11160 (OZ277). Antimicrob Agents Chemother 51:667–672
69. Creek DJ, Ryan E, Charman WN, Chiu FC, Prankerd RJ, Vennerstrom JL, Charman SA (2009) Stability of peroxide antimalarials in the presence of human hemoglobin. Antimicrob Agents Chemother 53:3496–3500
70. O'Neill PM, Posner GH (2004) A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 47:2945–2964
71. Araujo NC, Barton V, Jones M, Stocks PA, Ward SA, Davies J, Bray PG, Shone AE, Cristiano ML, O'Neill PM (2009) Semi-synthetic and synthetic 1,2,4-trioxaquines and 1,2,4-trioxolaquines: synthesis, preliminary SAR and comparison with acridine endoperoxide conjugates. Bioorg Med Chem Lett 19:2038–2043
72. WHO. http://www.who.int/malaria/publications/atoz/coa_website5.pdf. Accessed 19 Dec 2010
73. FDA. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm149559.htm. Accessed 19 Dec 2010
74. MMV. http://www.mmv.org/research-development/project-portfolio/phase-iia. Accessed 20 Dec 2010
75. Ranbaxy. http://www.ranbaxy.com/socialresposbility/mm.aspx. Accessed 20 Dec 2010
76. Charman SA, Arbe-Barnes S, Bathurst IC, Brun R, Campbell M, Charman WN, Chiu FC, Chollet J, Craft JC, Creek DJ et al (2011) Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. Proc Natl Acad Sci USA 108:4400–4405
77. http://www.corporatecitizenship.novartis.com/patients/access-medicines/intellectual-property/biodiversity.shtml. Accessed 05 Jan 2011

Combination Therapy in Light of Emerging Artemisinin Resistance

Harald Noedl

Abstract Within less than a decade virtually all malaria-endemic countries have adopted one of the WHO-recommended artemisinin-based combination therapies (ACTs) for the treatment of falciparum malaria. In 2006, the first cases of clinical artemisinin resistance were reported from the Thai–Cambodian border. A number of factors are likely to have contributed to the development of artemisinin resistance in Southeast Asia. However, current evidence suggests that artemisinin resistance is simply a natural consequence of the massive deployment of ACTs in the region. The potentially devastating implications of resistance to a drug class to which there is currently no real alternative call for cost-effective strategies to extend the useful life spans of currently available antimalarial drugs. At the same time, major efforts to develop novel combination therapies not based on artemisinins are required.

1 Introduction

"The history of malaria contains a great lesson for humanity – that we should be more scientific in our habit of thought, and more practical in our habits of government. The neglect of this lesson has already cost many countries an immense loss in life and prosperity" [1].

With almost 800,000 deaths and hundreds of millions of clinical cases every year, much of what Sir Ronald Ross expressed almost exactly 100 years ago still holds true today [2]. In spite of major advances in the development of new artemisinin-based combination therapies (ACTs), the fact that malaria control is almost entirely reliant on a single class of antimalarials makes malaria control more

H. Noedl (✉)
Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Kinderspitalgasse 15, Vienna 1090, Austria
e-mail: harald.noedl@meduniwien.ac.at

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_11,

vulnerable than ever before. Sir Ronald Ross was a British–Indian physician and entomologist, primarily noted for identifying the link between mosquitoes and malaria in the late nineteenth century for which he was awarded the Nobel Prize in Medicine in 1902. By that time, quinine was already firmly established in western medicine as the treatment of choice for malaria and the detection of the first cases of antimalarial drug resistance to quinine in South America was only a few years away. The discovery of the antimalarial properties of the bark of *Arbor febrifuga* (*Cinchona* spp.), a tree native to tropical South America, in the early seventeenth century had revolutionised malaria therapy. With the extraction of the main *Cinchona* alkaloids by Pelletier and Caventou in the early nineteenth century, the era of the "Peruvian bark" came to an end and the medicinal use of the bark was largely abandoned for the use of one of its main alkaloids, quinine [3]. Quinine was also the first antimalarial drug to which resistance was reported. In fact, the first reports of resistance (a series of treatment failures) emerged as early as in 1910 from South America [4, 5]. Surprisingly, throughout the twentieth century quinine resistance proved to have relatively little impact on the therapeutic use of the drug in most parts of the world and up till now it has never reached a level comparable to that seen with some of the synthetic antimalarials. Quinine is still widely used in malaria therapy and remains one of the most important partner drugs in antimalarial combination therapy. However, in recent years, the class of drugs that has drawn most of the attention and which is the basis for the majority of currently available combination therapies is the artemisinins.

Artemisinin is a sesquiterpene lactone extracted from sweet wormwood (*Artemisia annua* or Chinese: *qinghao*), a common plant native to temperate Asia, but naturalised and recently cultivated throughout the world. The first recorded use of the plant *qinghao* for the treatment of febrile illnesses dates back to the fourth century AD in China. Artemisinin was finally extracted and its antimalarial properties characterised in the early 1970s by Chinese scientists. Since then the use of the parent compound has largely been replaced by the use of its semisynthetic derivatives. Artesunate and artemether, the most commonly used artemisinin derivatives, are hydrolysed to dihydroartemisinin, which has a very short plasma half-life. This also means that virtually all artemisinin derivatives are likely to share an identical mode of action. Artemisinins are active against all asexual stages of malaria parasites and seem to exert some activity also against gametocytes [6]. Although the endoperoxide bridge seems to be vital for their antimalarial activity, the mechanism of action of the artemisinin compounds is still not fully understood [7].

More recently, fully synthetic peroxides have been developed as a promising alternative to currently used artemisinin derivatives. They contain the same peroxide bond that confers the antimalarial activity of artemisinins. One such peroxide, the ozonide OZ277 or arterolane, has recently entered Phase III clinical trials in the form of an arterolane maleate–piperaquine phosphate combination [8]. Originally, these compounds were developed as an alternative to circumvent the dependency on agricultural production of artemisinin. In the light of emerging artemisinin

resistance, their performance against artemisinin-resistant parasites may now decide their future more than anything else.

Unfortunately, the poor pharmacokinetic properties of artemisinins, particularly their short half-lives and unpredictable drug levels in individual patients, translate into substantial treatment failure rates when used as monotherapy, thereby suggesting their combination with longer half-life partner drugs. In the past decade, artemisinin and its semisynthetic derivatives have therefore become the most important basis for antimalarial combination therapies.

2 Combination Therapy

Combination therapy has a long history of use in the treatment of chronic and infectious diseases such as tuberculosis, leprosy, and HIV infections. More recently, it has also been applied to malaria treatment [9–11]. The theory underlying antimalarial combination therapy is that if two drugs are used with different modes of action, and ideally, also different resistance mechanisms, then the per-parasite probability of developing resistance to both drugs is the product of their individual per-parasite probabilities [12]. This is based on the assumption that throughout its history (e.g., chloroquine resistance has independently arisen only on a very limited number of occasions) this would make selection for resistance to a treatment combining two drugs with different modes of action extremely unlikely [13].

The WHO has recently defined antimalarial combination therapy as "the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite. The concept is based on the potential of two or more simultaneously administered schizontocidal drugs with independent modes of action to improve therapeutic efficacy and also to delay the development of resistance to the individual components of the combination" [6]. This definition specifically excludes a number of combinations commonly used in malaria therapy, such as atovaquone–proguanil or sulphadoxine–pyrimethamine, based on the assumption that the respective partners share similar modes of action and further reduces the number of currently available non-ACT combinations [14]. The WHO currently recommends five different ACTs (Table 1).

Compared to chloroquine, the cost of modern combination therapies is almost prohibitive. During the first years of deployment, the high cost of the new combination treatments therefore remained a major limiting factor. However, the past years have seen a major increase in donor funding. The Global Fund to Fight AIDS, Tuberculosis and Malaria alone has committed almost US $20 billion to support large-scale prevention, treatment and care programmes, including the massive deployment of combination therapies.

Table 1 List of ACTs recommended for the treatment of uncomplicated falciparum malaria by the World Health Organization [15]

Artemisinin derivative	Partner drug(s)	Formulation[a]	Resistance
Artemether	Lumefantrine	Coformulated	MDR
Artesunate	Amodiaquine	Coformulated	–
Artesunate	Mefloquine	Coblistered or codispensed	MDR
Artesunate	Sulfadoxine–pyrimethamine	Coblistered or codispensed	–
Dihydroartemisinin	Piperaquine	Coformulated	MDR

[a]The WHO recommends fixed-dose combinations over coblistered or codispensed formulations
MDR: recommended in areas of multidrug resistance (East Asia), artesunate plus mefloquine, or artemether plus lumefantrine or dihydroartemisinin plus piperaquine

3 Pharmacokinetic Mismatch and Compliance

In essence, the main concept behind combination therapy in malaria is to delay the development of resistance, to improve therapeutic efficacy, and to reduce malaria transmission. However, the optimal pharmacokinetic properties for an antimalarial drug (whether used in combination or as a single agent) have been a matter of debate. Ideally, antimalarial drugs should be present in the blood stream just long enough to cover the approximately three parasite life cycles (i.e., 6 days for *P. falciparum*) needed to eliminate all asexual parasites. In reality, this is difficult to achieve and a key to many limitations associated with ACTs seems to be the pharmacokinetic mismatch of the partner drugs [16]. A pharmacokinetic mismatch can also be a major factor contributing to resistance of the long-acting partner drug, which in the later stages of its presence in the blood stream is not protected by the short-acting artemisinins. This is not a problem as long as both drugs are fully efficacious on their own and as long as the drug levels of both drugs remain above the minimum inhibitory concentrations until all asexual parasites have been cleared. However, with a reasonable duration of drug administration, short half-life drugs will not be able to cover the minimum duration of drug exposure. At the same time, long half-life drugs will result inevitably in a long tail, during which the drug levels of the partner drugs will be below the minimum inhibitory concentrations and without protection from the artemisinin compound. This particularly applies to the use of ACTs in high transmission areas [17].

Compliance also remains a key factor in the rational use of antimalarial drugs. In many settings, directly observed therapy is not an option. While rapid elimination reduces the selective pressure by avoiding a long tail of subtherapeutic concentrations, antimalarial drugs with a half-life of less than 24 h (such as artemisinins or quinine) need to be administered for at least 7 days to be fully efficacious. Although compliance with malaria treatment is difficult to assess in study settings and shows significant variations across different studies, there is a general consensus that antimalarial treatment regimens lasting up to 3 days are likely to give good compliance [18].

4 Coformulation

Another potential problem of treating patients with more than one antimalarial drug is the fact that many currently available combination therapies are not coformulated, greatly increasing complexity of treatment and the chances of misuse. Currently, the only widely used coformulated combination is artemether–lumefantrine. More recently, coformulated combinations of artesunate–amodiaquine and dihydroartemisinin–piperaquine have become available but coformulated combinations of many other ACTs are still unavailable. Even though coformulated, artemether–lumefantrine remains a relatively complex regimen (with an adult dose of four tablets twice daily for 3 days) and compliance, and therefore programmatic effectiveness, is not optimal [19].

5 ACT and Antimalarial Drug Resistance

Throughout the past 100 years, drug resistance has emerged as one of the biggest challenges for malaria control. The extensive deployment of antimalarial drugs since the introduction of chloroquine in the 1940s has provided a remarkable selection pressure on malaria parasites to evolve resistance mechanisms to virtually all available antimalarial drugs. In essence, it is continuous drug pressure that results in the selection of parasite populations with genetically reduced drug sensitivity. The widespread and indiscriminate use of antimalarial drugs places a strong selective pressure on malaria parasites to develop resistance. Malaria parasites can acquire high levels of resistance, both in the individual parasite as well as on a population basis. The high degree of resistance expressed by malaria parasites is at least in part attributable to their high diversity and genetic complexity, resulting in a variety of potential mechanisms to evade drug activity. This way *P. falciparum* has developed resistance to virtually all antimalarials in current use, drugs that once were considered the front line against the disease. However, the geographical distribution and extent of resistance to any single antimalarial drug show major variations. Originally believed to be limited to *P. falciparum*, antimalarial drug resistance is now also known to affect other species [20]. *P. vivax* has rapidly developed resistance to sulfadoxine–pyrimethamine in many parts of the world, whereas high-level resistance to chloroquine remains confined largely to Indonesia, East Timor, Papua New Guinea and other parts of Oceania [6].

In the late 1990s, combination therapy was introduced to overcome the quickening pace of drug resistance development in Southeast Asia. However, by that time drug resistance had reached many of the potential partner drugs or at least drugs structurally related to those used in combination with artemisinins. This particularly applies to mefloquine, an arylaminoalcohol, which had previously been used extensively as monotherapy in Southeast Asia. Based on large-scale field trials starting in 1983 mefloquine was introduced as standard therapy by the

Thai Ministry of Public Health as early as 1985 to overcome increasing chloroquine and sulfadoxine–pyrimethamine resistance [21]. Interestingly, mefloquine was used in combination with sulfadoxine–pyrimethamine initially before being deployed as monotherapy. Rising numbers of failures with the standard-dose mefloquine (15 mg/kg) resulted in an increase of the dose to 25 mg/kg and in 1995 mefloquine monotherapy had to be replaced by the combination of mefloquine with artesunate, making this combination the first ACT to be deployed on a large scale in Southeast Asia.

In an attempt to limit the impact of increasing levels of resistance to traditional antimalarial drugs, in 2001 the World Health Organization recommended that all countries experiencing resistance to conventional monotherapies, such as chloroquine, sulfadoxine–pyrimethamine, or mefloquine should use combination therapies, preferably based on artemisinin derivatives for the treatment of uncomplicated falciparum malaria [21]. However, although ACTs have shown high efficacy in the treatment of malaria in Southeast Asia, where transmission is typically low, concerns remain about their long-term implementation as first-line therapy in high-transmission areas in Africa [19, 22].

6 Cross-resistance

The problem of antimalarial drug resistance is even further aggravated by the existence of cross-resistance among drugs with related chemical structures. This particularly applies to the 4-aminoquinolines (e.g. chloroquine–amodiaquine) and the arylaminoalcohols (e.g. mefloquine–lumefantrine) but also to artemisinin and its semisynthetic derivatives (e.g. artesunate–artemether). Malaria control has largely been relying on a small number of structurally related drugs essentially belonging to just a very few different classes. Once malaria parasites develop resistance to a single member of any of these classes, their sensitivity to most other antimalarials sharing a similar mode of action (i.e., typically belonging to the same class) is also compromised. This means that (e.g., in an area like Southeast Asia where the combination of artesunate and mefloquine is loosing its clinical efficacy) the introduction of ACTs using chemically related compounds, such as artemether–lumefantrine, may not be an option.

Interestingly, activity correlations derived from in vitro studies indicate that in spite of their different chemical structure there may be a certain level of cross sensitivity between artemisinin derivatives and certain arylaminoalcohols, currently the most commonly used partner drugs in ACTs [23]. The existence of this link is also supported by the potential role that the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene may be playing in simultaneously mediating the sensitivity to arylaminoalcohols and artemisinins [24].

7 Resistance to Partner Drugs

Preventing resistance to the partner drugs is obviously crucial. If the partner drug is not 100% successful in eliminating the parasites surviving the initial impact from the (subcurative) 3-day artemisinin treatment, ACTs are likely to select for artemisinin resistance. However, resistance has been reported against most of the commonly used partner drugs or at least to structurally closely related drugs resulting in activities considerably below 100%. Currently the most important partner drugs used in ACTs are lumefantrine, mefloquine, amodiaquine, and more recently piperaquine [22]. Mefloquine and lumefantrine are structurally related and belong to the class of the arylaminoalcohol antimalarials, whereas amodiaquine and piperaquine are both closely related to chloroquine.

Mefloquine is a synthetic antimalarial widely used throughout Southeast Asia as a combination partner for artesunate. It was introduced in Thailand in the mid 1980s. By the mid 1990s, resistance had reached a level that necessitated its combination with artesunate to reach adequate cure rates [21]. In spite of high levels of mefloquine resistance, particularly in Thailand, Cambodia, and Myanmar, mefloquine remains the most important ACT partner drug in the region.

Lumefantrine is commonly used coformulated with artemether and has never been used in monotherapy on any significant scale. Although clinical resistance to lumefantrine has not explicitly been reported, there is a strong indication of cross-resistance with mefloquine [25]. The use of lumefantrine is therefore not advisable in areas where high levels of mefloquine resistance have been reported.

Amodiaquine is a 4-aminoquinoline antimalarial originally developed in the 1940s. It is structurally closely related to chloroquine but due to its higher potency shows considerable activity also against chloroquine-resistant parasites. Resistance to both drugs also seems to be mediated by the same genetic mechanism [26]. Resistance to amodiaquine was reported soon after the advent of chloroquine resistance but has never reached its magnitude [27].

Although piperaquine, a bisquinolone antimalarial, is still considered to be a relatively new antimalarial drug throughout most of the malaria-endemic world, it has a long history of use in malaria treatment and prophylaxis in China [28]. Consequently, high levels of resistance have been reported from parts of southern China and resistance can relatively easily be induced in a *P. berghei* model [29].

8 Artemisinin Resistance

The statement "Resistance has arisen to all classes of antimalarials except, as yet, to the artemisinin derivatives" [6] in the WHO treatment guidelines from 2006 unfortunately does not hold true any longer. Clinical artemisinin resistance was first identified in 2006 in Ta Sanh, a small town in close proximity to the Thai–Cambodian border, a known hotspot of antimalarial drug resistance [30].

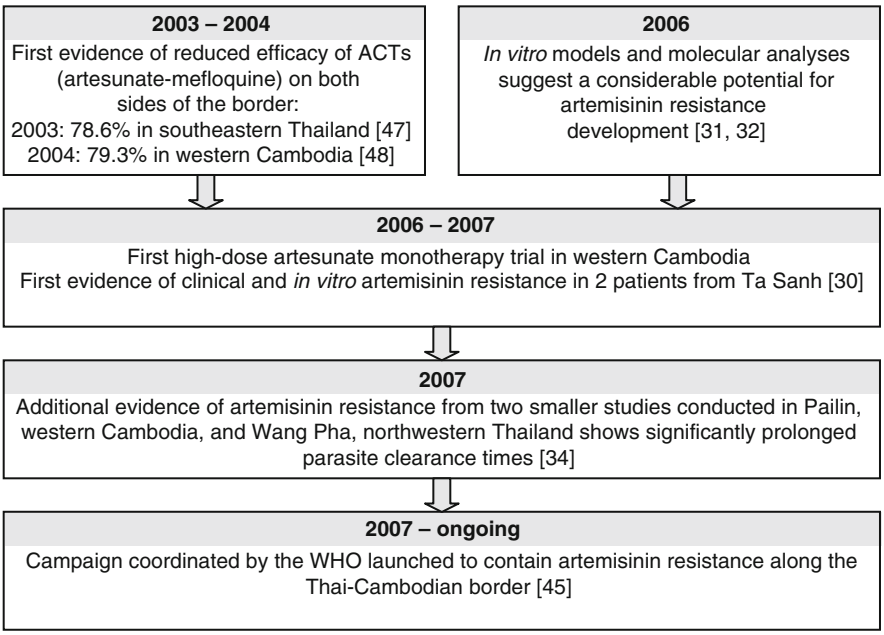


Fig. 1 Evidence of emerging artemisinin resistance along the Thai–Cambodian border

Even before the discovery of clinical resistance, in vitro models and molecular analysis had suggested a considerable potential for resistance development [7, 31, 32]. Moreover, ex vivo data and clinical treatment response seem to indicate that artemisinin sensitivity is also compromised in western Thailand [33, 34] (Fig. 1).

Not since the discovery of chloroquine resistance in the 1950s has malaria control been reliant upon the efficacy of a single class of drugs as much as it does currently. In the past 10 years, virtually all falciparum malaria-endemic countries have adopted some kind of ACT as first- or second-line therapy for uncomplicated falciparum malaria. In the absence of a defined artemisinin resistance mechanism and mechanism of action, as well as data from clinical trials using the new synthetic peroxides, it is hard to tell what impact the recent developments in Southeast Asia will have. However, in the current situation losing a single drug to resistance could potentially endanger virtually all malaria control efforts worldwide.

9 Defining Artemisinin Resistance

Defining artemisinin resistance remains a major challenge. The most commonly used definition of antimalarial drug resistance dates back to 1973 and defines resistance as “The ability of a parasite strain to survive and/or multiply despite

the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" [38]. Although, – with minor modifications, – this definition remains valid as of today, it was developed for traditional monotherapies and in its original version has therefore only limited applicability to modern combination treatments. The most obvious problem in defining resistance to combination therapies is the fact that, with two or more combination partners, resistance might arise to a single component without ever becoming clinically evident. Since in ACTs artemisinins are generally responsible for the initial reduction in the parasite burden, the most obvious clinical parameter indicating reduced susceptibility to artemisinins is prolonged parasite clearance [39, 40]. Although data from ACT trials can provide important initial data on compromised drug sensitivity, a detailed assessment of treatment response to artemisinins requires extensive controlled monotherapy studies with artemisinin derivatives (including basic pharmacokinetics and a reliable way of excluding reinfections). These studies are currently being conducted in Asia and Africa. Ex vivo drug sensitivity data can be extremely helpful in interpreting geographical or temporal trends but need to be interpreted in a broader context with clinical data [33]. In spite of a number of interesting leads, as yet reliable molecular markers of artemisinin resistance have not been identified [31]. Artemisinin resistance can have major implications for malaria control programs in the affected countries and should therefore only be considered after careful analysis of treatment response parameters and treatment success in relation to ex vivo drug sensitivity.

10 The Causes of Artemisinin Resistance

The general view has been that several factors protect artemisinins from the development of resistance: the short plasma half-life of both the parent compound and its active metabolite, the rapidity with which the drugs clear asexual parasites, and the presence of an effective partner drug from a different class of antimalarials, which is expected to protect the artemisinin component [41]. It has been hypothesised that the de novo emergence of antimalarial drug resistance can be prevented by use of combination therapies [12, 42]. The assumption is that because of the logarithmic distribution of parasite numbers in human malaria infections, inadequately treated high biomass infections are a major source of de novo emergence of resistance. However, the recent events suggest that the hope that resistance can actually be prevented through combinations may have been overly optimistic.

Much of the blame for emerging artemisinin resistance in Southeast Asia has been assigned to the extensive use of artemisinin monotherapy. Compliance issues and counterfeited or substandard tablets that contain smaller amounts of or less active ingredients are considered to be additional sources of drug pressure [43]. Specific epidemiological, pharmacokinetic, and parasite factors in Southeast Asia have also been implicated in the development of artemisinin resistance [44]. Consequently, even before artemisinin resistance had been reported for the first

time the WHO banned artemisinin monotherapy in an attempt to protect the artemisinins and to slow down the development of resistance. Interestingly Vietnam, a country with one of the longest histories of artemisinin monotherapy use in Southeast Asia, was by far not he first to report artemisinin resistance. In Vietnam, artemisinins have been used for malaria control since 1989 and although the current national guidelines recommend the use of ACTs, artemisinin and artesunate are still widely available as monotherapy through the private sector [45]. Vietnam is also one of the few countries where artemisinin monotherapy can be linked directly to a highly successful malaria control program. Between 1991 and 2006, malaria cases in the country have diminished from 1,672,000 clinical cases with 4,650 deaths, originally, to 91,635 with 43 deaths [46].

Although the development of artemisinin resistance is likely to have been a complex multifactorial event, the actual explanation for artemisinin resistance is likely to be rather simple. ACTs have simply gone down the same road that all previous antimalarials have. Artemisinin resistance is probably a natural consequence of the extensive deployment of ACTs and was bound to happen sooner or later. "Preventing" artemisinin resistance was never a real option.

11 Measures to Limit the Spread of Resistance

When the first evidence of artemisinin resistance became available in 2006, the World Health Organization launched an ambitious campaign to contain artemisinin resistance along the Thai–Cambodian border. These efforts involve the early detection and rapid treatment of all malaria infections on both sides of the border, preferably with a non-ACT combination therapy. In addition, the deployment of insecticide-treated nets to decrease malaria transmission and the screening and treatment of migrants were intensified, together with a more thorough mapping of the geographic boundaries of artemisinin resistance [15, 35]. Recently, Maude et al. concluded that containment of artemisinin-resistant malaria could also be achieved by eliminating malaria using ACT [47]. However, as ACTs are more effective against infections with artemisinin-sensitive parasites resulting in a relative increase in the proportion of artemisinin-resistant parasite isolates, this approach would require malaria elimination down to the last parasite. Unfortunately, this is unlikely to ever happen in a landlocked environment surrounded by malaria-endemic countries.

Although the history of malaria control teaches us that the Thai–Cambodian border has always been a hotspot of antimalarial drug resistance development, it also teaches us that sooner or later resistance is likely to emerge independently in other parts of the world. With the unprecedented deployment of ACTs throughout the malaria-endemic world, artemisinin resistance is likely to eventually emerge in other parts of the world.

The potentially devastating implications of resistance to a drug to which there is currently no real alternative calls for cost-effective strategies to extend the useful

life spans of currently available antimalarial drugs while at the same time investing into major efforts to develop novel compounds as a replacement for the artemisinins.

References

1. Ross R (1911) The prevention of malaria. John Murray, London
2. World Health Organization (2010) World Malaria Report 2009. WHO, Geneva
3. Bruce-Chwatt L (1988) History of malaria from prehistory to eradication. In: Wernsdorfer WH, McGregor IA (eds) Malaria. Principles and Practice of malariology. Churchill Livingstone, Edinburgh, pp 1–59
4. Nocht B, Werner H (1910) Beobachtungen ueber eine relative Chininresistenz bei Malaria aus Brasilien. Dtsch Med Wschr 36:1557–1560
5. Neiva A (1910) Ueber die Bildung einer chininresistenten Rasse des Malariaparasiten. Mem Inst Oswaldo Cruz 2:131–140
6. Chen PQ, Li GQ, Guo XB, He KR, Fu YX, Fu LC, Song Y (1994) The infectivity of gametocytes of *Plasmodium falciparum* from patients treated with artemisinin. Chin Med J 107:709–711
7. Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O (2006) Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. Trends Mol Med 12:200–205
8. Olliaro P, Wells TN (2009) The global portfolio of new antimalarial medicines under development. Clin Pharmacol Ther 85(6):584–595
9. Peters W (1969) Drug resistance in malaria–a perspective. Trans R Soc Trop Med Hyg 63:25–45
10. Li GQ, Arnold K, Guo XB, Jian HX, Fu LC (1984) Randomised comparative study of mefloquine, qinghaosu and pyrimethamine-sulfadoxine in patients with falciparum malaria. Lancet 2:1360–1361
11. Peters W (1990) The prevention of antimalarial drug resistance. Pharmacol Ther 47:499–508
12. White NJ, Pongtavornpinyo W (2003) The *de novo* selection of drug-resistant malaria parasites. Proc Biol Sci 270:545–554
13. Su X, Kirkman LA, Fujioka H, Wellems TE (1997) Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. Cell 91:593–603
14. Kremsner PG, Krishna S (2004) Antimalarial combinations. Lancet 364:285–294
15. Campbell CC (2009) Malaria control – addressing challenges to ambitious goals. N Engl J Med 361:522–523
16. Giao PT, de Vries PJ (2001) Pharmacokinetic interactions of antimalarial agents. Clin Pharmacokinet 40:343–373
17. Bloland PB, Ettling M, Meek S (2004) Combination therapy for malaria in Africa: hype or hope? Bull World Health Organ 78:1378–1388
18. Congpuong K, Bualombai P, Banmairuroi V, Na-Bangchang K (2010) Compliance with a three-day course of artesunate-mefloquine combination and baseline anti-malarial treatment in an area of Thailand with highly multidrug resistant falciparum malaria. Malar J 9:43
19. Bloland PB (2003) A contrarian view of malaria therapy policy in Africa. Am J Trop Med Hyg 68:125–126
20. Baird JK, Basri H, Purnomo BMJ, Subianto B, Patchen LC, Hoffman SL (1991) Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. Am J Trop Med Hyg 44:547–552
21. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR (2002) Epidemiology of drug-resistant malaria. Lancet Infect Dis 2:209–218

22. Eastman RT, Fidock DA (2009) Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. Nat Rev Microbiol 7:864–874
23. Noedl H, Wernsdorfer WH, Krudsood S, Wilairatana P, Viriyavejakul P, Kollaritsch H, Wiedermann G, Looareesuwan S (2001) *In vivo-in vitro* model for the assessment of clinically relevant antimalarial cross-resistance. Am J Trop Med Hyg 65:696–699
24. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA (2006) Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 194:528–535
25. Basco LK, Bickii J, Ringwald P (1998) *In vitro* activity of lumefantrine (benflumetol) against clinical isolates of *Plasmodium falciparum* in Yaoundé, Cameroon. Antimicrob Agents Chemother 42:2347–2351
26. Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Björkman A (2006) Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of *pfcrt* 76 T and *pfmdr1* 86Y. Infect Genet Evol 6(4):309–314
27. Olliaro P, Mussano P (2003) Amodiaquine for treating malaria. Cochrane Database Syst Rev CD000016
28. Tran TH, Dolecek C, Pham PM, Nguyen TD, Nguyen TT, Le HT, Dong TH, Tran TT, Stepniewska K, White NJ, Farrar J (2004) Dihydroartemisinin-piperaquine against multidrug-resistant *Plasmodium falciparum* malaria in Vietnam: randomised clinical trial. Lancet 363:18–22
29. Giao PT, de Vries PJ, le Hung Q, Binh TQ, Nam NV, Kager PA (2004) CV8, a new combination of dihydroartemisinin, piperaquine, trimethoprim and primaquine, compared with atovaquone-proguanil against falciparum malaria in Vietnam. Trop Med Int Health 9:209–216
30. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM; Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620
31. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O (2006) Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 366:1960–1963
32. Krishna S, Pulcini S, Fatih F, Staines H (2010) Artemisinins and the biological basis for the PfATP6/SERCA hypothesis. Trends Parasitol 26(11):517–23
33. Noedl H, Socheat D, Satimai W (2009) Artemisinin-resistant malaria in Asia. N Engl J Med 361:540–541
34. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
35. Enserink M (2008) Signs of drug resistance rattle experts, Trigger Bold Plan. Science 322:1776
36. Vijaykadga S, Rojanawatsirivej C, Cholpol S, Phoungmanee D, Nakavej A, Wongsrichanalai C (2006) *In vivo* sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. Trop Med Int Health 11:211–219
37. Denis MB, Tsuyuoka R, Poravuth Y, Narann TS, Seila S, Lim C, Incardona S, Lim P, Sem R, Socheat D et al (2006) Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. Trop Med Int Health 11:1360–1366
38. World Health Organization (1973) Chemotherapy of malaria and resistance to antimalarials. Report of a WHO Scientific Group. WHO Technical Report Series, Geneva, 529
39. Noedl H (2005) Artemisinin resistance: how can we find it? Trends Parasitol 21:404–405
40. Stepniewska K, Ashley E, Lee SJ, Anstey N, Barnes KI, Binh TQ, D'Alessandro U, Day NP, de Vries PJ, Dorsey G et al (2010) *In vivo* parasitological measures of artemisinin susceptibility. J Infect Dis 201:570–579

41. White NJ, Nosten F, Looareesuwan S, Watkins WM, Marsh K, Snow RW, Kokwaro G, Ouma J, Hien TT, Molyneux ME et al (1999) Averting a malaria disaster. Lancet 353:1965–1967
42. White NJ (1998) Preventing antimalarial drug resistance through combinations. Drug Resist Updat 1:3–9
43. Newton PN, Dondorp A, Green M, Mayxay M, White NJ (2003) Counterfeit artesunate antimalarials in southeast Asia. Lancet 362:169
44. Dondorp AM, Yeung S, White L, Nguon C, Day NP, Socheat D, von Seidlein L (2010) Artemisinin resistance: current status and scenarios for containment. Nat Rev Microbiol 8:272–280
45. Thanh NV, Toan TQ, Cowman AF, Casey GJ, Phuc BQ, Tien NT, Hung NM, Biggs BA (2010) Monitoring for *Plasmodium falciparum* drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998–2009. Malar J 9:181
46. Thang NDEA, le Hung X, le Thuan K, Xa NX, Thanh NN, Ky PV, Coosemans M, Speybroeck N, D'Alessandro U (2009) Rapid decrease of malaria morbidity following the introduction of community-based monitoring in a rural area of central Vietnam. Malar J 8:3
47. Maude RJ, Pontavornpinyo W, Saralamba S, Aguas R, Yeung S, Dondorp AM, Day NP, White NJ, White LJ (2009) The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. Malar J 8:31
48. World Health Organization (2006) Guidelines for the treatment of malaria. WHO, Geneva

New Medicines to Combat Malaria: An Overview of the Global Pipeline of Therapeutics

Timothy N.C. Wells

Abstract Over the last 5 years, there has been an increased investment in malaria medicines, with the emergence of a range of new fixed dose artemisinin combination therapies (FACTs). Of the six FACTS, two are now approved and prequalified by the WHO, with another two submitted for stringent regulator approval. Malaria treatments are therefore available to more than 160 million patients, which cure the disease in more than 98% of cases, with 3 days of therapy, and cost as little as $0.30 per treatment for infants. Molecules currently in the pipeline offer the possibility that this could be replaced by a single dose cure in the next few years. Many new compounds have entered the pipeline as a result of advances in phenotypic screening and new targets identified from the parasite genomes. Artemisinin resistance has been confirmed in Cambodia, and new classes of medicines will be needed in case artemisinin resistance spreads. The recent call for the eradication of malaria has set new objectives for the drug discovery and development field. There is an additional focus on having compounds, which can block transmission. In addition, safe medicines to kill liver stages, and block the relapse of *P. vivax* and *P. ovale* are needed, which are more convenient than the 14-day primaquine treatment, and better tolerated in G6PD-deficient patients. The next decade will be a defining one, as we implement new generations of therapies in the field, and bring forward the ones needed for the next stages of malaria eradication.

1 Introduction

The challenges of malaria drug discovery and development have never been greater. On the positive side, there is a wealth of activity all the way from early discovery through registration of new medicines through to optimizing their use in

T.N.C. Wells (✉)
Medicines for Malaria Venture, 20 route de Pré-Bois, Geneva CH-1215, Switzerland
e-mail: wellst@mmv.org

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_12,

Phase IV clinical trials. In the last 2 years, two classes of fixed dose artemisinin combination therapies have been approved by the World Health Authority, and treat more than 160 million patients. Two more are currently being reviewed by the European Medicines Agency (EMA), in the first step toward World Health Organization (WHO) prequalification. This brings the total number of fixed dose artemisinin combination therapies to six; each has its particular strengths (Table 1). There is such a wide choice for the "customer" – in most cases the national malaria control programs, is positive. These medicines are affordable – costing as little as $0.30 for a child dose and $1.20 for an adult dose. The approval by WHO of two of them means they can be supplied at subsidized prices with the help of international agencies, or for free in Africa. There is a continual need to build on this success by optimizing this therapeutics for their use in the clinic, especially with pediatric patients.

In the midst of this success, there is always the need to keep on improving the medicines we have. Resistance to malaria drugs is always a possibility. With the ongoing campaign to eradicate malaria [1], the urgency to have new classes of drugs is compounded since the more we use therapies, and the closer we get to eradication, the more likely resistance is to become evident [1].

Over the last 12 months, since the pipeline was last reviewed [2], there has been a lot of progress (Fig. 1 shows the current state of the global antimalarial drug portfolio, not simply the projects which are part of collaborations with Medicines for Malaria Venture). There are many challenges that any medicines must address [3]. First, to simplify treatment regimens, with the hope of bringing forward a single dose cure. Second, to have medicines that will block transmission of the parasite from one infected individual to another via the mosquito and thus break the cycle of infection. Third, to target dormant *P. vivax* and *P. ovale* forms in the liver, known as hypnozoites, which have the potential to relapse after a few weeks or even months, causing new outbreaks of disease, in the absence of an infectious bite. Finally, new medicines are urgently needed to overcome the potential threat of artemisinin resistance.

Malaria offers challenges beyond those seen in other therapeutic areas; most of the patients are children under 5 years of age, half of them are less than 2 years, and the disease is especially prevalent in expectant mothers. This places additional stringency on the safety profiles of the potential new medicines. The portfolio of projects, which we hope will address these challenges, comes from a variety of sources. Some are from rationally designed approaches using clinically validated targets, and as much as possible based on the technologies of rational drug design. Others are coming from new, innovative high-throughput screens where millions of compounds have been tested for their activity on the whole parasite. This has radically changed the face of discovery. We now have tens of thousands of starting points for medicinal chemistry active at submicromolar concentrations, where only 5 years ago there was just a handful. Taken together, there are big challenges ahead, but the next decade promises to be a very exciting one. There will be new medicines launched to help the short-term fight against the parasite, alongside bednets, insecticides and the potential contribution of vaccines; and new classes of

Table 1 Characteristics of fixed dose artemisinin combination therapies available or soon to be launched

Fixed dose ACT	Coartem-D	Coarsucam	Eurartesim	Pyramax	AS/MQ	Arco
Partner	Novartis/MMV	Sanofi/DNDi	Sigma-Tau/Pfizer/MMV	ShinPoong/MMV	Farmanguinhos DNDi; Mepha	Kunming Pharmaceutical
	Artemether Lumefantrine	Artesunate Amodiaquine	Dihydro artemesinin Piperaquine	Artesunate Pyronaridine	Artesunate Mefloquine	Artemisinin Naphthoquine
Launch	1Q'09	4Q'08	4Q'11	1Q'12	–	
Key selling point	Market leader safety: >300 million treatments Anti-Gametocyte?	First-line therapy in Francophone Africa; Once per day	Long half-life Once per day; Good posttreatment prophylaxis at 42 days	*P. vivax* clinical data (schizonticide) Potential combination with Primaquine?	First-line treatment in Thailand *P. vivax*	Single dose cure
Key weakness	Twice per day, strong food effect	resistance	Stability – interaction between DHA and PQP No pediatric formulation	Limited field use single supplier	Psychiatric, GI adverse events. No SRA or WHO approval. Expensive	Limited data available. No SRA or WHO approval. Expensive
Stability	24 months	36 months	18–24 months	24 months	No data	No data
Pediatric	formulation Dispersible taste masked	Dissolves no taste mask	Pediatric version under development for 2013	Sachet taste masked for 2012		
Comments			DHA-piperaquine marketed by Holley-Cotec but no SRA or WHO approval			

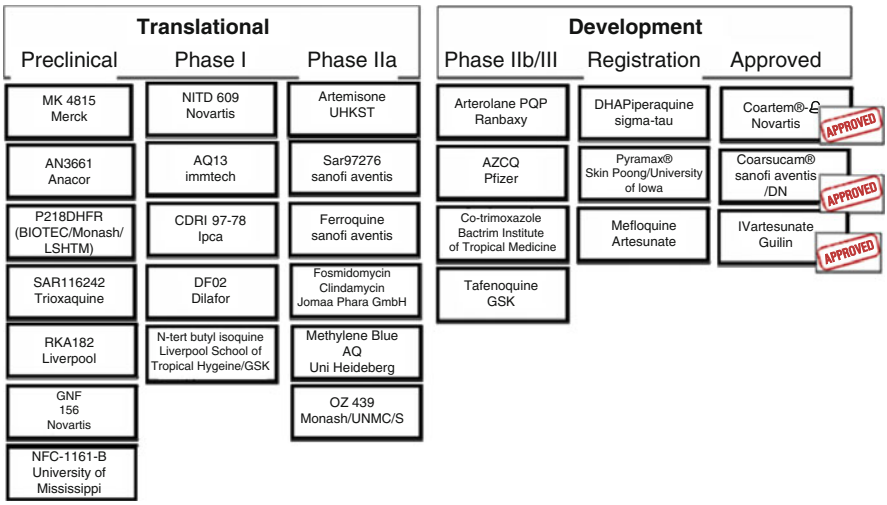


Fig. 1 Global portfolio of medicines under development for malaria March 2011. Projects are only reported which have already entered lead optimization. Preclinical refers to molecules in the process of obtaining GLP safety, toxicology and pharmacokinetic data

medicines being developed, which will help drive the long-term agenda leading to eradication.

2 The Next Generation of Artemisinin Combination Therapies: New Medicines About to Be Registered

Artemisinin was brought forward as a new treatment for malaria over 40 years ago [4] and has long been known to be an effective therapy against fevers. It acts rapidly to kill parasites, clearing fever and bringing parasitemia below the level of detection within 24 h. On its own, 7 days of treatment is needed for a cure in the majority of patients, and so a second medicine is needed to sustain the antiparasitic activity in a 3-day regimen. Artemisinin combination therapies are now extremely effective, surpassing the WHO's guidelines that 95% of patients should not have recrudesced after 28 days – many clinical trials showing efficacies of 98–99%. The combination partner also reduces the possibility of artemisinin resistance emerging, and to reinforce this value the WHO in January 2006 withdrew its recommendation for the use of artemisinin-based monotherapy for the treatment of uncomplicated malaria, and since has been campaigning for its complete withdrawal. Fixed dose combinations therefore offer two advantages, first a much simpler regimen, and second that artemisinin monotherapy is no longer an option for the patient (older combination therapies were coblistered, and field workers reported that often only the artemisinin tablet was taken). The last 2 years have marked a turning point for

these fixed dose combinations. Two have been prequalified by the WHO, or by Stringent Regulatory Authorities (Tables 1 and 2). Coartem and the pediatric taste-masked dispersible form Coartem-D (artemether–lumefantrine, by Novartis in collaboration with MMV), and Coarsucam (artesunate–amodiaquine, by sanofi-aventis in collaboration with Drugs for Neglected Diseases Initiative, DND*i*), as has been discussed in chapter "Artemisinins: Artemisinin, Dihydroartemisinin, Artemether and Artesunate". Enough of these WHO prequalified medicines, and their generic versions were purchased by disease endemic countries to treat more than 160 million patients in 2010. This represents almost two thirds of the people who need treatment. In the past, cost has often been cited as a barrier to health care. Current medicines are produced with the cost of an adult course of treatment being generally around US $1.20, and a pediatric dose being a quarter of this price, and this represents a significant drop from the cost of manufacture of around $2.50 10 years ago. Several generic versions of artemether–lumefantrine are now being produced, which brings additional price competition into the market. WHO prequalification is a necessary control step here – generic medicines which have not been approved by a stringent regulatory agency must show evidence that they are manufactured to Good Manufacturing Practice standards, and also show clinical data to confirm bioequivalency to the innovator medicine (see http://www.ema.europa.eu/pdfs/human/qwp/140198enrev1.pdf for more details).

A second wave of fixed dose artemisinin combination therapies has progressed through pivotal clinical trials in the last 2 years, with two more being submitted to regulatory agencies in 2010. The first is dihydroartemisinin–piperaquine, (provisionally planned to be marketed as Eurartesim). This has been developed by sigma-tau in collaboration with MMV, which was submitted for review by the EMA, in July 2009 with approval currently anticipated in the summer of 2011. This combination is now included in the revised Malaria Treatment Guidelines [5], and would be submitted for WHO prequalification as soon as it is registered. This project was originally a collaboration with Holley-Cotec, who have their own version of the medicine launched in China and many other countries, which is being used to treat around two million patients a year. So far this medicine has not been approved by a stringent regulatory authority or prequalified by the WHO, but this situation may now change with the inclusion of DHA–piperaquine in the WHO's treatment guidelines for malaria in 2010. The second fixed dose ACT currently under regulatory review is pyronaridine–artesunate, which is being developed by Shin Poong in collaboration with MMV, and has an adult form and a pediatric taste-masked granule form for children less than 15 kg. This was submitted to the EMA in March 2010, this time under the new article 58 legislation. This allows the EMA to give scientific advice that can be used to support the use of the medicine in countries outside of Europe. The issue is that if a medicine is approved by the EMA under the normal orphan legislation, it must actually be marketed in Europe or else the authorization will be withdrawn. Article 58 allows the stringent review by the EMA, but without the need to actually market and sell the drug in Europe. A decision is anticipated at the end of in 2012. Pyronaridine–artesunate is not included in the current WHO treatment guidelines, but will be discussed over the

Table 2 The pipeline of Medicines in Development for treating malaria – April 2010

Active Ingredients	Partnership	Comments
Products marketed, registered, in submission or in pivotal clinical trials (with product names under the active ingredient)		
Artemether 20 mg/Lumefantrine 120 mg	Novartis/ MMV	Four Dose strengths are registered in 83 countries. Coartem-D is a special dispersible formulation with taste masking, developed for children. Over 350 million Coartem treatments have been used, of which in the first year of launch 53 million were Coartem-D. Generic versions, prequalified by WHO as meeting acceptable quality are available from Ajanta Pharma Ltd (India), Ipca Laboratories Ltd (India) and Cipla , Ltd (India)
Artesunate 50 mg/ Amodiaquine 135mg (Coarsucam, Artesunate-Amodiaquine Winthrop®)	Sanofi-aventis/DNDi MMV	Developed by sanofi-aventis and DNDi. Registered in Morocco in March 2007, and in 24 other Afrian countries prequalified by WHO in October 2008. Manufactured by sanofi-aventis (MAPHAR Laboratories, Morocco), with 45 million treatments at no-profit price in 2009 Other prequalified versions of the medicine, are loose combinations – from Arsuamoon® (Guilin, China), Larimal (Ipca, India) and Falcimon (Cipla, India)
Dihydroartemisinin 10 mg/ Piperaquine 80 mg	Sigma-tau/MMV Holley-Cotec	Submitted registration dossier to the EMEA in July 2009, with an expected approval date over the summer of 2011 Holley-Cotec has registered Duo-Cotexin (40 mg Dihydroartemisinin, 320 mg Piperaquine phosphate) in China, 18 African countries, plus Pakistan Cambodia and Myanmar. Other generic versions are now being produced following the inclusion into the WHO treatment guidelines in 2010
Artesunate 60 mg/ Pyronaridine 180 mg (Pyramax)	Shinpoong Pharmaceutical Co., Ltd/ MMV	Developed in two formulations a tablet for patients over 15 kg, and a sachet of granules (20 mg artesunate/60 mg pyronaridne) for patients > 5 kg. Submitted to the EMA in March 2010 with approval for adult form anticipated mid-2012

Artesunate 100 mg/ Mefloquine 220 mg	Farmanguinhos /DNDi	Product registered in Brazil in June 2008. Not prequalified by WHO, or approved by stringent regulatory authorities Cipla agreed to supply the drug in South East Asia in April 2008 Mepha has a version which is available in the premium markets in Africa, treating around 300 000 patients per year
Naphthoquine phosphate 78.3 mg/ Artemisinin 125 mg (ARCO)	Kunming Pharmaceutical Corp.(KPC)	Marketed as a single dose cure of 8 tablets: representing a dose of 20 mg/kg artemisinin. Naphthoquine phosphate represents 50 mg of free base. No ICH-GCP studies have been published to date, although clinical studies are starting now to be publishd
Azithromycin 250mg/ Chloroquine 150 mg	Pfizer/ MMV	Fixed dose combinations developed for prevention of infection in pregnancy. The treatment dose was established by an adult Phase III study, completed in September 2007. The study on intermittent preventive treatment in Pregnancy (IPTp) is currently being designed by Pfizer/MMV and the London School of Hygeine and Tropical Medicine and is now actively recruiting patients
Arterolane malate 150 mg/ Piperaquine phosphate 750 mg	Ranbaxy/ MMV	Arterolane (Rbx11160, OZ277): fully synthetic endoperoxide, originally discovered by an MMV consortium. Phase IIa showed a low exposure in patients, but this was reversed by combination partner, piperaquine. Ranbaxy started a Phase III targeting Indian registration in 2011
Sulphamethoxazole 800 mg/ Trimethoprim 160 mg (Co-trimoxazole Bactrim)	Institute of Tropical Medicine, Belgium	Testing as a preventative medicine in pregnancy (IPTp) as an alternative to SP. Trial targeted both HIV infected and non-infected pregnant women with CD4$\geq$ 200/μL. Study to complete April 2011
Products in Phase II – defining dose for pivotal trials		
Tafenoquine 200 mg	GlaxoSmith Kline	8-aminoquinoline (WR-238605; SB-252263) for the radical cure (relapse prevention) of *P. vivax*. Tafenoquine induces hemolysis in patients with a G6PD deficiency, and a study is ongoing to determine the safe dose in G6PD A- patients. A combination Phase II/III study has been discussed with US FDA and WHO and will start in 2011

(continued)

Table 2 (continued)

Active Ingredients	Partnership	Comments
Ferroquine	Sanofi-aventis	Ferroquine (SSR-97193 - originally from the University of Lille) completed Phase IIa in asymptomatic adult malaria patients in combination with artesunate in October 2008. The Phase IIb study of ferroquine in adolescents and children with symptomatic malaria started in October 2009. Efficacy was deemed insufficient, and so additional Phase II trials with Ferroquine have been discussed
Albitiazolium bromide	Sanofi-aventis	Albitiazolium bromide (SAR-97276, TE3) from Henri Vial at the University of Montpellier, is a choline uptake inhibitor. A multicentre open lablel efficacy and safety Phase II study with 180 patients started in August 2008. and was terminated due to insufficient level of efficacy. Further clinical studies are planned increasing the dose, with a view to providing a treatment for severe malaria in the case of artemisinin resistance
Fosmidomycin/ Clindamycin (Fosclin)	Jomaa Pharma GmbH	Fosclin is an oral fixed-dose combination of fosmidomycin (a 1-deoxy-D-xylulose 5-phosphate reductoisomerase inhibitor) and clindamycin (a lincosamide ribosomal protein synthesis inhibitor). Clinically it has a rapid parasite clearance time (median 18 h) primarily driven by fosmidomycin. A Phase II study in 40 patients with uncomplicated *P falciparum* malaria was initiated in November 2009. Alternative partners for Fosmidomycin are also in discussion
Artemisone	Hong Kong University of Science and Technology	Artemisone (BAY 44–9585) is a semi-synthetic artemsinin derivative, developed originally by Bayer, the University of Hong Kong University of Science and Technology and MMV. It has been completed Phase II studies in adult malaria patients, and is efficacious. It is being discussed for treatment for Artemisinin resistant patients in the Thai-Cambodia border region, although the current formulation is not sufficiently stable to allow these studies to take place

Methylene Blue/ Amodiaquine	Ruprects-Karls- University Heidelberg SFB544	Shown to be active in a Phase II study of 180 patients in Burkina Faso (Zoungrana et al., 2008). Methylene blue 10mg/kg/day and Amodiaquine 4 mg/kg/day for 3 days resulted in 95% efficacy at day 28 (PCR corrected), Vomitting and dysurea were significantly higher than with Amodiaquine-Artesunate
OZ439	MMV (Nebraska Monash Swiss Tropical Institute)	Synthetic 1,2,4 trioxalane - completed Phase I, with 800 mg dose giving plasma exposure over 60 hours, supporting its use as part of the long sought after single dose cure. Phase IIa studies started in late 2010 to confirm this result in patients, and the drug-drug interaction studies with potential partners are currently being planned
Phase I		
AQ-13	Immtech/ Tulane	Immtech is developing AQ-13 for the treatment of malaria and malaria prophylaxis in travelers (Form 10Q 2009). Additional genotoxicology studies are required. Tulane has independently shown that 700 mg AQ13 gives the same exposure as 600 mg Cholorquine in human volunteers
CDRI 97/78	Ipca	1,2,4 trioxane originally developed by India's Central Drug Research Institute. Started human volunteer studies in 2008 for safety evaluation. Human pharmacokinetic measurements are planned for a later study
GSK 932121	GlaxoSmith Kline/ MMV	GSK-932121A is 4(1H)-pyridone, mitochondrial electron transport blocker. It entered a Phase I trial in December 2008, and this trial was suspended in April 2009 because of safety concerns from studies of a pro-drug
N-tert butyl isoquine (GSK-369796)	Liverpool School of Tropical Hygeine/ GSK / MMV	N-tert butyl isoquine started Phase I in 2008, and showed a maximum tolerated dose of 1000 mg. Currently the clinical project is on hold. More supporting data is being obtained by the Liverpool group as to whether this represents an acceptable therapeutic window in man are obtained

(continued)

Table 2 (continued)

Active Ingredients	Partnership	Comments
Late stage Preclinical		
BCX 4208	Biocryst/ MMV/ Albert Einstein College of Medicine	Purine nucleoside phosphorylase inhibitor, in preclinical evaluation for treatment of P falciparum infection.. BCX 4208 has already been tested in man for other diseases (gout), and is being evaluated
MK 4815	MMV/ Merck	MK4815 has been shown to be active against P falciparum in vivo. Preclinical studies in rodents and dogs suggested additional safety studies in primates prior to commencing Phase I. Concerns about the therapeutic window have slowed down the progression of this molecule
Trioxaquine SAR116242	Sanofi-aventis/ Palumed	Trioxaquine SAR-116242 (PA-1103) is a fusion compound containing a cholorquine moiety and a trioxane endoperoxide – currently in GLP preclinical evaluation
RKA-182	Liverpool School of Tropical Medicine/ University of Liverpool	Synthetic endoperoxide containing tetroxane active group. Currently undergoing preclinical safety evaluation. Phase I studies could be expected to start in late 2011
NITD-609	Novartis led consortium/ MMV	A Novartis led consortium (with Swiss Tropical Insititute and the Biomedical Primate Research Institute) identified a spiroindolone, KAE609, optimized from a high throughput screening hit. This entered Phase I in late 2010
P218 DHFR inhibitor	MMV	P218 is a Dihydrofolate reductase inhibitor, active the resistant quadruple mutant of plasmodium DHFR. Identified by a consortium led by Thailand's BIOTEC with Monash University and London School of Hygeine and Tropical Medicine. It entered preclinical development in the spring of 2010
NPC-1161-B	University of Mississippi	Next Generation 8-aminoquinoline for the radical cure of P vivax. NPC1161B is the (−) enantiomer of the 8-aminoquinoline racemic mixture of NPC1161C retaining activity, but with potentially less hematological side effects. Partnership with Cumberland Pharmaceuticals (Nashville, TN)

DHODH inhibitors	Three consortia in the MMV portfolio	Three ongoing collaborations – one led by University of Texas, one by the Genzyme, and the other by GlaxoSmithKline. All three are being optimized using high resolution three dimensional structures. The Texas project is funded through an NIH grant. A preclinical candidate was defined in June 2011
Aminoindoles	Genzmye, Broad Institute/ MMV	Series of aminoindole inhibitors based on a whole cell screening hit, Genz 644442. Current compounds are active *in vivo* and are in late lead optimization
Imidazolidinediones	Walter Reid Army Institute of Research	WR182393 has radical curative and causal prophylactic activity in Rhesus infected with *P. cynomolgi* given intramuscularly, but not orally
dUTP'ase inhibitors	Medivir AB, University of York	Medivir is leading a project to identify dUTP'ase inhibitors, active against Plasmodium and other protozoal parasites. Current compounds are active in vivo, and expected to enter preclinical safety assessment within the next 18 months
Cell based lead	MerckSerono, WHO/TDR	MerckSerono has been collaborating with WHO TDR to develop inhibitors originally identified from cell based screening. A lead series was approved in late 2000 aiming for a preclinical candidate within 24 months
Cell based lead	Pfizer, WHO/TDR	In 2006 Pfizer started a program with WHO/TDR to screen 12 000 selected compounds against a variety of protozoan parasites. The best compounds are in lead optimization and a preclinical candidate is expected in the next 12 months
GNF-156 series	Novartis-led consortium/ MMV	This is the second compound optimized a whole cell active from HTS of the Novartis corporate collection, being developed by the consortium that includes the Swiss Tropical and Public Health Insitute and the Biomedical Primate Research Centre. It hasentered into preclinical development by the end of 2010, and could enter Phase I early in 2012
AN3661	Anacor/ MMV	AN3661 is the first of a novel class of boron containing compounds to be moved to preclinical development for the treatment of malaria. Phase I trials are anticipated to start in early 2012

(continued)

Table 2 (continued)

Active Ingredients	Partnership	Comments
Stopped in the last 12 months		
Tinidazole	Walter Reed Army Institute of Research	Based on a previously published Indian report, this compound was tested directly in patients. However no benefit on preventing relapse was observed
AD452	Treague/MMV	AD452 is the (+)erythro isomer of mefloquine. Based on rodent data it was proposed that the psychiatric and gastric adverse events experienced by patients receiving mefloquine may be caused by the (−) isomer. Pure (+)erythro-mefloquine was tested in a Phase I study with detailed psychiatric profiling, in comparison with the racemate and no significant difference was seen

Details of the prequalified medicines were downloaded from the WHO web-site at http://apps.who.int/prequal/ on January 14th 2010

next year based on recent clinical data and publications. A fixed dose combination of mefloquine–artesunate was developed by DND*i* and Farminguinhos of Brazil, and launched in 2008. This is currently being used only in the Brazilian market, although there are plans to license the technology to Ipca in India and expand its use in south-east Asia, where it is often the first-line therapy. Another version of mefloquine–artesunate is produced by Mepha, and this has been successfully marketed in the private market in Africa. One issue here is the relative price of artesunate–mefloqine, which at $2.50 is over twice as expensive as the other treatments. For the newer ACTs, the major cost component comes from the partner drug, since a larger dose is required. The cost per kilogram of the partner drugs is often higher than artesunate (currently costing $300/kg). Mefloquine is a good illustration: the dose of drug needed is three times that of artesunate, and the bulk price for mefloquine can be as high as $1,200/kg. New approaches to synthesizing mefloquine have been developed by MMV and our partners as a result of the (+)-mefloquine isomer project (see below), and there is no reason why in the future the cost of mefloquine–artesunate fixed dose combinations should not be at the same price as other ACTs. One remaining ACT is naphthoquine–artemisinin, marketed as ARCO by Kunming Pharmaceuticals. The combination tablet was initially developed by the Academy of Military Medical Sciences (AMMS), Beijing, China and is currently administered as a single-dose regimen. Although clinical experiences and clinical data are very limited, available preclinical data suggest that naphthoquine is relatively potent [6, 7]. The major concerns are those of safety (most 4-aminoquiniolines have safety issues if the entire dose is given at once), and the efficacy of artemisinin given as a single dose. Some clinical studies have been published, but they are lacking validation according to ICH Good Clinical Practice, and the safety database does not include the 2,000 patients normally required for registration.

Beyond ACTs, there are two other combination treatments in development. Artemisinin derivatives cause embryotoxicity in rats, and therefore have been contraindicated in the first trimester of pregnancy [8]. Pregnant women are especially at risk of malaria, since the parasite adheres to the placenta. Azithromycin and chloroquine are both known to be safe in pregnancy, and the combination has two other advantages. First, there appears to be clinical synergy, in that azithromycin overcomes chloroquine resistance. Second, the use of an antibacterial in pregnant women will reduce the incidence of sexually transmitted infections, with a concomitant effect on survival of the baby [9]. The second combination is sulphamethoxazole 800 mg with trimethoprim (known as Co-trimoxazole), which is being tested in HIV-infected pregnant women, and also has an impact on the malaria infection.

3 Next Generation Therapies: Overcoming Artemisinin Resistance

The major challenge of any anti-infective program is that sooner or later the infectious agent will develop resistance to therapy. Presently, the mechanism of action of and possible mechanisms of resistance to artemisinins are not fully understood. Thus far, the development of artemisinin resistance has been relatively slow considering the number of treatments that have been used, and the fact that monotherapies are still available in many disease endemic countries. The first reports of such resistance are now emerging [10, 11], where there is an increase in the parasite clearance time from patients along the Thai–Cambodia border. This is also reflected in some cases in a small change in sensitivity of parasite isolates to drugs, but so far no stable isolates of resistant parasites have been identified.

Unless the mechanism of resistance is an enzyme which specifically degrades endoperoxides, it is likely that the new semi- and fully synthetic endoperoxides: Arterolane [12] – formerly known as OZ277 or Rbx11160, OZ439 [13], Artemisone [14], RKA182 (a 1,2,4,5 tetraoxane) [15], CDRI 97/78 [16] and Trioxaquine [17] (Table 2) will have clinical activity against these resistant parasites. Since cell biology is unable to give an unambiguous answer to the question of the mechanism of resistance, the only route forward at this present time is to test these medicines in artemisinin resistance areas, and measure parasite clearance times. Such studies are now planned, and would be possible for compounds that have already been tested in uncomplicated malaria patients, such as Arterolane, Artemisone, and OZ439. Despite repeated attempts to start clinical studies in artemisinin insensitive patients, we have so far been unable to commence such studies. The relative difficulty in obtaining patients should be seen as a positive sign about the relative numbers of resistant patients, and the focal nature of the resistance to date. In addition, these studies are always difficult to interpret since parasite clearance times are also dependent on the role of the immune response in parasite infection. If these new generations of endoperoxide therapy are shown to be clinically active in artemisinin-resistant patients, then a 3-day combination therapy could be developed and registered within 5–7 years, perhaps faster depending on local need and local regulatory requirements. It is most unlikely that the entire class is compromised by an artemisinin resistance mechanism – amodiaquine is active against chloroquine resistance strains, for example. The next generation endoperoxide OZ439 offers an additional advantage over artemisinin in that it has a long half life, and is predicted to be able to maintain a therapeutically useful concentration in patients for as long as 96 h from a single dose.

In the case that artemisinin resistance destroyed the entire endoperoxide class of medicines, we will have to fall back on the antibiotic classes. Fosmidomycin–clindamycin, a mixture of an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase and an antibiotic, is currently in Phase IIa clinical trials and seems to have a rapid parasite clearance time, driven by the parasite reduction ratio of fosmidomycin [18]. If the speed of parasite killing can be confirmed in other

studies, then it would be possible to imagine a combination with other partner medicines, including the 4-aminoquinoline family members such as piperaquine.

Other new approaches are still progressing through preclinical development. NITD609 is the first antimalarial to come from the phenotypic screen of more than two million compounds, carried out by Novartis. It moved from the initial high-throughput screen to a first administration in human volunteers in less than 4 years, which is extremely fast. Recently, AN3661 entered preclinical development. This is an extremely exciting new compound with unknown mechanism of action discovered by Anacor based on its activity in whole parasite assays. Its active moiety contains an oxaborole ring, and it has excellent drug-like properties. However, both of these are still at least 7 years away from being launched, and the complications of combination therapy and funding issues make this more likely to be closer to 10 years. Also, it is worth remembering that historically the chances that a molecule entering Phase I for anti-infective disease will make it all the way through to registration are at best two out of seven, and that this ratio falls as more and more innovative classes of drugs are tested (data from CMR International).

The second generation endoperoxide OZ439 is also exciting, since it has a half life of 12–24 h in man – and this means that currently a single dose in volunteers is enough to keep the plasma exposure above that required to kill the parasite for up to 60 h. It thus represents the first step toward having a single dose cure for malaria. This would be a dramatic step forward from the current 3 days of treatment – not only in terms of patient compliance, but also the ability for the health worker to observe treatment directly. The challenge over the next 2 years will be to find a partner for OZ439 in the combination. Most of the 4-aminoquinolines, aminoalcohols and related molecules have very long half lives, and so would be ideal; however, they cannot be given as a single dose because of the side effects. The hunt is therefore on for a long-acting partner where all the treatment can be given as a single administration. This also has to be a more potent medicine on a milligrams per patient basis, since the current dose of most partner drugs is as mush as 2 g. Some of the newer drugs such as naphthoquine or ferroquine [19] (a 4-aminoquinoline developed by sanofi-aventis) may have potential here.

4 Finding New Starting Points: Genomes and Screens

Two events over the last few years have helped the community find new starting points for antimalarial medicines. The first is technological. The development of high-throughput screening platforms, and their associated robotics and image processing reached such a state that by 2007 it was possible to consider testing compounds for their actions against the erythrocytic stages of *Plasmodium falciparum* whole parasites in 384 and 1,536 well formats [20, 21] by Novartis and GlaxoSmithKline. The high density of these formats has the advantage that the wells are smaller and use less compound and less cells, making the assays significantly cheaper. The cost of assays has come down by almost 100-fold in the last few

years, and the throughput has gone up by two orders of magnitude – to date MMV and our partners have screened almost five million compounds. The availability of a high-throughput assay in a university context (at the Eskitis Institute, in Queensland, Australia) has meant that we have been able to screen the collections of companies who for various reasons do not want to have parasites or human blood in their HTS facility. This approach has also been adopted by other academic collaborators [22, 23]. Overall, the results have been impressive, and we are seeing a hit rate of around 0.5% of compounds which reproducibly have shown the ability to inhibit or kill the parasite at a concentration of less than 1 μM. This offers a major repository of structures of new potential antimalarials, which can be the start of medicinal chemistry programs. The recent decision to publish the structures in the public domain from their high-throughput screens now gives a map of "whole cell screening space" for the world to work from. In addition, Medicines for Malaria Venture has a program for making representative compounds available. The 25,000 currently identified hits have been clustered into around 1,000 families, and plates of these compounds will be available to malaria researchers in the second half of 2011. The availability of all these information should help groups to focus on areas where they have a unique contribution, and reduce duplication in hits to leads programs. The speed at which these molecules can be brought forward is illustrated by NITD609, which came from the Novartis screen, and moved from the original identification to preclinical evaluation in less than 3 years, a speed that is considered state of the art for commercial product development. However, the challenge will be to do this as efficiently as possible, and for this we will have to have a unique blend of using industry experience, academic insight and working with partners in countries such as India, South Africa, Brazil, and China to reduce costs.

The second major step forward has been the availability of the plasmodial genome sequences, allowing us to know a great many of the potential targets in the parasite [24]. Based on the structural knowledge of what makes good drug targets, these potential targets can be prioritized [25]. It also allows an "ortholog" approach to be followed by some groups, and this work has been pioneered by sanofi-aventis. The idea here is that among the targets identified in the parasite, many will have orthologs in the human genome, and these will already have been the subject of a human inhibitor search (more than 1,000 targets from the human genome have been worked on by the major pharmaceutical companies). By selecting compounds known to be active against the human target, and a representative set of structurally related inactives, then the hit-rate can be further improved to an astonishing 20–25%. In addition, much is already known about these compounds, such as their pharmacokinetics, metabolism and potential safety issues, meaning that the subsequent optimization is much faster. Often this work is further aided by the availability of high-resolution three-dimensional structures, so that compounds can be optimized by structure-based design [26]. These techniques have been used to great advantage on two of the projects listed in Table 2 – the identification of dihydrofolate reductase inhibitors, which overcome trimethoprim resistance [27], and the rational design of dihydro orotate dehydrogenase inhibitors [28].

5 Challenges of the Eradication Era of Gametocytes and Hypnozoites

The announcement in 2007 of a plan to eradicate malaria by the WHO and the Bill and Melinda Gates Foundation [29], raised the need for new classes of medicines. Two areas are most clearly highlighted in the search for new medicines. First, there is a need for a medicine that would destroy the dormant forms of *P. vivax* and *P. ovale,* known as hypnozoites [30]. When an infected mosquito bites, the first major parasite target is the liver, and in the case of these two parasite species, some of the parasites remain in the liver as small dormant forms – for anywhere between a few days and several years. The only registered therapy against hypnozoites is primaquine, a medicine developed during the Korean war. This has two major issues, first it requires 14 days of therapy, and so compliance is virtually nonexistent in the field, to the extent that some countries do not even have 14 days therapy on their treatment guidelines. Second, it causes hemolysis in patients who have a deficiency in the enzyme glucose 6-phosphate 1-dehydrogenase [31] (G6PD) – a condition that is as common as 15–20% in some malaria endemic regions. Tafenoquine was discovered originally by the Walter Reed Army Institute of Research, and has been tested in several thousand soldiers by the US army, and has the advantage of a longer half life, meaning that the course of treatment could be reduced to a much more manageable 3 days [32]. Currently, GlaxoSmithKline is undergoing studies to determine the safe dose in G6PD deficient individuals, as a prelude to a pivotal development program, which is planned to start in early 2011. This study will include women, and children, and should allow a registration of the product with the US FDA within 5 years. Beyond Tafenoquine, there is one other 8-aminoquinoline, NPC-1161C [33], but it is not clear that this offers an advantage in terms of potential safety in G6PD deficient patients, and this will require further *in vivo* assessment. There are currently no other chemical series in clinical development for antirelapse therapy. This is because up to now, there has been no cell biological model of the hypnozoite, and all compounds had to be tested in primates, which is extremely laborious and time consuming. The hypnozoites themselves are difficult to detect, and although primary hepatocyte systems have been available for 25 years [34], it has not been easy to produce cell lines, which support infection and produce hypnozoites. To date, the most robust system is from the related parasite *P. cynomolgi*, which is able to produce small stable forms when used to infect primary rhesus hepatocytes [35]. This gives a starting point for the evaluation of new compounds, although currently only perhaps hundreds can be tested per year.

The other challenge of the eradication era is to have molecules that will stop the transmission of the parasite to the mosquito vector. It is known that only the gametocytes (the sexual forms) of the parasite are transmitted, and that *P. falciparum* produces gametocytes in response to stress, including drug stress. The assays for gametocyte maturation and for the development of the ookinete inside the insect gut are well described [36]. Already we have been able to test the known malaria portfolio (all of the compounds listed in Table 2, plus all the

compounds which are currently marketed antimalarials, and all the leads, compounds which are currently too early to be described in this review). Putting together a complete data set of the activity of compounds against the erythrocytic stages, the gametocyte and insect stages, and indeed the liver stages is part of what MMV has termed "Malaria Lifecycle Fingerprinting." The advantage of having all the data collected from identical assays carried out in identical conditions should not be underestimated. For the transmission blocking targets, a number of genes have already been identified as being involved from gene disruption (knock out) studies either in *P. falciparum* or *P. berghei* [37–39]. By selecting targets which inhibit gametocytes and transmission, it may be possible to identify molecules only active on these stages. The advantage would be that the transmission stages are relatively rare (there are less than five ookinetes, compared with as many as 10^{12} parasites at the erythrocytic stages). This means that development of resistance should be much less likely for the parasite.

The ultimate medicine would therefore be one which combined all the best attributes of a single dose cure of malaria, with something that was able to block transmission and also prevent relapses of disease caused by the hypnozoites. Such a medicine has been termed the "single exposure, radical cure" by the Malaria Eradication research working party (malERA) [40].

6 Conclusions

The last decade has seen considerable progress in the pipeline of antimalarial medicines. The prioritization of fixed dose artemisinin combination therapies, and their registration with stringent regulatory authorities and the WHO means now that the National Malaria Control Programs in disease endemic countries have a choice of treatment, and more importantly an evidence base on which to make their choice of treatment. Beyond this, the technologies which have come from high-throughput screening and from the genomic revolution have led to an acceleration of discovery efforts, and a wide array of new and exciting starting points for medicinal chemistry. The focus of molecules of the future cannot simply be about treating the patient – and coming up with the next generation of therapies to overcome resistance. The key to the future is the development of medicines, which are better suited to the needs of the patient – a single dose cure for *P. falciparum* malaria, and a shorter and safer course of treatment for destroying the hypnozoite reservoirs of *P. vivax*. Finally by having the tools that will prevent the infection of the insect vector, the community will be in a position to break the cycle of transmission, leading to the potential of the ultimate eradication of this devastating disease.

Acknowledgments I would like to thank the Medicines for Malaria Research and Development team for all their insights into this article, and for the many advisors, especially our External Scientific Advisory Committee, who have done so much to transform the pipeline of antimalarial therapeutics.

References

1. Maude RJ, Pontavornpinyo W, Saralamba S, Aguas R, Yeung S, Dondorp AM, Day NP, White NJ, White LJ (2009) The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. Malar J 8:31
2. Oliaro P, Wells TN (2009) The global portfolio of new antimalarial medicines under development. Clin Pharm Ther 85:584–595
3. Wells TN, Alonso PL, Gutteridge WE (2009) New medicines to improve control and contribute to the eradication of malaria. Nat Rev Drug Discov 8:879–891
4. White NJ (2008) Qinghaosu (artemisinin): the price of success. Science 320:330–334
5. World Health Organization. Malaria treatment guidelines, June 2010. http://www.who.int/malaria/publications/atoz/9789241547925/en/index.html. Accessed 22 May 2011
6. Tun T, Tint HS, Lin K, Kyaw TT, Myint MK, Khaing W, Tun ZW (2009) Efficacy of oral single dose therapy with artemisinin-naphthoquine phosphate in uncomplicated falciparum malaria. Acta Trop 111:275–278
7. Qu HY, Gao HZ, Hao GT, Li YY, Li HY, Hu JC, Wang XF, Liu WL, Liu ZY (2010) Single-dose safety, pharmacokinetics, and food effects studies of compound naphthoquine phosphate tablets in healthy volunteers. J Clin Pharmacol 50:1310–1318
8. Clark RL (2009) Embryotoxicity of the artemisinin antimalarials and potential consequences for use in women in the first trimester. Reprod Toxicol 28:285–296
9. Chico RM, Pittrof R, Greenwood B, Chandramohan D (2008) Azithromycin-chloroquine and the intermittent preventive treatment of malaria in pregnancy. Malar J 7:255
10. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM; Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620
11. Dondorp AM, Nosten F, Yi P, Das D, Phyo PA, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
12. Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, Dong Y, Dorn A, Hunziker D, Matile H et al (2004) Identification of an antimalarial synthetic trioxolane drug development candidate. Nature 430:900–904
13. Charman SA, Arbe-Barnes S, Bathurst IC, Brun R, Campbell M, Charman WN, Chiu FC, Chollet J, Craft JC, Creek DJ et al (2011) Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. Proc Natl Acad Sci USA 108:4400–4405
14. Nagelschmitz J, Voith B, Wensing G, Roemer A, Fugmann B, Haynes RK, Kotecka BM, Rieckmann KH, Edstein MD (2008) First assessment in humans of the safety, tolerability, pharmacokinetics, and *ex vivo* pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. Antimicrob Agents Chemother 52:3085–3091
15. O'Neill PM et al. (2010) http://www.a-star.edu.sg/Portals/0/media/Press%20Release/PressRelease_UKSIN_FINAL_media.pdf
16. Singh RP, Sabarinath S, Gautam N, Gupta RC, Singh SK (2009) Liquid chromatographic tandem mass spectrometric assay for quantification of 97/78 and its metabolite 97/63: A promising trioxane antimalarial in monkey plasma. J Chromatogr B 877:2074–2080
17. Coslédan F, Fraisse L, Pellet A, Guillou F, Mordmüller B, Kremsner PG, Moreno A, Mazier D, Maffrand JP, Meunier B (2008) Selection of a trioxaquine as an antimalarial drug candidate. Proc Natl Acad Sci USA 105:17579–17584
18. Na-Bangchang K, Ruengweerayut R, Karbwang J, Chauemung A, Hutchinson D (2007) Pharmacokinetics and pharmacodynamics of fosmidomycin monotherapy and combination therapy with clindamycin in the treatment of multidrug resistant falciparum malaria. Malar J 6:70
19. Dive D, Biot C (2008) Ferrocene conjugates of chloroquine and other antimalarials: the development of ferroquine, a new antimalarial. ChemMedChem 3:383–391

20. Plouffe D, Brinker A, McNamara C, Henson K, Kato N, Kuhen K, Nagle A, Adrián F, Matzen JT, Anderson P et al (2008) *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. Proc Natl Acad Sci USA 105:9059–9064
21. Gamo FJ, Sanz LM, Vidal J, Cozar C, Alarez E, Lavandera JL, Vanderwall DE, Green DVS, Kumar V, Hasan S et al (2010) Thousands of chemical starting points for antimalarial lead identification. Nature 465:305–310
22. Baniecki ML, Wirth DF, Clardy J (2007) High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery. Antimicrob Agents Chemother 51:716–723
23. Guiguemde WA, Shelat AA, Bouck D, Duffy S, Crowther GJ, Davis PH, Smithson DC, Connelly M, Clark J, Zhu F et al (2010) Chemical genetics of *Plasmodium falciparum*. Nature 465:311–315
24. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S et al (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498–511
25. Aguero F, Al-Lazikani B, Aslett M, Berriman M, Buckner FS, Campbell RK, Carmna S, Carruthers IM, Chan AW, Chen F et al (2008) Genomic-scale prioritization of drug targets: the TDR Targets database. Nat Rev Drug Discov 7:900–907
26. Grundner C, Perrin D, Hooft van Huijsduijnen R, Swinnen D, Gonzalez J, Gee CL, Wells TN, Alber T (2007) Structural basis for selective inhibition of *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpB. Structure 15:499–509
27. Yuthavong Y, Yuvaniyama J, Chitnumsub P, Vanichtanankul J, Chusacultanachai S, Tarnchompoo B, Vilaivan T, Kamchonwongpaisan S (2005) Malarial (*Plasmodium falciparum*) dihydrofolate reductase-thymidylate synthase: structural basis for antifolate resistance and development of effective inhibitors. Parasitology 130:249–259
28. Deng X, Gujjar R, El Mazouni F, Kaminsky W, Malmquist NA, Goldsmith EJ, Rathod PK, Phillips MA (2009) Structural plasticity of malaria dihydroorotate dehydrogenase allows selective binding of diverse chemical scaffolds. J Biol Chem 284:26999–27009
29. Roberts L, Enserink M (2007) Malaria. Did they really say ... eradication? Science 318:1544–1545
30. Wells TN, Burrows J, Baird JK (2010) Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination. Trend Parasitol 26:1471–1477
31. Beutler E, Duparc S; G6PD Deficiency Working Group (2007) Glucose-6-phosphate dehydrogenase deficiency and antimalarial drug development. Am J Trop Med Hyg 77:779–789
32. Kitchener S, Nasveld P, Edstein MD (2007) Tafenoquine for the treatment of recurrent *Plasmodium vivax* malaria. Am J Trop Med Hyg 76:494–496
33. Nanayakkara NP, Ager AL Jr, Bartlett MS, Yardley V, Croft SL, Khan IA, McChesney JD, Walker LA (2008) Antiparasitic activities and toxicities of individual enantiomers of the 8-aminoquinoline 8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate. Antimicrob Agents Chemother 52:2130–2137
34. Mazier D, Landau I, Druilhe P, Miltgen F, Guguen-Guillouzo C, Baccam D, Baxter J, Chigot JP, Gentilini M (1984) Cultivation of the liver forms of *Plasmodium vivax* in human hepatocytes. Nature 307:367–369
35. Dembele L, Gego A, Zeeman AM, Franetich JF, Silvi O, Rametti A, LeGrand R, Dereuddre-Bosquet N, Sauerwein R, van Gemert GJ (2011) Towards an *in vitro* model of *Plasmodium* hypnozoites suitable for drug discovery. PLoS One 6:e18162
36. Sinden RE (1998) Gametocytes and sexual development. In: Sherman IW (ed) Malaria: parasite biology, pathogenesis and protection. ASM, Washington, DC, pp 25–48
37. Dorin-Semblat D, Sicard A, Doerig C, Ranford-Cartwright L, Doerig C (2008) Disruption of the PfPK7 gene impairs schizogony and sporogony in the human malaria parasite *Plasmodium falciparum*. Eukaryot Cell 7:279–285
38. Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. Science 327:1644–1648

39. McRobert L, Taylor CJ, Deng W, Fivelman QL, Cummings RM, Polley SD, Billker O, Baker DA (2008) Gametogenesis in malaria parasites is mediated by the cGMP-dependent protein kinase. PLoS Biol 6:e139
40. Alonso PL, Djimde A, Kremsner P, Magill A, Milman J, Nájera J, Plowe CV, Rabinovich R, Wells T, Yeung S (2011) The malERA Consultative Group on Drugs, A research agenda for malaria eradication: drugs. PLoS Med 8:e1000402

Molecular Markers of *Plasmodium* Resistance to Antimalarials

Andrea Ecker, Adele M. Lehane, and David A. Fidock

Abstract Investigations into the molecular basis of *Plasmodium* parasite resistance to antimalarial drugs have made strong progress in defining key determinants. Mutations in the digestive vacuole transmembrane proteins *P. falciparum* chloroquine resistance transporter (PfCRT) and *P. falciparum* multidrug resistance protein 1 (PfMDR1) are important drivers of parasite resistance to several quinoline-based drugs including chloroquine, amodiaquine, and to a lesser extent quinine. Amplification of *pfmdr1* can also mediate resistance to mefloquine and impact lumefantrine efficacy. Parasite resistance to antifolates has been mapped to point mutations in the target enzymes dihydrofolate reductase and dihydropteroate synthase, and mutations in cytochrome *b* have been found to ablate atovaquone efficacy. Antibiotic resistance has been associated with mutations that preclude drug inhibition of protein translation in the parasite apicoplast. The study of resistance to artemisinin derivatives and several partner drugs used in artemisinin-based combination therapies is an area of active research that has yet to define clearly how in vitro resistance can translate into predictions of treatment failures. Research in this area is important not only for its ability to generate molecular markers of

A. Ecker
Department of Microbiology and Immunology, Columbia University Medical Center, Room 1502, Hammer Health Sciences Center, 701 West 168th Street, New York, NY 10032, USA

A.M. Lehane
Department of Microbiology and Immunology, Columbia University Medical Center, Room 1502, Hammer Health Sciences Center, 701 West 168th Street, New York, NY 10032, USA

Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia

D.A. Fidock (✉)
Department of Microbiology and Immunology, Columbia University Medical Center, Room 1502, Hammer Health Sciences Center, 701 West 168th Street, New York, NY 10032, USA

Division of Infectious Diseases, Department of Medicine, Columbia University Medical Center, New York, NY 10032, USA
e-mail: df2260@columbia.edu

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_13,

treatment failure but also for the insights it can provide into drug mode of action and the development of chemical and pharmacological strategies to overcome resistance mechanisms.

1 Introduction

The ability to monitor and predict parasite drug resistance is crucial to the rational implementation of antimalarial treatment policies. The determination of resistance in vivo is costly and confounded by the effects of host immunity, pharmacokinetic variables, and the possibility of reinfection, among other factors. Similarly, determination of drug resistance of field isolates in vitro presents many technical caveats associated with performing drug susceptibility assays on non-culture-adapted and frequently polyclonal isolates from patients who may have an unknown treatment history. Not surprisingly, results from these in vitro assays may not always correlate with clinical outcome. Alternatively, molecular markers can serve as sentinels in the surveillance of drug resistance. However, the clinical relevance of a putative molecular marker must first be established.

While a wealth of studies attempting to correlate molecular markers and treatment outcome have been published, the lack of standardization of trial design and data reporting (as recommended in [1, 2]) can render comparison between trials difficult, and trials may fail to find an existing correlation due to lack of statistical power, if too few patients are included or the prevalence of a mutation is near saturation. Ideally, the contribution of a molecular marker to drug responses should further be determined in defined genetic backgrounds by allelic exchange. Importantly, with few exceptions, resistance phenotypes are multifactorial, and conflicting results in different genetic backgrounds, or experimental or geographical settings are therefore to be expected. The reader is referred to recent reviews for a general introduction to genetic mapping of drug resistance [3, 4].

In the following sections we will discuss molecular markers for drug resistance as they relate to *P. falciparum* (Table 1). Molecular markers for drug resistance in *P. vivax* are summarized at the end of this chapter.

2 Molecular Markers for Resistance to Quinoline-Based Drugs

2.1 P. falciparum *Chloroquine Resistance Transporter*

The primary determinant of chloroquine (CQ) resistance (CQR), *P. falciparum* chloroquine resistance transporter (*pfcrt*), was identified through a genetic cross between the CQ-sensitive and CQ-resistant clones HB3 (Honduras) and Dd2 (Indochina), respectively. In the progeny of this cross, inheritance of verapamil-reversible CQR segregated with a single genetic locus on chromosome 7, and was

Table 1 Summary of the major molecular markers for clinical resistance that have been identified for antimalarial drugs

Drug	Molecular marker for resistance	Comments
Amodiaquine	K76T mutation in PfCRT and N86Y mutation in PfMDR1	Other mutations in *pfcrt* and *pfmdr1* also affect amodiaquine response; the South American 7G8 *pfcrt* allele confers greater resistance to monodesethylamodiaquine (the primary metabolite) as compared to other *pfcrt* alleles investigated to date
Atovaquone	Y268S/C/N mutation in cytochrome *b*	This mutation mediates atovaquone resistance; it has been shown in the murine parasite *P. yoelii* that mutations in cytochrome *b* that cause atovaquone resistance also ablate the synergistic effects of the atovaquone–proguanil combination
Azithromycin	G76V mutation in the ribosomal protein L4	Based on an in vitro study; azithromycin is under investigation with partner drugs in malaria treatment trials
Chloroquine	K76T mutation in PfCRT	This mutation is accompanied by other mutations in PfCRT; polymorphisms in *pfmdr1* and perhaps *pfmrp* may augment or otherwise play a role in *pfcrt*-mediated chloroquine resistance
Clindamycin	A1875C mutation in apicoplast 23S rRNA	A2058C mutation at equivalent position in *E. coli* 23S rRNA is known to mediate resistance to clindamycin
Cycloguanil	Mutations in DHFR: A16V and S108T	Certain combinations of pyrimethamine-resistance-conferring mutations in DHFR also reduce parasite susceptibility to cycloguanil
Fosmidomycin	*pfdxr* amplification	Based on an in vitro study; fosmidomycin has not been registered for malaria treatment
Lumefantrine	*pfmdr1* amplification	The wild-type *pfcrt* allele (K76), and possibly also *pfmdr1* N86, confer a reduction in parasite susceptibility to lumefantrine as compared to other *pfcrt* and *pfmdr1* alleles
Mefloquine	*pfmdr1* amplification	Most important predictor of mefloquine resistance discovered to date; however mefloquine resistance has also been observed in the absence of *pfmdr1* amplification
Pyrimethamine	Mutations in DHFR: S108N, N51I, C59R and I164L	Not all of these mutations need be present; the extremely resistant quadruple mutant is widespread in Southeast Asia, whereas the triple mutant lacking I164L is prevalent in Africa
Quinine	No reliable marker identified	Quinine resistance is multifactorial; mutations in *pfcrt*, *pfmdr1* and *pfnhe-1*, and copy number variations in *pfmdr1*, may play a role

(continued)

Table 1 (continued)

Drug	Molecular marker for resistance	Comments
Sulfadoxine	Mutations in DHPS: S436A/F, A437G, K540E, A581G and A613S/T	Not all mutations need be present; the A437G mutation is the most commonly observed

ultimately mapped to mutations in the highly interrupted 13-exon *pfcrt* gene [5]. *pfcrt* accounts for >95% of the CQ response variation among the progeny, as revealed by quantitative trait loci (QTL) analysis [6] (Fig. 1). A strong association of *pfcrt* with parasite response to CQ has also been confirmed in a recent genome-wide association study [7]. *pfcrt* encodes a 49 kDa putative transporter of unknown function that localizes to the membrane of the parasite's digestive vacuole (DV) [5]. Based on bioinformatic analyses, PfCRT is a member of the drug/metabolite transporter superfamily [8].

Allelic exchange experiments have demonstrated unequivocally that mutant *pfcrt* can confer verapamil-reversible CQR to sensitive parasites [9]. This may to some extent depend on the genetic background as in the D10 (Papua New Guinea) strain the introduction of mutant *pfcrt* only resulted in a moderate CQ tolerance phenotype [10]. Importantly, a single mutation, resulting in the amino acid change K76T, has been shown to be essential for CQR, as removal of this mutation from CQ-resistant strains (Dd2 and 7G8, representative of resistant parasites from Southeast Asia and South America, respectively) resulted in complete loss of the resistance phenotype [11]. Replacement of the positively charged lysine (K) residue with a neutral threonine (T) may enable PfCRT to transport diprotonated CQ out of the DV, either through active transport or via facilitated diffusion, thereby decreasing access to its heme target in the DV (reviewed in [12]). This transport activity of mutant PfCRT was also studied in heterologous expression systems such as yeast [13], *Dictyostelium discoideum* [14] and *Xenopus laevis* oocytes [15]. In the latter study, mutant PfCRT was shown directly to mediate CQ transport, while no transport activity was seen for the wild type form.

Notably, K76T is always accompanied by at least three and up to eight additional polymorphisms. These are mainly found in or immediately adjacent to one of the ten transmembrane domains and may compensate for altered PfCRT function, due to the K76T polymorphism, and/or modulate drug susceptibility [5]. Amino acid sequences surrounding K76T (position 72–76) distinguish African and Southeast Asian (predominantly CVIET) and South American, Philippino, and Papua New Guinean strains (predominantly SVMNT) from the invariant wild type, CQ-sensitive haplotype (CVMNK) [16]. All together, 16 variant residues have been identified, rendering PfCRT an extraordinarily polymorphic protein (Table 2) [5, 17–28].

An association of *pfcrt* K76T and in vitro CQR has also been confirmed in many, although not all, studies using field isolates (reviewed in [29]). This might reflect the requirements for additional genes in drug resistance, an interplay with other undetected *pfcrt* polymorphisms, or technical caveats associated with determining the susceptibility of non-culture-adapted, frequently polyclonal patient isolates.

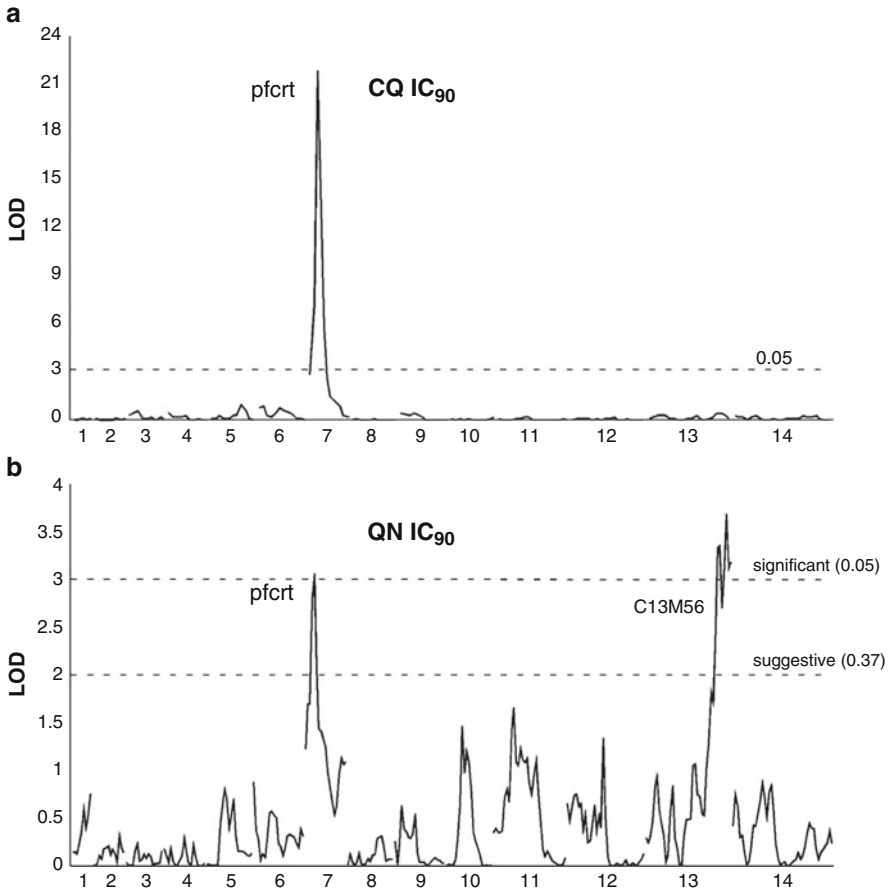


Fig. 1 Quantitative trait loci (QTL) analysis of a cross between the chloroquine (CQ)- and quinine (QN)-sensitive HB3 strain and the CQ-resistant and low-level QN-resistant Dd2 strain. *pfcrt* accounts for >95% of the variation in CQ responses amongst the progeny, evident as a sharp peak on chromosome 7 with a log of differences (LOD) score of >20 (**a**), however this gene has a more modest effect on QN responses (**b**). Most of the QN response variation can be attributed to two main additive QTL: 35% to a chromosome 13 peak centered on the C13M56 marker (thought to be a result of mutations in *pfnhe-1* or a neighboring gene [6, 90]), and another 30% to a chromosome 7 peak centered on *pfcrt*. This figure was reproduced in modified form with permission from [6]

pfcrt K76T has also been associated with CQ treatment failure in clinical settings (e.g. [30–32]). A recent review and meta-analysis of 25 studies confirmed that *pfcrt* K76T increased the risk of therapeutic failure after CQ treatment [1]. This validates the usefulness of *pfcrt* K76T as a sensitive molecular marker for CQR in the field. Nevertheless, *pfcrt* K76T has proven less predictive of clinical failure in patient populations with high pre-existing partial immunity; this "premunition" allows

Table 2 PfCRT polymorphisms

Region of origin[a]	Isolates (examples)	CQ response[b]	PfCRT position and encoded amino acid[c,d]																
			72	74	75	76	97	144	148	160	194	220	271	326	333	334	356	371	Ref.
Wild type haplotype																			
All regions	HB3	S	C	M	N	K	H	A	L	L	I	A	Q	N	T	S	I	R	[5]
Mutant haplotypes																			
A	106/1[e]	S	C	I	E	K	H	A	L	L	I	S	E	S	T	S	I	I	[5]
A, SEA	PAR, FCB	R	C	I	E	T	H	A	L	L	I	S	E	S	T	S	I	I	[5]
A, SEA	102/1, Dd2	R	C	I	E	T	H	A	L	L	I	S	E	S	T	S	T	I	[5]
SEA	field isolate	n.d.	C	I	E	T	H	A	–	L	–	S	E	–	–	–	–	R	[28]
SEA	Cam742	R	C	I	E	T	H	A	L	L	I	S	E	N	T	S	I	I	[19]
SEA	Cam783	R	C	I	E	T	H	A	L	L	I	S	E	N	T	S	T	I	[19]
SEA	TM93-C1088	R	C	I	E	T	L	A	–	L	–	S	E	S	–	–	T	I	[18]
SEA	field isolate	n.d.	C	I	E	T	H	Y	–	L	–	A	E	–	–	–	–	R	[28]
SEA	field isolate	n.d.	C	I	D	T	H	Y	–	L	–	A	E	–	–	–	–	R	[28]
SEA	field isolate	n.d.	C	I	D	T	H	Y	–	L	–	A	E	–	–	–	–	I	[28]
SEA	Cam738	R	C	I	D	T	H	A	I	L	T	S	E	N	S	S	I	R	[19]
SEA	Cam734	R	C	I	D	T	H	F	I	L	T	S	E	N	S	S	I	R	[19]
SEA	PH1	R	C	M	N	T	H	T	L	Y	I	A	Q	D	T	S	I	R	[18]
SEA	PH2	n.d.	S	M	N	T	H	T	–	Y	–	A	Q	D	–	–	I	R	[18]
SEA	PNG4	R	S	M	N	T	H	–	–	–	–	A	Q	D	–	–	L	R	[27]
SA	7 G8	R	S	M	N	T	H	A	L	L	I	S	Q	D	T	S	L	R	[5]
SA	Ecu1110	R	C	M	N	T	H	–	–	–	–	S	Q	D	–	–	L	R	[5]
SA	TU741	R	C	M	N	T	H	A	L	L	I	S	Q	D	T	N	L	R	[20]
SA	Jav	R	C	M	E	T	Q	A	L	L	I	S	Q	N	T	S	I	T	[5]
SA	TA4641	R	C	M	E	T	Q	A	L	L	I	S	E	N	T	S	I	–	[20]
SA	TA4640	R	C	M	E	T	Q	A	L	L	I	S	Q	N	S	S	I	I	[20]

[a] *A* Africa, *SEA* Southeast Asia, *SA* South America

[b] *S* CQ-sensitive, *R* CQ-resistant, *n.d.* not determined

[c] Gray shading indicates residues that differ from the wild type allele. Residues that were not reported are indicated by a dash

[d] Additional partial haplotypes for position 72–76 have been reported in [17, 21–26]

[e] Revertant?

some patients with infections carrying *pfcrt* T76 to respond adequately to CQ treatment [31, 33].

pfcrt K76T has also been used as an epidemiological tool to monitor parasite populations in response to changes in drug policy, as exemplified in a study that surveyed *pfcrt* genotypes after discontinuation of CQ use in Malawi. Strikingly, CQ-resistant strains disappeared within 10 years of CQ withdrawal, which suggests that the CVIET-type mutant *pfcrt* allele imparts a substantial fitness cost in this high transmission setting [34]. Nevertheless, minority *pfcrt* K76T genotypes may still be present in Malawi, as standard PCRs may fail to correctly identify these in polyclonal infections. They can be detected with greater sensitivity using a multiple site-specific heteroduplex tracking assay [35].

In addition to their central role in parasite response to CQ, *pfcrt* mutations can significantly alter the degree of susceptibility to a number of structurally unrelated, clinically important drugs, including several of the partner drugs used in artemisinin-based combination therapy. Introduction of mutant *pfcrt* into the CQ-sensitive GC03 strain conferred a slight increase in resistance to amodiaquine (AQ) and to its primary and active metabolite desethylamodiaquine (DEAQ) [9]. Field studies have also demonstrated selection of *pfcrt* K76T in recrudescences and reinfections following AQ treatment, although the presence or absence of this mutation before treatment was not predictive of treatment outcome [36]. *pfcrt* mutations, particularly in the 3′ region of the gene, were enriched in Colombian field isolates with decreased susceptibility to AQ and DEAQ [20]. Indeed, the analysis of two genetic crosses revealed that South American *pfcrt* alleles, in combination with South American *pfmdr1* alleles (see below for a discussion of *pfmdr1*), mediate high DEAQ resistance but only moderate CQR [37]. AQ has been used in South America since the late 1940s [37]. Thus, historically DEAQ may have been the main driving force in selection of these PfCRT haplotypes.

In contrast, the *pfcrt* K76T mutation was reported to increase parasite susceptibility to lumefantrine, and was selected against in reinfections following artemether–lumefantrine treatment [38] (note, it is generally assumed that these reinfections represent reinoculations and that selective pressure is exerted by subtherapeutic levels of the long half-life artemisinin partner drug, in this case lumefantrine). *pfcrt* mutations, particularly at position 76, can also increase parasite susceptibility to quinine, mefloquine, halofantrine, and artemisinin and its derivatives [9, 39]. In the case of quinine, the effects of *pfcrt* mutations depend on the parasite genetic background [11], and the pattern of cross-resistance with its diastereomer quinidine is complex [39, 40]. This suggests that *pfcrt* is but one component of a multifactorial basis of quinine resistance [6].

2.2 P. falciparum *Multidrug Resistance Protein 1*

P. falciparum multidrug resistance protein 1 (*pfmdr1*) encodes an ATP-binding cassette (ABC) transporter orthologous to mammalian P-glycoproteins that mediate

verapamil-reversible multidrug resistance in mammalian cancer cells. PfMDR1, also known as P-glycoprotein homologue 1 (Pgh-1), is composed of two homologous halves, each with six predicted transmembrane domains and a conserved nucleotide-binding domain [12]. PfMDR1 localizes to the DV membrane and may function in importing solutes into the DV [41]. Together with PfCRT, PfMDR1 is likely to be an important regulator of drug accumulation in this organelle.

pfmdr1 was identified based on the similarity of CQR and tumor multidrug resistance and the observation that CQR can be reversed by the calcium channel blocker verapamil [42], which suggested the involvement of an *mdr* ortholog [43, 44]. However, the analysis of the HB3 × Dd2 genetic cross revealed that CQR was not linked to *pfmdr1* [45], and led to the identification of *pfcrt* as the major determinant of resistance [5].

Nevertheless, it was subsequently shown that *pfmdr1* plays a central role in parasite responses to many drugs. Amplification of *pfmdr1* was selected in vitro by mefloquine pressure [44, 46, 47], and was shown to correlate with increased transcript [43, 46, 47] and protein [46] abundance. The mefloquine-selected lines displayed cross-resistance to halofantrine and quinine [46, 47] and vice-versa [48]. This cross-resistance has also been observed in the field [49] and may explain why *pfmdr1* amplifications were observed in field isolates obtained before mefloquine was in clinical use [46].

Increased *pfmdr1* copy number was also associated with reduced in vitro susceptibility to mefloquine, halofantrine, quinine, dihydroartemisinin, and artesunate in Thai isolates [49–51]. Importantly, *pfmdr1* gene amplification was shown to be the most important predictor for treatment failure following mefloquine monotherapy [49, 52] or artesunate–mefloquine combination therapy [49, 53]. Increased *pfmdr1* copy number was also associated with a four-fold increase in the risk of treatment failure of a 4-dose artemether–lumefantrine regimen, while the 6-dose regimen remained effective in the presence of *pfmdr1* amplification [54]. Nevertheless, some mefloquine-resistant parasites lack *pfmdr1* amplification, illustrating that mefloquine (and halofantrine) cross-resistance can also be mediated by other mechanisms [48, 49, 55]. It is worth noting that *pfmdr1* transcript levels are reportedly rapidly upregulated in vitro following parasite exposure to CQ, mefloquine or quinine, but not to pyrimethamine [56]. While *pfmdr1* amplification was originally reported in CQ-resistant lines [43], several subsequent studies have demonstrated an inverse relationship between *pfmdr1* copy number and the level of CQR, suggesting that *pfmdr1* overexpression tends to reduce the level of CQR [46, 47, 57].

The genetic disruption of one of the two *pfmdr1* copies in the drug resistant FCB line increased susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin [58], further illustrating the central role that *pfmdr1* copy number plays in parasite responses to multiple antimalarials. However, no change in IC_{50} values for CQ was observed, suggesting that the effect of *pfmdr1* copy number on CQR may be strain-specific or depend on specific combinations of *pfcrt* and *pfmdr1* polymorphisms [58].

In addition to amplification, *pfmdr1* polymorphisms have also been associated with drug susceptibility. These occur in several different haplotypes (Table 3) [18, 20, 37, 59–67]. The mutations likely alter the substrate specificity of PfMDR1, as illustrated by the observation that PfMDR1 S1034C/N1042D failed to complement yeast deficient for a mating factor export molecule, while wild type PfMDR1 could [68]. Furthermore, it was shown that expression of wild type PfMDR1 in mammalian cells increased CQ uptake, while PfMDR1 S1034C/N1042D had no effect [69]. Similarly, in heterologous expression studies in *Xenopus* oocytes wild type PfMDR1 transported CQ and quinine but not halofantrine, whereas PfMDR1 variants transported halofantrine but not the other two drugs [70]. Of note, with the exception of Y184F, mutations are generally absent from *pfmdr1* alleles that have also undergone amplification [49, 54].

An association of *pfmdr1* mutations and response to CQ has been reported in some, but not other studies using field isolates (reviewed in [29, 71, 72]). A recent review and meta-analysis of 29 studies supported a role for *pfmdr1* N86Y in CQ and AQ treatment failure, although the association with CQ was weak [1]. Other than N86Y, D1246Y may also be involved in AQ/DEAQ resistance. In East Africa, D1246Y (together with N86Y and Y184) was selected in recrudescent infections after AQ therapy [73], and in a set of Colombian field isolates the parasites with

Table 3 PfMDR1 polymorphisms

Region of origin	Isolates (examples)	PfMDR1 position and encoded amino acid[a,b]					Reference
		86	184	1034	1042	1246	
Wild type							
All regions	D10	N	Y	S	N	D	
Major mutant haplotypes							
K1 haplotype (predominant in Asia and Africa)	Dd2	Y	Y	S	N	D	[37, 62]
7G8 haplotype (predominant in South America)	7G8	N	F	C	D	Y	[37, 62]
Minor mutant haplotypes							
Africa, Southeast Asia	Ghana, Nigeria 2	N	F	S	N	D	[62, 65, 67]
Southeast Asia	Field isolates	N	F	C	N	D	[67]
South America, Southeast Asia	Jav, Ecu1110, HB3, BT3	N	F	S	D	D	[20, 37, 59, 62, 67]
South America, Southeast Asia	PH4	N	F	C	D	D	[18, 59, 67]
South America	Field isolates	N	F	S	D	Y	[20, 64]
Africa	Field isolates	N	F	–	N	Y	[60]
Africa	Field isolates	N	Y	–	N	Y	[60]
Africa	GB4, Nigeria 32	Y	F	S	N	D	[37, 62]
Africa	Field isolates	Y	Y	S	N	Y	[63, 66]
Africa	Field isolates	Y	F	–	N	Y	[60]
Africa	Field isolates	F	Y	S	N	D	[61]

[a]Gray shading indicates residues that differ from the wild type allele. – not determined
[b]Additional polymorphisms may be present at other positions

pfmdr1 D1246Y tended to have the highest AQ IC_{50} values [20]. In South America, the 7G8-type *pfcrt* and *pfmdr1* alleles appear to interact to confer high-level AQ/DEAQ resistance [37]. *pfmdr1* N1042D was strongly associated with lumefantrine IC_{50} values in isolates from the Thai–Myanmar border [74]. Furthermore, in two studies in Africa the wild type N86 and the Y184F and 1246D mutations had significantly higher allelic frequencies in reinfections following artemether–lumefantrine treatment [75, 76], suggesting that *pfmdr1* polymorphisms may prove useful as a molecular marker for lumefantrine resistance. In contrast, while certain *pfmdr1* point mutations did alter susceptibility of field isolates to mefloquine and artesunate in vitro [49, 50, 74], they were not predictive for treatment outcome following mefloquine monotherapy or mefloquine–artesunate combination therapy [49, 52, 77].

Allelic exchange studies have confirmed the influence of 3′ *pfmdr1* point mutations on parasite responses to numerous drugs. In the CQ-sensitive D10 and CQ-resistant 7G8 parasite strains, the S1034C/N1042D/D1246Y mutations were sufficient to confer a significant decrease in quinine susceptibility [78]. A strong influence of position 1042 on quinine response was confirmed in the GC03 and 3BA6 genetic backgrounds, representing progeny of the HB3 × Dd2 genetic cross, although the three mutations were insufficient to confer quinine resistance to GC03 when compared with the wild type allele [72]. These findings corroborate a QTL analysis of the same genetic cross that attributed 10% of the total variation in quinine response to a region on chromosome 5 containing *pfmdr1* [6], and a recent genome-wide association study that reported an association of *pfmdr1* with quinine and, more weakly, CQ responses [7]. In contrast, the presence of the S1034C/N1042D/D1246Y mutations was correlated with increased susceptibility to artemisinin, mefloquine, and halofantrine in all genetic backgrounds used in these allelic exchange studies (D10, 7G8, GC03, and 3BA6) [72, 78]. These findings corroborate the linkage between *pfmdr1* mutations and increased susceptibility to mefloquine, halofantrine, lumefantrine, artemisinin, artemether, and arteflene (Ro 42–1611) that had previously been observed in the genetic cross between HB3 and the CQ-sensitive 3D7 strain [79].

The role of 3′ *pfmdr1* polymorphisms in parasite responses to CQ has been less clear. While reversal of S1034C/N1042D/D1246Y in a CQ-resistant clone (7G8) halved the level of CQR, introduction of the same mutations into another CQ-resistant clone (3BA6) and two sensitive clones (D10 and GC03) had no effect on parasite susceptibility to CQ [72, 78]. These findings suggested that *pfmdr1* polymorphisms can enhance CQR in a strain-dependent manner, but are insufficient to confer CQR to a CQ-sensitive strain. *pfmdr1* may contribute to CQR directly by transporting drug or by influencing physiological parameters that influence drug accumulation or parasite susceptibility to the toxic consequences of drug interference with heme detoxification. Alternatively, *pfmdr1* polymorphisms might be beneficial indirectly by compensating for physiological perturbations induced by the expression of mutant *pfcrt* [29]. This epistatic interaction may in part explain the observed linkage disequilibrium between *pfcrt* and *pfmdr1* (reviewed in [29]).

2.3 P. falciparum *Multidrug Resistance-Associated Protein and Other Transporters*

In addition to *pfcrt* and *pfmdr1*, many other transporters have earlier been reported to associate with parasite responses to quinine and CQ in a study that genotyped 97 culture-adapted isolates for SNPs in 49 genes encoding predicted or known transporters and transport regulatory proteins [80]. The identified SNPs included two point mutations in the plasma membrane ABC transporter *P. falciparum* multidrug resistance-associated protein (PfMRP) (PFA0590w), which were weakly associated with CQR in Asia (Y191H) and the Americas (A437S), and quinine resistance in the Americas (Y191H, A437S) [80]. However, studies on *P. falciparum* isolates from French travelers returning from Africa [81], and on clinical isolates from the Thailand–Myanmar border [74], failed to find significant associations between SNPs in these transporter genes and drug responses, with the single exception of a borderline significant association of a 3 bp insertion in the ABC transporter G7 (PF13_0371) and increased artesunate IC_{50} values [74], the relevance of which remains to be determined. Of note, PfMRP Y191H could not be analyzed in the Thai–Myanmar isolates due to insufficient variation [74]. Recent studies have also reported evidence for the possible selection of certain PfMRP alleles in reinfections following artemether–lumefantrine treatment (those with 876I) [82], and in recrudescence following sulfadoxine–pyrimethamine treatment (those with 1466K) [83].

Disruption of PfMRP in the CQ-resistant strain W2 rendered the parasite more susceptible to CQ and quinine, possibly as a result of reduced efflux of these drugs by the knockout parasite [84]. PfMRP may transport multiple chemically unrelated drugs, as illustrated by the reduced IC_{50} values that were also reported for artemisinin, piperaquine, and primaquine. PfMRP-deficient parasites also accumulated more glutathione, indicating that PfMRP may transport glutathione out of the cell. Overall the observed reductions in IC_{50} values were modest (38–57%), suggesting that PfMRP might serve as a secondary determinant to modulate parasite resistance to these antimalarials.

2.4 P. falciparum *Na^+/H^+ Exchanger 1*

Earlier QTL mapping of 35 progeny of a cross between the low-level quinine-resistant Dd2 clone and the quinine-sensitive HB3 clone identified five genomic regions with additive or pairwise effects on quinine response [6]. Most of the quinine response was attributed to two additive loci, a chromosome 7 peak that was centered around *pfcrt* (30% of the variation) and a peak on chromosome 13 (35% of the variation) that interacted with a locus on chromosome 9 (Fig. 1). Within

the chromosome 13 region, the authors identified the *P. falciparum* Na^+/H^+ exchanger 1 (*pfnhe-1*) gene that encodes a putative Na^+/H^+ exchanger as a potential candidate gene and demonstrated an association of a D- and N-rich repeat polymorphism (microsatellite ms4760-1) with quinine response in 71 isolates from Africa, South east Asia, and Central and South America [6]. Of note, this locus is highly polymorphic, with >30 different genotypes reported thus far [85, 86]. However, subsequent studies have reported conflicting results on the association of this locus with quinine responses, with increasing repeat number associated with increased quinine IC_{50} values in one study [86], but decreased IC_{50} values in another study [87].

Some strains with higher levels of quinine resistance were reported to possess elevated Na^+/H^+ exchange activity and either a higher cytosolic pH or vacuolar-to-cytosolic pH gradient [88], although the experimental basis of these observations has been called into question in other studies [89, 90]. Importantly, a genetic knockdown of *pfnhe-1* expression through truncation of the 3′ untranslated region resulted in a statistically significant ~30% decrease in quinine mean IC_{50} values in the CQ- and (moderately) quinine-resistant 1BB5 and 3BA6 parasite lines, but not in the CQ-sensitive GC03 line [90]. These data support a role for *pfnhe-1* in contributing to quinine susceptibility in a strain-specific manner but also imply that other parasite factors are required for the parasite to be resistant to quinine.

3 Molecular Markers for Resistance to Antifolates

Antifolates that have been used for malaria treatment include pyrimethamine, sulfadoxine, and proguanil. In the case of proguanil, it is the cycloguanil metabolite that interferes with folate metabolism. Resistance of *P. falciparum* parasites to all of these agents is widespread [91]. Pyrimethamine and cycloguanil inhibit the parasite dihydrofolate reductase (DHFR) and sulfadoxine inhibits a downstream enzyme in the folate biosynthetic pathway, dihydropteroate synthase (DHPS). The sulfadoxine–pyrimethamine combination was used extensively as a first-line treatment against *P. falciparum* in many malaria-endemic regions when CQ lost its efficacy [92]. The efficacy of this combination is highly dependent on synergy between the components; when resistance to either component exists, the efficacy of the combination is severely reduced [93]. Readers are referred to Gregson and Plowe [94] for a comprehensive review on the mechanisms of antifolate action and resistance in *P. falciparum*.

Resistance of *P. falciparum* parasites to antifolates is mediated by mutations in the target proteins – DHFR in the case of pyrimethamine and cycloguanil and DHPS in the case of sulfadoxine. Evidence for an association between mutations in DHFR/DHPS and resistance to the antifolates came from sequencing of the *dhfr* and *dhps* genes in strains with varying susceptibilities to antifolate drugs [95–100]. A causal effect for mutations in these proteins was subsequently established by transfecting parasites with mutant forms of the genes [101, 102]. The effects of

mutations in DHFR and DHPS on enzymatic activity and sensitivity to antifolate-mediated inhibition have also been ascertained with recombinant proteins from *P. falciparum* or by characterizing corresponding mutations in the DHFR ortholog from *Toxoplasma gondii* [103–105].

A number of different mutant forms of DHFR have been identified in the field. Pyrimethamine-resistant isolates contain a S108N mutation, and more highly resistant isolates possess additional mutations at codons 51 (N51I), 59 (C59R), and sometimes 164 (I164L). An A16V/S108T form has also been discovered and confers greater resistance to cycloguanil than to pyrimethamine [97]. The other forms confer a greater degree of resistance to pyrimethamine than to cycloguanil with the exception of the "quadruple mutant" (the S108N/N51I/C59R/I164L combination), which confers high-level resistance to both drugs [97, 98]. The quadruple mutant is of particular concern as it has been associated with very high levels of sulfadoxine–pyrimethamine treatment failure [106]. It is widespread in Southeast Asia but remains rare in Africa [107]. Studies in which *P. falciparum* DHFR variants were expressed in yeast or *E. coli* revealed that the addition of the I164L mutation to the triple mutant form of DHFR augmented resistance to pyrimethamine but also had a negative impact on enzyme activity (as inferred from the rate of yeast/bacterial growth) [108, 109]. Thus, quadruple mutant parasites may be at a fitness disadvantage, and more fit, less resistant parasites may have the upper hand in Africa where asymptomatic untreated infections are common [94]. Five mutations associated with sulfadoxine resistance have been identified in DHPS: S436A/F, A437G, K540E, A581G, and A613S/T. Among these mutations, the A437G change (either alone or in combination with other mutations) is observed most often in field isolates [93].

A recent study analyzed the literature (consisting predominantly of studies performed in Africa within the past decade) on the effect of mutations in DHFR on treatment outcome with sulfadoxine–pyrimethamine [1]. When considered in isolation and without knowledge of whether other DHFR mutations were present, the mutations at positions 108, 51, and 59 gave odds ratios (ORs; the proportion of therapeutic failures with mutation-bearing parasites divided by the proportion of therapeutic failures with wild type parasites) of 2.1, 1.7, and 1.9, respectively. For the 51 + 59 + 108 triple mutant, the OR was 4.3 [1]. No data were provided for the quadruple mutant, presumably because fewer studies on its association with treatment failure have been performed.

From studies investigating the influence of DHPS mutations on sulfadoxine–pyrimethamine treatment outcome, Picot et al. determined an OR of 1.5 for the A437G mutation and an OR of 3.9 for the 437 + 540 double mutant. For "quintuple mutants" with mutations at positions 51, 59, and 108 in DHFR and 437 and 540 in DHPS, the OR was 5.2 [1]. Two studies reported that the presence of two mutations, DHFR C59R and DHPS K540E, was highly predictive of the presence of the quintuple mutation set [110, 111]. Thus, monitoring just these two mutations could be sufficient to enable predictions of sulfadoxine–pyrimethamine efficacy in a given area [94]. As with all antimalarial treatment regimens, parasite genotype is not the only determinant of treatment outcome. Host factors including immunity,

nutritional status (including blood folate concentrations), and drug metabolism rates will also impact on patient response.

Although the move towards artemisinin-based combination therapies has lessened the use of sulfadoxine–pyrimethamine for malaria treatment, the latter continues to occupy an important place in the "intermittent preventative treatment" of pregnant women. A recent study investigated whether this choice of treatment of pregnant women was of benefit in an area of Tanzania in which mutant *dhfr* and *dhps* alleles are prevalent. Worryingly, the authors found that women who received treatment in this area were more likely to have a higher parasitemia and increased level of inflammation in the placenta than those who did not [112]. This would imply that the monitoring of sulfadoxine–pyrimethamine resistance in areas in which it is being considered for use in intermittent preventative treatment is of utmost importance for informing policy choices.

There is no evidence for an amplification of the genes encoding DHFR or DHPS in antifolate-resistant *P. falciparum* field isolates [94]. However, the amplification of a gene upstream in the folate biosynthesis pathway, *gtp-cyclohydrolase I*, has been observed in certain strains [113] and has been reported to associate with the I164L mutation in DHFR [114]. An increase in the level of GTP-cyclohydrolase I (the rate-limiting enzyme in the folate biosynthesis pathway) might be an adaptation to compensate for a reduced efficiency of 164L-containing DHFR [114].

4 Molecular Markers for Resistance to Artemisinins

Clinical resistance to artemisinin derivatives has not yet emerged, although data from artemisinin monotherapy treatment studies in west Cambodia provide evidence of delayed parasite clearance times, which might presage the emergence of bona fide resistance leading to treatment failures [115, 116]. Thus there is a clear imperative to identify molecular markers of artemisinin resistance. The major candidate that has been proposed is the ATP-consuming calcium-dependent *P. falciparum* SERCA ortholog, PfATP6. Initial studies in transfected *Xenopus laevis* oocytes provided evidence that PfATP6 could be specifically inhibited by artemisinin, an effect that could be antagonized by the mammalian SERCA inhibitor thapsigargin [117]. When expressed and purified from *Saccharomyces cerevisiae*, PfATP6 incorporated into lipid vesicles could not be inhibited by artemisinin and was only partially sensitive to high concentrations of the SERCA inhibitor thapsigargin [118]. The latter suggests that caution should be taken when interpreting these results, which do not rule out the PfATP6 hypothesis (reviewed in [119]). Modeling studies have predicted that introducing an L263E mutation into PfATP6 could result in a loss of artemisinin binding, which was subsequently confirmed in oocyte assays [120]. This mutation has now been engineered into the *pfatp6* locus in *P. falciparum* [121]. Assays with cultured asexual blood stage parasites found no difference between L263E and wild type lines in their IC_{50} values with artemisinin, dihydroartemisinin, and artesunate. However, L263E

mutant lines showed a clear difference in the distribution of their IC_{50} values, and IC_{50} values normalized to wild type controls showed a significant increase to artemisinin and dihydroartemisinin that was particularly evident in the D10 (Papua New Guinea) genetic background.

Studies have not observed the L263E mutation in field isolates. However, multiple other mutations have been observed, including the S769N mutation that was detected in a few isolates from French Guiana that yielded high IC_{50} values when tested against artemether [119, 122, 123]. Studies to define their impact in *P. falciparum* should shed further light on the relationship between PfATP6 and parasite susceptibility to artemisinins.

The search for molecular markers for artemisinin resistance via a genetic approach of selecting for resistance and identifying mutations that might be causal for resistance has been severely hampered by the difficulties in selecting stably resistant lines in vitro. In genetically modified parasite lines, *pfcrt* and *pfmdr1* mutations were observed to increase parasite susceptibility to artemisinin and its derivatives [9, 39, 72, 78], but the observed changes were small and neither gene therefore appears to be a major resistance determinant. *pymdr1* amplification was reported in unstably artemisinin-resistant *P. yoelii* [124], and some – but not all – artemisinin- or artelinic acid-selected *P. falciparum* lines had also increased their *pfmdr1* copy number [125, 126]. However, when the artelinic-acid resistant *P. falciparum* line was cultured in the absence of drug, parasites rapidly deamplified *pfmdr1*, concomitant with only a partial reversal of drug resistance [127]. Stable resistance to artemisinin and its derivatives has to date only been obtained in *P. chabaudi*; this was achieved in the absence of mutations or copy number changes in the *pfcrt* and *pfmdr1* orthologs and other genes previously associated with the mechanism of action of artemisinins [128].

Linkage group selection performed with the uncloned progeny of a genetic cross between the artemisinin-resistant *P. chabaudi* line and a sensitive clone subsequently identified a region on chromosome 2 that appeared to be selected by artemisinin [129]. Within this region, the authors detected two nonsynonymous mutations within a gene encoding a putative deubiquitinating protease termed ubiquitin-specific protease-1 (UBP-1) or ubiquitin carboxyl terminal hydrolase (UBCTH) (the ortholog of *P. falciparum* MAL1P1.34b). These mutations arose during selection with CQ and with CQ and mefloquine (V2728F), or with CQ and artesunate (V2697F), respectively [130]. This puzzling observation of similar mutations in the same gene occurring under selection with two different drugs could be explained if (1) mutant *pcubp1* compensated for a fitness cost in the last common progenitor instead of directly contributing to resistance to either drug; or (2) if both drugs compromised a common cellular function, such as the regulation of oxidative stress; or (3) if UBP-1 mediated the posttranslational modification of a target that modulated parasite responses to both drugs [129]. Of note, no mutations in *pfubp1* were found in unstably artemisinin- or artelinic acid-resistant *P. falciparum* [125, 129]. Without allelic exchange studies, one cannot rule out that artemisinin and artesunate resistance in *P. chabaudi* is instead caused by a different gene, either physically linked or epistatic to *pcubp1*.

Unstably artemisinin-resistant *P. yoelii* parasites were also shown to overexpress translationally controlled tumor protein homolog (TCTP) [131], a protein previously identified as potentially interacting with dihydroartemisinin [132]. However, no change in PfTCTP expression level was observed in artemisinin- or artelinic acid-resistant *P. falciparum* [125]. There is to date no evidence that TCTP can contribute to reduced parasite susceptibility to artemisinin.

Parasite responses to the artemisinin combination therapy partner drugs lumefantrine, AQ, and mefloquine can be impacted by *pfcrt* and *pfmdr1*, and the reader is referred to the respective section of this chapter. Resistance to piperaquine has been reported following its use as monotherapy in China, but the genetic resistance determinant has not been identified [133]. Resistance to pyronaridine has not yet been observed.

5 Molecular Markers for Resistance to Antibiotics

Doxycycline is a tetracycline antibiotic that is used widely for antimalarial chemoprophylaxis. Doxycycline and tetracycline can also be used in combination with quinine, quinidine, or artesunate [91]. In bacteria, tetracyclines exert their toxic effects by binding to the 30S subunit of the 70S ribosome (comprising the 16S rRNA and ribosomal proteins) and thereby inhibiting protein translation [134]. It is likely that in *P. falciparum* they target the bacterial-like protein translation machinery in the apicoplast [135]. There have been reports of prophylactic failures with doxycycline, although this could relate to inadequate doses or to poor compliance. Clinical resistance has not been demonstrated.

In a recent study, the doxycycline susceptibilities of 747 *P. falciparum* isolates from different African countries were tested in vitro with the [^{3}H]-hypoxanthine incorporation method [136]. The strains were classified into three different phenotypic groups, with IC_{50} values for doxycycline stratified as 4.9 $\pm$ 2.1, 7.7 $\pm$ 1.2 or 17.9 $\pm$ 1.4 μM. The drug assays were performed over 42 h (<1 complete asexual cycle), despite previous evidence showing that doxycycline becomes markedly more potent in the second cycle of parasite proliferation [137]. A follow-up study reported that increases in the copy number of *pfmdt* (PFE0825w; a putative drug/metabolite transporter) and *pftetQ* (PFL1710c; a putative tetQ family GTPase) were more frequent in parasites in the "higher doxycycline IC_{50} group" than in parasites from the other two groups [138]. Furthermore, the number of KYNNNN amino acid motif repeats in TetQ was on average slightly lower in the higher doxycycline IC_{50} group [138]. It is likely that the second-cycle potency of doxycycline is more relevant to its clinical activity than its first-cycle potency, thus it would be premature to consider *pftetQ* or *pfmdt* as potential molecular markers for reduced parasite susceptibility to doxycycline without first knowing whether a higher first-cycle IC_{50} value is predictive of a higher second-cycle IC_{50} value.

Clindamycin is a lincosamine antibiotic that, like the tetracyclines, inhibits prokaryotic protein translation, but in this case by binding to the 50S ribosomal subunit, which comprises the 23S rRNA, the 5S rRNA, and ribosomal proteins [134]. Clindamycin is also recommended for malaria treatment in combination with quinine, quinidine, or artesunate, and unlike the tetracyclines is deemed safe for pregnant women and small children [139]. The existence of clindamycin-resistant *P. falciparum* parasites was first uncovered in 2010, when genomic analyses of isolates from the Peruvian Amazon led to the discovery of two point mutations in the apicoplast 23S rRNA, one of which (A1875C) had a similar location to mutations known to confer clindamycin resistance in various bacteria [140]. In vitro drug susceptibility testing revealed that the A1875C mutation was associated with a marked decrease in parasite sensitivity to clindamycin (a >100-fold increase in IC_{50}).

The erythromycin derivative azithromycin, which is being investigated as a potential component of an antimalarial combination therapy, also acts against bacteria by binding to the 50S ribosomal subunit [134]. It binds in the polypeptide exit tunnel adjacent to the peptidyl transferase center and hence interferes with the translocation of nascent polypeptides through this tunnel [141]. The binding site includes the 23S rRNA and the proteins L4 and L22 [142–144].

In a recent study, *P. falciparum* parasites resistant to azithromycin were generated through in vitro drug pressure [145]. The drug-selected lines had IC_{50} values for azithromycin that were 16- to 17-fold higher than those of the parental lines (7G8 and Dd2). Studies on the mechanisms of azithromycin action and resistance in bacteria allowed Sidhu and colleagues to take a candidate gene approach to determining the molecular basis of azithromycin resistance. Genes encoding the *P. falciparum* orthologs of the 23S rRNA and the proteins L4 and L22 were PCR-amplified from the azithromycin-resistant lines and sequenced. A single point mutation was found in the *Pfrpl4* gene (encoding L4) in the azithromycin-resistant lines selected from the 7G8 and Dd2 lines, and not in the parent lines themselves. This mutation was identical in both azithromycin-resistant lines and would result in a glycine to valine substitution at position 76 in the L4 protein. Similarly-placed L4 mutations have been reported in macrolide-resistant bacteria, and the region of the protein in which they reside appears to be key in determining the access of drug to the binding site in the ribosome (reviewed in [146]) (Fig. 2). Thus, this study strongly suggests that azithromycin acts against *P. falciparum* by inhibiting protein translation in the apicoplast and that, like in bacteria, resistance can be mediated by alterations in the components that are predicted to form the drug-binding site.

Another antibiotic that has shown some promise in clinical studies is fosmidomycin [147–149]. Fosmidomycin inhibits 1-deoxy-D-xylulose 5-phosphate (DOXP) reductoisomerase (DXR), the second enzyme in the DOXP pathway for isoprenoid biosynthesis [139]. A recent study investigated whether *P. falciparum* parasites resistant to this antibiotic could be generated through in vitro drug

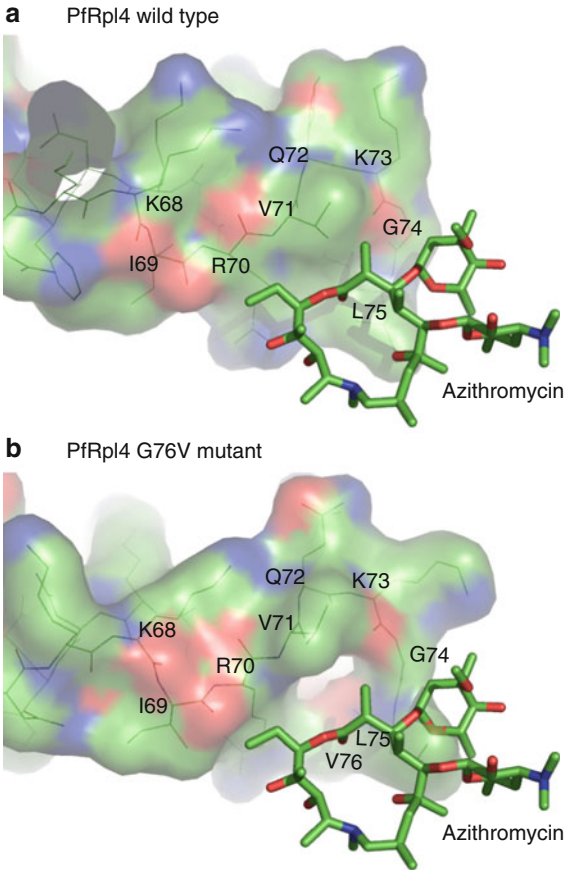


Fig. 2 Structural models of (**a**) wild type and (**b**) the G76V mutant Pfrpl4 with azithromycin. These models, derived using the *Deinococcus radiodurans* crystal structure of protein L4 as a template, revealed a steric clash between the side chain of 76V in the G76V mutant and azithromycin, consistent with this mutation conferring azithromycin resistance. Modeling was performed by Qingan Sun and James C. Sacchettini (Texas A&M University, College Station, TX). This figure was originally published in [145]. © The American Society for Biochemistry and Molecular Biology

pressure, and if so, whether the resistance mechanism could be elucidated using a high-density tiling microarray [150]. Fosmidomycin-resistant parasites (with an ~8-fold elevated IC_{50} value) were generated by sequentially increasing the drug concentration applied to Dd2 parasites. DNA from fosmidomycin-resistant clones and the parental line was then hybridized onto the microarrays, and a single large amplification event on chromosome 4 of the fosmidomycin-resistant clones was observed. The amplified region was ~100 kb in size and contained 23 genes, the first of which was *pfdxr*, the gene encoding the putative target of fosmidomycin. Subsequent analyses revealed a ~3.8-fold increase in the level of *pfdxr* transcript and a ~3-fold increase in the gene copy number in a fosmidomycin-resistant clone compared with the Dd2 parental line, with no changes in the gene sequence [150].

Thus, potential molecular markers for resistance of *P. falciparum* parasites to azithromycin and fosmidomycin have been identified before these compounds have been approved for use as antimalarial drugs. The identification of these markers was aided greatly by previous studies on antibiotic action and resistance in bacteria.

6 Molecular Markers for Resistance to Atovaquone

Atovaquone was developed in the 1980s and found to possess activity against a number of parasites including *Plasmodium* [151]. When tested as a monotherapy for *P. falciparum* malaria, atovaquone treatment was associated with a high risk of parasite recrudescence (~30%) [152–154]. Recrudescent parasites isolated from patients treated with atovaquone were found to be highly resistant to the drug, with a >1,000-fold elevated IC_{50} value when tested in vitro [153]. Despite atovaquone's shortcomings when used as a single agent, the synergistic combination of atovaquone and proguanil (Malarone) has proven very effective against malaria, and is used for treatment and prophylaxis [155]. This synergy is attributable to proguanil, not its DHFR-inhibiting metabolite cycloguanil [156]. A study with the murine malaria parasite *P. yoelii* revealed that once parasites are resistant to atovaquone, synergy with proguanil is lost [157].

Studies on the mechanism of action of atovaquone have revealed that the drug inhibits mitochondrial electron transport by interacting with the cytochrome *bc*1 complex [158]. It was recently shown, by generating transgenic parasites expressing the *S. cerevisiae* dihydroorotate dehydrogenase enzyme in the cytosol, that the parasite's electron transport chain is essential only for the regeneration of ubiquinone, which is the required electron acceptor for the parasite (but not the *S. cerevisiae*) dihydroorotate dehydrogenase [159]. The transgenic parasites were found to be highly resistant to atovaquone and other electron transport inhibitors, yet remained highly susceptible to the atovaquone–proguanil combination. The authors hypothesized that the maintenance of a membrane potential across the mitochondrial membrane is essential for parasite viability, and that in addition to the electron transport chain, a proguanil-sensitive pathway for the generation of a mitochondrial membrane potential exists in the parasite. Atovaquone alone did not abrogate the membrane potential in either the parental or transgenic parasites; however, the atovaquone–proguanil combination was highly effective in dissipating the membrane potential in both cases [159].

There is evidence to support the use of the cytochrome *b* gene (encoded on the mitochondrial genome) as a molecular marker to monitor atovaquone resistance. Mutations in this gene were found in each of nine independent atovaquone-resistant *P. yoelii* lines selected using suboptimal treatment of *P. yoelii*-infected mice [157]. The mutations in cytochrome *b* observed in the atovaquone-resistant *P. yoelii* lines all fell within a 15 amino acid region that forms part of the catalytic domain, or Q_o site, where ubiquinol oxidation occurs [160]. Mutations in the Q_o region of the cytochrome *b* gene have subsequently been reported in atovaquone-resistant *P. falciparum* [161], *P. berghei* [162], and *T. gondii* [163] parasites.

Atovaquone–proguanil treatment failures have been associated with the mutation of Y268 in *P. falciparum* cytochrome *b* to S, C or N (Y268S/C/N; [164, 165], and references therein). It has been shown that parasites bearing a mutation at position 268 can arise and spread within individual patients receiving atovaquone–proguanil treatment [166]. Introducing a mutation in the bacterial

cytochrome *b* at the position corresponding to residue 268 in *P. falciparum* (Y302C in the bacterial protein) was found to render the bacterial cytochrome *bc*1 complex much less sensitive to inhibition by atovaquone [167], thereby establishing a causal effect for this mutation in the phenomenon of atovaquone resistance.

7 Molecular Markers for Drug Resistance in *P. vivax*

The lack of a long term in vitro culture system for *P. vivax* remains a major obstacle to the study of drug resistance in this important human pathogen. Insights into the genetic basis of resistance therefore remain limited and have been gained mainly by comparison with *P. falciparum*.

Orthologs of *pfcrt* and *pfmdr1* have been identified in the *P. vivax* genome. Multiple polymorphisms have been reported in these genes, *pvcrt-o* (also known as *pvcg10*) and *pvmdr1*, albeit not at positions homologous to the mutations found in the *P. falciparum* genes [168, 169]. Moreover, *pvcrt-o* and *pvmdr1* mutations did not associate with CQR in patient isolates or monkey-adapted lines [168, 170–172]. One exception may be *pvmdr1* Y976F, which was found to associate with higher CQ IC_{50} values in Thai isolates [173]. However, this mutation had reached near fixation in the studied parasite isolates from Papua, Indonesia [173], Madagascar [172], and Brazil [174], despite low rates of CQ treatment failure reported in the latter two studies, and may thus be of limited value as a molecular marker. These findings suggest that CQR may have a different genetic basis in *P. vivax*. It is worth noting, however, that expression of wild-type *pvcrt-o* in *P. falciparum* increased the CQ IC_{50} value by 2.2-fold, and in *D. discoideum* resulted in reduced CQ accumulation in acid endosomes [175], suggesting that *pvcrt-o* could nevertheless play some role in CQR in *P. vivax*.

A mechanism of drug resistance that may more readily translate from *P. falciparum* is the role of *mdr1* amplification in mefloquine resistance. Two studies have reported increased *pvmdr1* copy numbers in *P. vivax* isolates from Tak province in Thailand, where mefloquine has been heavily used, but not in isolates from regions where parasites have not been exposed to this drug (other Thai provinces, as well as Laos, Myanmar, Papua) [169, 173].

There is also emerging evidence that mutations in *dhfr* and *dhps* are associated with a reduced parasite sensitivity and a higher risk of treatment failure to sulfadoxine–pyrimethamine in *P. vivax* (reviewed in [176]). More than 20 *dhfr* alleles have been identified in *P. vivax* [176]. This includes the pyrimethamine-resistance-conferring S58R/S117N form, whose crystal structure has recently been elucidated, along with wild type *P. vivax* DHFR ([177]; Fig. 3). The S58R/S117N mutations are homologous to the C59R/S108N mutations in PfDHFR that cause moderate resistance to pyrimethamine. The residue at position 58 in PvDHFR was found not to interact with pyrimethamine, and it was suggested that it may be involved in binding the natural substrate. The mutation at codon 117 was found to perturb the

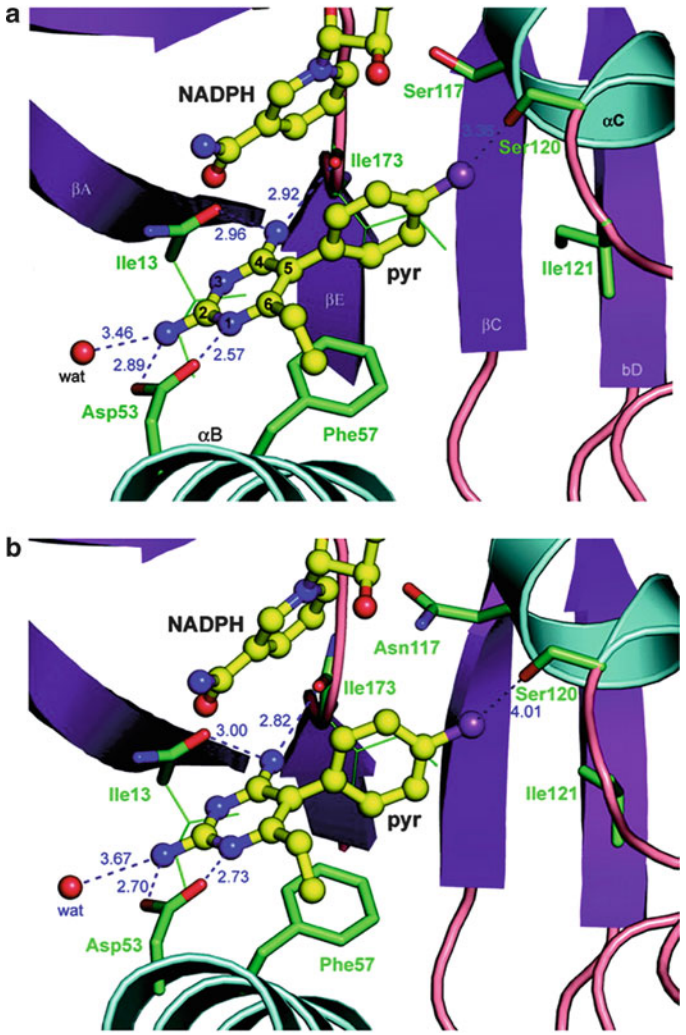


Fig. 3 Binding of pyrimethamine in the active site of *Plasmodium vivax* DHFR. The pyrimethamine (pyr) and NADPH cofactor are shown as *balls* and *sticks* with carbon, nitrogen, and chlorine colored *yellow*, *blue*, and *magenta*, respectively. (**a**) Pyr binding with the wild type *P. vivax* DHFR. Interactions between the enzyme and the pyrimidine ring of the inhibitor include electrostatic interactions and H-bonds indicated by dotted lines. *Numbers* next to the lines indicate distances in Å. (**b**) Pyr binding with the S58R/S117N double-mutant enzyme. X-ray crystallography revealed that the interactions around the pyrimidine ring were similar to the wild type enzyme. The mutation at amino acid 117 from S to N increases a steric factor in the active site. As a result, the positions of both NADPH and pyr were perturbed from their optimum binding, reducing the efficiency of pyr by as much as 300-fold. The mutant R residue at position 58 did not directly interact with the inhibitor and was proposed to affect substrate binding. Data were published by [177]. These crystal structure images were reproduced with kind permission from Yongyuth Yuthavong and PNAS

binding of pyrimethamine [177], as shown previously for the S108N mutation in the *P. falciparum* enzyme [178].

Acknowledgments Funding for this work was provided in part by the NIH (R01 AI50234 to D.A.F.) and an Investigator in Pathogenesis of Infectious Diseases Award from the Burroughs Wellcome Fund (to D.A.F.). A. M. L. is supported by an Australian NHMRC Overseas Biomedical Fellowship (585519) and A.E. is supported by a Human Frontier Science Program Long Term Postdoctoral Fellowship.

References

1. Picot S, Olliaro P, de Monbrison F, Bienvenu AL, Price RN, Ringwald P (2009) A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. Malar J 8:89
2. Sibley CH, Barnes KI, Watkins WM, Plowe CV (2008) A network to monitor antimalarial drug resistance: a plan for moving forward. Trends Parasitol 24:43–48
3. Hayton K, Su XZ (2008) Drug resistance and genetic mapping in *Plasmodium falciparum*. Curr Genet 54:223–239
4. Ekland EH, Fidock DA (2007) Advances in understanding the genetic basis of antimalarial drug resistance. Curr Opin Microbiol 10:363–370
5. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW et al (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 6:861–871
6. Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 52:985–997
7. Mu J, Myers RA, Jiang H, Liu S, Ricklefs S, Waisberg M, Chotivanich K, Wilairatana P, Krudsood S, White NJ et al (2010) *Plasmodium falciparum* genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs. Nat Genet 42:268–271
8. Martin RE, Kirk K (2004) The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. Mol Biol Evol 21:1938–1949
9. Sidhu AB, Verdier-Pinard D, Fidock DA (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 298:210–213
10. Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, Fidock DA (2010) Identification of a mutant PfCRT-mediated chloroquine tolerance phenotype in *Plasmodium falciparum*. PLoS Pathog 6:e1000887
11. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, Muhle RA, Alakpa GE, Hughes RH, Ward SA, Krogstad DJ et al (2005) A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J 24:2294–2305
12. Valderramos SG, Fidock DA (2006) Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci 27:594–601
13. Zhang H, Paguio M, Roepe PD (2004) The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. Biochemistry 43:8290–8296
14. Naude B, Brzostowski JA, Kimmel AR, Wellems TE (2005) *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. J Biol Chem 280:25596–25603

15. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, Kirk K (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science 325:1680–1682
16. Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA (2005) Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. Mol Microbiol 56:323–333
17. Best Plummer W, Pinto Pereira LM, Carrington CV (2004) *Pfcrt* and *pfmdr1* alleles associated with chloroquine resistance in *Plasmodium falciparum* from Guyana, South America. Mem Inst Oswaldo Cruz 99:389–392
18. Chen N, Kyle DE, Pasay C, Fowler EV, Baker J, Peters JM, Cheng Q (2003) *pfcrt* allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. Antimicrob Agents Chemother 47:3500–3505
19. Durrand V, Berry A, Sem R, Glaziou P, Beaudou J, Fandeur T (2004) Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and their relationship to chloroquine resistance *in vitro*. Mol Biochem Parasitol 136:273–285
20. Echeverry DF, Holmgren G, Murillo C, Higuita JC, Bjorkman A, Gil JP, Osorio L (2007) Polymorphisms in the *pfcrt* and *pfmdr1* genes of *Plasmodium falciparum* and *in vitro* susceptibility to amodiaquine and desethylamodiaquine. Am J Trop Med Hyg 77:1034–1038
21. Hatabu T, Iwagami M, Kawazu S, Taguchi N, Escueta AD, Villacorte EA, Rivera PT, Kano S (2009) Association of molecular markers in *Plasmodium falciparum crt* and *mdr1* with *in vitro* chloroquine resistance: a Philippine study. Parasitol Int 58:166–170
22. Huaman MC, Yoshinaga K, Suryanatha A, Suarsana N, Kanbara H (2004) Polymorphisms in the chloroquine resistance transporter gene in *Plasmodium falciparum* isolates from Lombok, Indonesia. Am J Trop Med Hyg 71:40–42
23. Lim P, Chy S, Ariey F, Incardona S, Chim P, Sem R, Denis MB, Hewitt S, Hoyer S, Socheat D et al (2003) *pfcrt* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. Antimicrob Agents Chemother 47:87–94
24. Menard D, Yapou F, Manirakiza A, Djalle D, Matsika-Claquin MD, Talarmin A (2006) Polymorphisms in *pfcrt*, *pfmdr1*, *dhfr* genes and *in vitro* responses to antimalarials in *Plasmodium falciparum* isolates from Bangui, Central African Republic. Am J Trop Med Hyg 75:381–387
25. Nagesha HS, Casey GJ, Rieckmann KH, Fryauff DJ, Laksana BS, Reeder JC, Maguire JD, Baird JK (2003) New haplotypes of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene among chloroquine-resistant parasite isolates. Am J Trop Med Hyg 68:398–402
26. Restrepo E, Carmona-Fonseca J, Maestre A (2008) *Plasmodium falciparum*: high frequency of *pfcrt* point mutations and emergence of new mutant haplotypes in Colombia. Biomedica 28:523–530
27. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ (2002) Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature 418:320–323
28. Yang Z, Zhang Z, Sun X, Wan W, Cui L, Zhang X, Zhong D, Yan G (2007) Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China. Trop Med Int Health 12:1051–1060
29. Waller KL, Lee S, Fidock DA (2004) Molecular and cellular biology of chloroquine resistance in *Plasmodium falciparum*. In: Waters AP, Janse CJ (eds) Malaria parasites: genomes and molecular biology. Caister Academic, Wymondham, pp 501–540
30. Pillai DR, Labbe AC, Vanisaveth V, Hongvangthong B, Pomphida S, Inkathone S, Zhong K, Kain KC (2001) *Plasmodium falciparum* malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. J Infect Dis 183:789–795
31. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA et al (2001) A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344:257–263

32. Babiker HA, Pringle SJ, Abdel-Muhsin A, Mackinnon M, Hunt P, Walliker D (2001) High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfcrt* and the multidrug resistance gene *pfmdr1*. J Infect Dis 183:1535–1538
33. Wellems TE, Plowe CV (2001) Chloroquine-resistant malaria. J Infect Dis 184:770–776
34. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimde AA, Kouriba B, Taylor TE, Plowe CV (2003) Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J Infect Dis 187:1870–1875
35. Juliano JJ, Kwiek JJ, Cappell K, Mwapasa V, Meshnick SR (2007) Minority-variant *pfcrt* K76T mutations and chloroquine resistance, Malawi. Emerg Infect Dis 13:872–877
36. Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Bjorkman A (2006) Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of *pfcrt* 76T and *pfmdr1* 86Y. Infect Genet Evol 6:309–314
37. Sa JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE (2009) Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci USA 106:18883–18889
38. Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Fidock DA, Gil JP (2009) *In vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible *pfcrt* K76 allele after treatment with artemether-lumefantrine in Africa. J Infect Dis 199:750–757
39. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol Pharmacol 61:35–42
40. Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X, Ferdig MT (2007) Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Mol Microbiol 63:270–282
41. Rohrbach P, Sanchez CP, Hayton K, Friedrich O, Patel J, Sidhu AB, Ferdig MT, Fidock DA, Lanzer M (2006) Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum*. EMBO J 25:3000–3011
42. Martin SK, Oduola AM, Milhous WK (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science 235:899–901
43. Foote SJ, Thompson JK, Cowman AF, Kemp DJ (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell 57:921–930
44. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF (1989) Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. Science 244:1184–1186
45. Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A, Krogstad DJ (1990) Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. Nature 345:253–255
46. Cowman AF, Galatis D, Thompson JK (1994) Selection for mefloquine resistance in *Plasmodium falciparum* is linked to amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. Proc Natl Acad Sci USA 91:1143–1147
47. Peel SA, Bright P, Yount B, Handy J, Baric RS (1994) A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (*pfmdr*) of *Plasmodium falciparum in vitro*. Am J Trop Med Hyg 51:648–658
48. Ritchie GY, Mungthin M, Green JE, Bray PG, Hawley SR, Ward SA (1996) *In vitro* selection of halofantrine resistance in *Plasmodium falciparum* is not associated with increased expression of Pgh1. Mol Biochem Parasitol 83:35–46
49. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ et al (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 364:438–447

50. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F, Krishna S (1999) The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob Agents Chemother 43:2943–2949
51. Wilson CM, Volkman SK, Thaithong S, Martin RK, Kyle DE, Milhous WK, Wirth DF (1993) Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. Mol Biochem Parasitol 57:151–160
52. Nelson AL, Purfield A, McDaniel P, Uthaimongkol N, Buathong N, Sriwichai S, Miller RS, Wongsrichanalai C, Meshnick SR (2005) *pfmdr1* genotyping and *in vivo* mefloquine resistance on the Thai-Myanmar border. Am J Trop Med Hyg 72:586–592
53. Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Ariey F et al (2007) *Pfmdr1* and *in vivo* resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. Am J Trop Med Hyg 76:641–647
54. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S et al (2006) Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin Infect Dis 42:1570–1577
55. Chaiyaroj SC, Buranakiti A, Angkasekwinai P, Looressuwan S, Cowman AF (1999) Analysis of mefloquine resistance and amplification of *pfmdr1* in multidrug-resistant *Plasmodium falciparum* isolates from Thailand. Am J Trop Med Hyg 61:780–783
56. Myrick A, Munasinghe A, Patankar S, Wirth DF (2003) Mapping of the *Plasmodium falciparum* multidrug resistance gene 5′-upstream region, and evidence of induction of transcript levels by antimalarial drugs in chloroquine sensitive parasites. Mol Microbiol 49:671–683
57. Barnes DA, Foote SJ, Galatis D, Kemp DJ, Cowman AF (1992) Selection for high-level chloroquine resistance results in deamplification of the *pfmdr1* gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. EMBO J 11:3067–3075
58. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA (2006) Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 194:528–535
59. Bacon DJ, McCollum AM, Griffing SM, Salas C, Soberon V, Santolalla M, Haley R, Tsukayama P, Lucas C, Escalante AA et al (2009) Dynamics of malaria drug resistance patterns in the Amazon basin region following changes in Peruvian national treatment policy for uncomplicated malaria. Antimicrob Agents Chemother 53:2042–2051
60. Bonizzoni M, Afrane Y, Baliraine FN, Amenya DA, Githeko AK, Yan G (2009) Genetic structure of *Plasmodium falciparum* populations between lowland and highland sites and antimalarial drug resistance in Western Kenya. Infect Genet Evol 9:806–812
61. Dlamini SV, Beshir K, Sutherland CJ (2010) Markers of anti-malarial drug resistance in *Plasmodium falciparum* isolates from Swaziland: identification of *pfmdr1*-86 F in natural parasite isolates. Malar J 9:68
62. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. Nature 345:255–258
63. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJ, Mutabingwa TK, Sutherland CJ, Hallett RL (2007) Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum mdr1* gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother 51:991–997
64. Mehlotra RK, Mattera G, Bockarie MJ, Maguire JD, Baird JK, Sharma YD, Alifrangis M, Dorsey G, Rosenthal PJ, Fryauff DJ et al (2008) Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite *Plasmodium falciparum*. Antimicrob Agents Chemother 52:2212–2222
65. Mita T, Kaneko A, Hombhanje F, Hwaihwanje I, Takahashi N, Osawa H, Tsukahara T, Masta A, Lum JK, Kobayakawa T et al (2006) Role of *pfmdr1* mutations on chloroquine resistance

in *Plasmodium falciparum* isolates with *pfcrt* K76T from Papua New Guinea. Acta Trop 98:137–144
66. Nsobya SL, Dokomajilar C, Joloba M, Dorsey G, Rosenthal PJ (2007) Resistance-mediating *Plasmodium falciparum pfcrt* and *pfmdr1* alleles after treatment with artesunate-amodiaquine in Uganda. Antimicrob Agents Chemother 51:3023–3025
67. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR (2003) Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr1*. Antimicrob Agents Chemother 47:2418–2423
68. Volkman SK, Cowman AF, Wirth DF (1995) Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA 92:8921–8925
69. van Es HH, Karcz S, Chu F, Cowman AF, Vidal S, Gros P, Schurr E (1994) Expression of the plasmodial *pfmdr1* gene in mammalian cells is associated with increased susceptibility to chloroquine. Mol Cell Biol 14:2419–2428
70. Sanchez CP, Rotmann A, Stein WD, Lanzer M (2008) Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in *Plasmodium falciparum*. Mol Microbiol 70:786–798
71. Duraisingh MT, Cowman AF (2005) Contribution of the *pfmdr1* gene to antimalarial drug-resistance. Acta Trop 94:181–190
72. Sidhu AB, Valderramos SG, Fidock DA (2005) *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 57:913–926
73. Holmgren G, Hamrin J, Svard J, Martensson A, Gil JP, Bjorkman A (2007) Selection of *pfmdr1* mutations after amodiaquine monotherapy and amodiaquine plus artemisinin combination therapy in East Africa. Infect Genet Evol 7:562–569
74. Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F (2005) Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob Agents Chemother 49:2180–2188
75. Dokomajilar C, Nsobya SL, Greenhouse B, Rosenthal PJ, Dorsey G (2006) Selection of *Plasmodium falciparum pfmdr1* alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother 50:1893–1895
76. Sisowath C, Ferreira PE, Bustamante LY, Dahlstrom S, Martensson A, Bjorkman A, Krishna S, Gil JP (2007) The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. Trop Med Int Health 12:736–742
77. Pillai DR, Hijar G, Montoya Y, Marouino W, Ruebush TK 2nd, Wongsrichanalai C, Kain KC (2003) Lack of prediction of mefloquine and mefloquine-artesunate treatment outcome by mutations in the *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) gene for *P. falciparum* malaria in Peru. Am J Trop Med Hyg 68:107–110
78. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403:906–909
79. Duraisingh MT, Roper C, Walliker D, Warhurst DC (2000) Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the *pfmdr1* gene of *Plasmodium falciparum*. Mol Microbiol 36:955–961
80. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC et al (2003) Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 49:977–989
81. Cojean S, Noel A, Garnier D, Hubert V, Le Bras J, Durand R (2006) Lack of association between putative transporter gene polymorphisms in *Plasmodium falciparum* and chloroquine resistance in imported malaria isolates from Africa. Malar J 5:24
82. Dahlstrom S, Ferreira PE, Veiga MI, Sedighi N, Wiklund L, Martensson A, Farnert A, Sisowath C, Osorio L, Darban H et al (2009) *Plasmodium falciparum* multidrug resistance protein 1 and artemisinin-based combination therapy in Africa. J Infect Dis 200:1456–1464

83. Dahlstrom S, Veiga MI, Martensson A, Bjorkman A, Gil JP (2009) Polymorphism in PfMRP1 (*Plasmodium falciparum* multidrug resistance protein 1) amino acid 1466 associated with resistance to sulfadoxine-pyrimethamine treatment. Antimicrob Agents Chemother 53:2553–2556
84. Raj DK, Mu J, Jiang H, Kabat J, Singh S, Sullivan M, Fay MP, McCutchan TF, Su XZ (2009) Disruption of a *Plasmodium falciparum* multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. J Biol Chem 284:7687–7696
85. Vinayak S, Alam MT, Upadhyay M, Das MK, Dev V, Singh N, Dash AP, Sharma YD (2007) Extensive genetic diversity in the *Plasmodium falciparum* Na^+/H^+ exchanger 1 transporter protein implicated in quinine resistance. Antimicrob Agents Chemother 51:4508–4511
86. Andriantsoanirina V, Menard D, Rabearimanana S, Hubert V, Bouchier C, Tichit M, Bras JL, Durand R (2010) Association of microsatellite variations of *Plasmodium falciparum* Na^+/H^+ exchanger (Pfnhe-1) gene with reduced *in vitro* susceptibility to quinine: lack of confirmation in clinical isolates from Africa. Am J Trop Med Hyg 82:782–787
87. Henry M, Briolant S, Zettor A, Pelleau S, Baragatti M, Baret E, Mosnier J, Amalvict R, Fusai T, Rogier C et al (2009) *Plasmodium falciparum* Na^+/H^+ exchanger 1 transporter is involved in reduced susceptibility to quinine. Antimicrob Agents Chemother 53:1926–1930
88. Bennett TN, Patel J, Ferdig MT, Roepe PD (2007) *Plasmodium falciparum* Na^+/H^+ exchanger activity and quinine resistance. Mol Biochem Parasitol 153:48–58
89. Spillman NJ, Allen RJ, Kirk K (2008) Acid extrusion from the intraerythrocytic malaria parasite is not via a $Na(^+)/H(^+)$ exchanger. Mol Biochem Parasitol 162:96–99
90. Nkrumah LJ, Riegelhaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, Wellems TE, Akabas MH, Fidock DA (2009) Probing the multifactorial basis of *Plasmodium falciparum* quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. Mol Biochem Parasitol 165:122–131
91. Vinetz JM, Clain J, Bounkeua V, Eastman RT, Fidock DA (2011) Chemotherapy of malaria. In: Brunton LL, Chabner B, Parker KL (eds) Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill Medical, New York, pp 1383–1418
92. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR (2002) Epidemiology of drug-resistant malaria. Lancet Infect Dis 2:209–218
93. Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Nzila AM (2001) Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol 17:582–588
94. Gregson A, Plowe CV (2005) Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev 57:117–145
95. Peterson DS, Walliker D, Wellems TE (1988) Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA 85:9114–9118
96. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ (1988) Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA 85:9109–9113
97. Foote SJ, Galatis D, Cowman AF (1990) Amino acids in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. Proc Natl Acad Sci USA 87:3014–3017
98. Peterson DS, Milhous WK, Wellems TE (1990) Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA 87:3018–3022
99. Triglia T, Cowman AF (1994) Primary structure and expression of the dihydropteroate synthetase gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA 91:7149–7153
100. Brooks DR, Wang P, Read M, Watkins WM, Sims PF, Hyde JE (1994) Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines

of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. Eur J Biochem 224:397–405
101. Wu Y, Kirkman LA, Wellems TE (1996) Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci USA 93:1130–1134
102. Triglia T, Wang P, Sims PF, Hyde JE, Cowman AF (1998) Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. EMBO J 17:3807–3815
103. Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, Santi DV (1997) Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc Natl Acad Sci USA 94:1124–1129
104. Triglia T, Menting JG, Wilson C, Cowman AF (1997) Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc Natl Acad Sci USA 94:13944–13949
105. Reynolds MG, Roos DS (1998) A biochemical and genetic model for parasite resistance to antifolates. *Toxoplasma gondii* provides insights into pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. J Biol Chem 273:3461–3469
106. Kublin JG, Witzig RS, Shankar AH, Zurita JQ, Gilman RH, Guarda JA, Cortese JF, Plowe CV (1998) Molecular assays for surveillance of antifolate-resistant malaria. Lancet 351:1629–1630
107. Kiara SM, Okombo J, Masseno V, Mwai L, Ochola I, Borrmann S, Nzila A (2009) *In vitro* activity of antifolate and polymorphism in dihydrofolate reductase of *Plasmodium falciparum* isolates from the Kenyan coast: emergence of parasites with Ile-164-Leu mutation. Antimicrob Agents Chemother 53:3793–3798
108. Cortese JF, Plowe CV (1998) Antifolate resistance due to new and known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. Mol Biochem Parasitol 94:205–214
109. Lozovsky ER, Chookajorn T, Brown KM, Imwong M, Shaw PJ, Kamchonwongpaisan S, Neafsey DE, Weinreich DM, Hartl DL (2009) Stepwise acquisition of pyrimethamine resistance in the malaria parasite. Proc Natl Acad Sci USA 106:12025–12030
110. Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam RA, Rogerson SJ, Lescano AG, Molyneux ME et al (2002) Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. J Infect Dis 185:380–388
111. Kyabayinze D, Cattamanchi A, Kamya MR, Rosenthal PJ, Dorsey G (2003) Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria. Am J Trop Med Hyg 69:247–252
112. Harrington WE, Mutabingwa TK, Muehlenbachs A, Sorensen B, Bolla MC, Fried M, Duffy PE (2009) Competitive facilitation of drug-resistant *Plasmodium falciparum* malaria parasites in pregnant women who receive preventive treatment. Proc Natl Acad Sci USA 106:9027–9032
113. Kidgell C, Volkman SK, Daily J, Borevitz JO, Plouffe D, Zhou Y, Johnson JR, Le Roch K, Sarr O, Ndir O et al (2006) A systematic map of genetic variation in *Plasmodium falciparum*. PLoS Pathog 2:e57
114. Nair S, Miller B, Barends M, Jaidee A, Patel J, Mayxay M, Newton P, Nosten F, Ferdig MT, Anderson TJ (2008) Adaptive copy number evolution in malaria parasites. PLoS Genet 4: e1000243
115. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ et al (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467
116. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM (2008) Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619–2620

117. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. Nature 424:957–961
118. Cardi D, Pozza A, Arnou B, Marchal E, Clausen JD, Andersen JP, Krishna S, Moller JV, le Maire M, Jaxel C (2010) Purified E255L mutant SERCA1a and purified PfATP6 are sensitive to SERCA-type inhibitors but insensitive to artemisinins. J Biol Chem 285: 26406–26416
119. Krishna S, Pulcini S, Fatih F, Staines H (2010) Artemisinins and the biological basis for the PfATP6/SERCA hypothesis. Trends Parasitol 26:517–523
120. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK et al (2005) A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 12:628–629
121. Valderramos SG, Scanfeld D, Uhlemann AC, Fidock DA, Krishna S (2010) Investigations into the role of the *Plasmodium falciparum* SERCA (PfATP6) L263E mutation in artemisinin action and resistance. Antimicrob Agents Chemother 54:3842–3852
122. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T et al (2005) Resistance of *Plasmodium falciparum* field isolates to *in vitro* artemether and point mutations of the SERCA-type PfATPase6. Lancet 366:1960–1963
123. Jambou R, Martinelli A, Pinto J, Gribaldo S, Legrand E, Niang M, Kim N, Pharath L, Volnay B, Ekala MT et al (2010) Geographic structuring of the *Plasmodium falciparum* sarco(endo) plasmic reticulum Ca^{2+} ATPase (PfSERCA) gene diversity. PLoS One 5:e9424
124. Ferrer-Rodriguez I, Perez-Rosado J, Gervais GW, Peters W, Robinson BL, Serrano AE (2004) *Plasmodium yoelii*: identification and partial characterization of an MDR1 gene in an artemisinin-resistant line. J Parasitol 90:152–160
125. Chavchich M, Gerena L, Peters J, Chen N, Cheng Q, Kyle DE (2010) Induction of resistance to artemisinin derivatives in *Plasmodium falciparum*: role of *Pfmdr1* amplification and expression. Antimicrob Agents Chemother 54:2455–2464
126. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F (2010) Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob Agents Chemother 54:1872–1877
127. Chen N, Chavchich M, Peters JM, Kyle DE, Gatton ML, Cheng Q (2010) De-amplification of *pfmdr1*-containing amplicon on chromosome 5 in *Plasmodium falciparum* is associated with reduced resistance to artelinic acid *in vitro*. Antimicrob Agents Chemother 54:3395–3401
128. Afonso A, Hunt P, Cheesman S, Alves AC, Cunha CV, do Rosario V, Cravo P (2006) Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes *atp6* (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), *tctp*, *mdr1*, and *cg10*. Antimicrob Agents Chemother 50:480–489
129. Hunt P, Afonso A, Creasey A, Culleton R, Sidhu AB, Logan J, Valderramos SG, McNae I, Cheesman S, do Rosario V et al (2007) Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. Mol Microbiol 65:27–40
130. Hunt P, Martinelli A, Modrzynska K, Borges S, Creasey A, Rodrigues L, Beraldi D, Loewe L, Fawcett R, Kumar S et al (2010) Experimental evolution, genetic analysis and genome re-sequencing reveal the mutation conferring artemisinin resistance in an isogenic lineage of malaria parasites. BMC Genomics 11:499
131. Walker DJ, Pitsch JL, Peng MM, Robinson BL, Peters W, Bhisutthibhan J, Meshnick SR (2000) Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii*. Antimicrob Agents Chemother 44:344–347
132. Bhisutthibhan J, Pan XQ, Hossler PA, Walker DJ, Yowell CA, Carlton J, Dame JB, Meshnick SR (1998) The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. J Biol Chem 273:16192–16198
133. Eastman RT, Fidock DA (2009) Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. Nat Rev Microbiol 7:864–874

134. Poehlsgaard J, Douthwaite S (2005) The bacterial ribosome as a target for antibiotics. Nat Rev Microbiol 3:870–881
135. Dahl EL, Rosenthal PJ (2008) Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics. Trends Parasitol 24:279–284
136. Briolant S, Baragatti M, Parola P, Simon F, Tall A, Sokhna C, Hovette P, Mamfoumbi MM, Koeck JL, Delmont J et al (2009) Multinormal *in vitro* distribution model suitable for the distribution of *Plasmodium falciparum* chemosusceptibility to doxycycline. Antimicrob Agents Chemother 53:688–695
137. Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ (2006) Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. Antimicrob Agents Chemother 50:3124–3131
138. Briolant S, Wurtz N, Zettor A, Rogier C, Pradines B (2010) Susceptibility of *Plasmodium falciparum* isolates to doxycycline is associated with *pftetQ* sequence polymorphisms and *pftetQ* and *pfmdt* copy numbers. J Infect Dis 201:153–159
139. Wiesner J, Reichenberg A, Heinrich S, Schlitzer M, Jomaa H (2008) The plastid-like organelle of apicomplexan parasites as drug target. Curr Pharm Des 14:855–871
140. Dharia NV, Plouffe D, Bopp SE, Gonzalez-Paez GE, Lucas C, Salas C, Soberon V, Bursulaya B, Kochel TJ, Bacon DJ et al (2010) Genome scanning of Amazonian *Plasmodium falciparum* shows subtelomeric instability and clindamycin-resistant parasites. Genome Res 20:1534–1544
141. Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA (2002) The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol Cell 10:117–128
142. Douthwaite S, Hansen LH, Mauvais P (2000) Macrolide-ketolide inhibition of MLS-resistant ribosomes is improved by alternative drug interaction with domain II of 23 S rRNA. Mol Microbiol 36:183–193
143. Gabashvili IS, Gregory ST, Valle M, Grassucci R, Worbs M, Wahl MC, Dahlberg AE, Frank J (2001) The polypeptide tunnel system in the ribosome and its gating in erythromycin resistance mutants of L4 and L22. Mol Cell 8:181–188
144. Hansen LH, Mauvais P, Douthwaite S (1999) The macrolide-ketolide antibiotic binding site is formed by structures in domains II and V of 23 S ribosomal RNA. Mol Microbiol 31:623–631
145. Sidhu AB, Sun Q, Nkrumah LJ, Dunne MW, Sacchettini JC, Fidock DA (2007) *In vitro* efficacy, resistance selection, and structural modeling studies implicate the malarial parasite apicoplast as the target of azithromycin. J Biol Chem 282:2494–2504
146. Franceschi F, Kanyo Z, Sherer EC, Sutcliffe J (2004) Macrolide resistance from the ribosome perspective. Curr Drug Targets Infect Disord 4:177–191
147. Borrmann S, Adegnika AA, Matsiegui PB, Issifou S, Schindler A, Mawili-Mboumba DP, Baranek T, Wiesner J, Jomaa H, Kremsner PG (2004) Fosmidomycin-clindamycin for *Plasmodium falciparum* infections in African children. J Infect Dis 189:901–908
148. Borrmann S, Adegnika AA, Moussavou F, Oyakhirome S, Esser G, Matsiegui PB, Ramharter M, Lundgren I, Kombila M, Issifou S et al (2005) Short-course regimens of artesunate-fosmidomycin in treatment of uncomplicated *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 49:3749–3754
149. Borrmann S, Issifou S, Esser G, Adegnika AA, Ramharter M, Matsiegui PB, Oyakhirome S, Mawili-Mboumba DP, Missinou MA, Kun JF et al (2004) Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. J Infect Dis 190:1534–1540
150. Dharia NV, Sidhu AB, Cassera MB, Westenberger SJ, Bopp SE, Eastman RT, Plouffe D, Batalov S, Park DJ, Volkman SK et al (2009) Use of high-density tiling microarrays to identify mutations globally and elucidate mechanisms of drug resistance in *Plasmodium falciparum*. Genome Biol 10:R21
151. Hudson AT, Dickins M, Ginger CD, Gutteridge WE, Holdich T, Hutchinson DB, Pudney M, Randall AW, Latter VS (1991) 566 C80: a potent broad spectrum anti-infective agent with

activity against malaria and opportunistic infections in AIDS patients. Drugs Exp Clin Res 17:427–435
152. Chiodini PL, Conlon CP, Hutchinson DB, Farquhar JA, Hall AP, Peto TE, Birley H, Warrell DA (1995) Evaluation of atovaquone in the treatment of patients with uncomplicated *Plasmodium falciparum* malaria. J Antimicrob Chemother 36:1073–1078
153. Looareesuwan S, Chulay JD, Canfield CJ, Hutchinson DB (1999) Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Am J Trop Med Hyg 60:533–541
154. Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ (1996) Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am J Trop Med Hyg 54:62–66
155. Nakato H, Vivancos R, Hunter PR (2007) A systematic review and meta-analysis of the effectiveness and safety of atovaquone proguanil (Malarone) for chemoprophylaxis against malaria. J Antimicrob Chemother 60:929–936
156. Srivastava IK, Vaidya AB (1999) A mechanism for the synergistic antimalarial action of atovaquone and proguanil. Antimicrob Agents Chemother 43:1334–1339
157. Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB (1999) Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites. Mol Microbiol 33:704–711
158. Fry M, Pudney M (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566 C80). Biochem Pharmacol 43:1545–1553
159. Painter HJ, Morrisey JM, Mather MW, Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 446:88–91
160. Vaidya AB, Mather MW (2000) Atovaquone resistance in malaria parasites. Drug Resist Updat 3:283–287
161. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q (2000) Mutations in *Plasmodium falciparum* cytochrome *b* that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother 44:2100–2108
162. Syafruddin D, Siregar JE, Marzuki S (1999) Mutations in the cytochrome *b* gene of *Plasmodium berghei* conferring resistance to atovaquone. Mol Biochem Parasitol 104:185–194
163. McFadden DC, Tomavo S, Berry EA, Boothroyd JC (2000) Characterization of cytochrome *b* from *Toxoplasma gondii* and Q(o) domain mutations as a mechanism of atovaquone-resistance. Mol Biochem Parasitol 108:1–12
164. Sutherland CJ, Laundy M, Price N, Burke M, Fivelman QL, Pasvol G, Klein JL, Chiodini PL (2008) Mutations in the *Plasmodium falciparum* cytochrome *b* gene are associated with delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil. Malar J 7:240
165. Perry TL, Pandey P, Grant JM, Kain KC (2009) Severe atovaquone-resistant *Plasmodium falciparum* malaria in a Canadian traveller returned from the Indian subcontinent. Open Med 3:e10–e16
166. Musset L, Le Bras J, Clain J (2007) Parallel evolution of adaptive mutations in *Plasmodium falciparum* mitochondrial DNA during atovaquone-proguanil treatment. Mol Biol Evol 24:1582–1585
167. Mather MW, Darrouzet E, Valkova-Valchanova M, Cooley JW, McIntosh MT, Daldal F, Vaidya AB (2005) Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. J Biol Chem 280:27458–27465
168. Orjuela-Sanchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alecrim MG, del Portillo HA (2009) Analysis of single-nucleotide polymorphisms in the *crt-o* and *mdr1* genes of *Plasmodium vivax* among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrob Agents Chemother 53:3561–3564
169. Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, Nosten F, Anderson TJ, Dondorp A, Day NP et al (2008) Gene amplification of the multidrug

resistance 1 gene of *Plasmodium vivax* isolates from Thailand, Laos, and Myanmar. Antimicrob Agents Chemother 52:2657–2659
170. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF et al (2001) Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J Infect Dis 183:1653–1661
171. Sa JM, Nomura T, Neves J, Baird JK, Wellems TE, del Portillo HA (2005) *Plasmodium vivax*: allele variants of the *mdr1* gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol 109:256–259
172. Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, Menard D (2008) *Plasmodium vivax* resistance to chloroquine in Madagascar: clinical efficacy and polymorphisms in *pvmdr1* and *pvcrt-o* genes. Antimicrob Agents Chemother 52:4233–4240
173. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A et al (2007) Chloroquine resistant *Plasmodium vivax*: *in vitro* characterisation and association with molecular polymorphisms. PLoS One 2:e1089
174. Gama BE, Oliveira NK, Souza JM, Daniel-Ribeiro CT, Ferreira-da-Cruz Mde F (2009) Characterisation of *pvmdr1* and *pvdhfr* genes associated with chemoresistance in Brazilian *Plasmodium vivax* isolates. Mem Inst Oswaldo Cruz 104:1009–1011
175. Sa JM, Yamamoto MM, Fernandez-Becerra C, de Azevedo MF, Papakrivos J, Naude B, Wellems TE, Del Portillo HA (2006) Expression and function of *pvcrt-o*, a *Plasmodium vivax* ortholog of *pfcrt*, in *Plasmodium falciparum* and *Dictyostelium discoideum*. Mol Biochem Parasitol 150:219–228
176. Hawkins VN, Joshi H, Rungsihirunrat K, Na-Bangchang K, Sibley CH (2007) Antifolates can have a role in the treatment of *Plasmodium vivax*. Trends Parasitol 23:213–222
177. Kongsaeree P, Khongsuk P, Leartsakulpanich U, Chitnumsub P, Tarnchompoo B, Walkinshaw MD, Yuthavong Y (2005) Crystal structure of dihydrofolate reductase from *Plasmodium vivax*: pyrimethamine displacement linked with mutation-induced resistance. Proc Natl Acad Sci USA 102:13046–13051
178. Yuvaniyama J, Chitnumsub P, Kamchonwongpaisan S, Vanichtanankul J, Sirawaraporn W, Taylor P, Walkinshaw MD, Yuthavong Y (2003) Insights into antifolate resistance from malarial DHFR-TS structures. Nat Struct Biol 10:357–365

Prevention of Malaria

Patricia Schlagenhauf and Eskild Petersen

Motto/Epigraph. Travellers to malaria-endemic areas need:

- Information on malaria risk, mode of transmission of the disease, incubation period and symptoms
- Advice on measures against mosquito bites
- Chemoprophylaxis for high-risk areas such as sub-Saharan Africa
- Advice regarding prompt diagnosis and self-treatment of malaria if appropriate

Abstract An estimated 80–90 million travellers visit malaria-endemic areas annually. Not all travellers have a similar risk. The risk of acquiring malaria will depend on many factors including the type and intensity of malaria transmission at the destination, the duration and style of travel, prevention measures used and individual characteristics. Primary prevention strategies are mosquito bite prevention measures (such as insecticide impregnated bednets, repellents and insecticides) and chemoprophylaxis. The three priority antimalaria chemoprophylactic regimens for travellers to areas with a high risk of *Plasmodium falciparum* malaria are: atovaquone/proguanil, doxycycline and mefloquine. In some countries, the strategy of stand-by emergency self-treatment is recommended for travellers to areas with a low risk of malaria. Malaria prevention advice and strategies should as far as possible be evidence based using sound epidemiological data when these are available. This chapter focuses on the chemoprevention and self-treatment of malaria in travellers.

P. Schlagenhauf
University of Zurich Centre for Travel Medicine, WHO Collaborating Centre for Travellers' Health, Institute for Social and Preventive Medicine, University of Zürich, Zurich, Switzerland

E. Petersen (✉)
Department of Infectious Diseases, Aarhus University Hospital Skejby, Brendstrupggardsvej 100 DK-8200 Aarhus N. Aarhus, Denmark
e-mail: eskildp@dadlnet.dk

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_14,

1 Chemoprophylaxis

The decision to use chemoprophylaxis will depend on a risk benefit analysis weighting the risk of malaria against the risk of possible adverse drug reactions. The risk of acquiring malaria will depend on many factors including the type and intensity of malaria transmission at the destination, the duration and style of travel, prevention measures used and individual characteristics [1–3]. This risk is difficult to quantify, and new risk areas may emerge [4], while risk at traditional traveller destinations can decline [5, 6]. Even though malaria imported to non-endemic countries is a notifiable disease, the population at risk (*i.e.* the exact number of travellers to a specific destination) is often impossible to ascertain.

Therefore, it has been proposed to use data on malaria endemicity in the indigenous population and then extrapolate the risk to travellers [7], although many travellers will have a lower risk of malaria than local populations in endemic areas because they have shorter exposition periods of exposure and higher standards of accommodation. The exceptions here are travellers visiting friends and relatives (VFR) who have a malaria risk comparable to or higher than the local communities.

With regard to tolerability, several studies quantify the rate of adverse events in short-term travellers using malaria chemoprophylaxis but the methodologies used are rarely comparable so that incidence rates can differ enormously between studies. The recent Cochrane Review of the tolerability of antimalarial drugs and the comments on this review highlight the need for more targeted and controlled studies to address this topic [8]. Data on the long-term use and tolerability of drugs for chemoprophylaxis are lacking. There is also a lack of data on the safety and efficacy of strategies for vulnerable populations such as pregnant/lactating women and small children.

2 Low Risk of Malaria Infection

One of the most difficult questions in malaria prophylaxis today is how to advise travellers who visit low-risk areas, often areas with unstable transmission and a changing malaria epidemiology. Few studies report the absolute risk for specific areas, but these data are necessary to allow a rational decision on whether to recommend chemoprophylaxis or not, balanced against the risk of side effects. Travellers should not be exposed to a “substantial risk” (discussed in the next section) of adverse events from malaria chemoprophylaxis in areas where the risk of malaria infection is very low.

Can we quantify the risk of contracting malaria and weigh this against the risk of adverse events from chemoprophylaxis?

A study from Sweden found the risk of malaria to travellers in West Africa, South Africa, South America and Thailand to be 302, 46, 7.2 and 2 per 100,000 visitors, respectively [9].

Studies from returning travellers to the United Kingdom two decades ago found that 1 in 77 or 1,300 per 100,000 persons VFR in Ghana contracted malaria falling to 1 per 100,000 travellers in South America [10]. A study of malaria in returning travellers to Denmark found that 158, 76 and 1.7 per 100,000 contracted malaria while visiting. The Gambia, Indonesia and Thailand, respectively [11]. A declining risk of malaria in travellers to South America has also been reported. The total number of imported malaria cases reported to surveillance bodies annually for the years 2000 to 2005 inclusive, fell from 395 to 209 cases of which 69% were non-falciparum in 2005 [12].

A study comparing the risk of side effects from chemoprophylaxis with risk of malaria infection [7] assumed that the risk of malaria is the same in travellers and in the indigenous population, which may not always be true. The risk in the indigenous population should therefore be seen as a maximal risk situation for travellers with high standards of overnight accommodation. An endemicity level of 20 cases per 1,000 of the indigenous population would result in an estimated one death per 100,000 travellers per year without chemoprophylaxis. This allows an estimation of the numbers of travellers to receive chemoprophylaxis to prevent one case and prevent one fatal outcome.

3 Risk of Adverse Events from Malaria Chemoprophylaxis

The risk of being infected with malaria should be balanced against the risk of adverse events from malaria chemoprophylaxis. Adverse events can be divided into frequently occurring events, usually mild, self-limiting and affecting large percentages of users and rare serious events, which are seen much less frequently and which are often recognised only after millions of users have taken the drug. Rare events are usually not discovered in Phase III trials prior to licencing of a drug and rely on post-marketing surveillance after licencing, which make the true incidence very uncertain as unreported events are likely. Amodiaquine was withdrawn due to serious side effects recognized after release and the same happened to the combination of dapsone/pyrimethamine (Maloprim).

There is only one double blind, randomised controlled trial which compared all current malaria prophylactic regimens and associated adverse events in 623 travellers randomised to atovaquone/proguanil (Malarone®), mefloquine (Lariam®), doxycycline and chloroquine/proguanil (as Savarine) [13]. Of the chloroquine/proguanil, mefloquine, doxycycline and atovaquone/proguanil users, 45, 42, 33, and 32% reported mild-to-moderate adverse events, respectively. Severe adverse events (that interfered with daily activity) were reported in 11, 12, 6 and 7% of mefloquine, chloroquine/proguanil, doxycycline and atovaquone/proguanil users, respectively [13]. It should be emphasised that an adverse event is not necessarily attributable to the antimalarial drug, but reflects all inter-current events experienced during the use of the drug.

A recent detailed review [14] found that despite widespread reports of the adverse effects of mefloquine, controlled studies (involving >5,000 subjects) have found a low incidence of serious adverse events. Studies of minor adverse events have, however, highlighted the neuropsychiatric/neuropsychological profile of this antimalarial showing an excess of such events in women.

In summary, because travellers are healthy persons and side effects do occur, it is important that the physician prescribing malaria chemoprophylaxis discuss the risk of side effects with the traveller and obtain consent of the need for malaria chemoprophylaxis and the drugs chosen.

4 Prophylaxis for Short-Term Visitors

Short-term travellers are persons visiting a malaria-endemic area for weeks or a few months. This group includes most tourists and the main reason for travel is business and leisure. The travellers usually have little prior knowledge of disease including malaria risk at their destination. Because of the incubation period of malaria, many short-term travellers who acquire the disease will be diagnosed after return. The actual profile of imported species varies among countries primarily reflecting the geographic origin of the infection. Malaria deaths are due almost exclusively to infection with *P. falciparum* [15].

The key problem for travel health practitioners, at present, is when to advise use of chemoprophylaxis for travellers to low-risk endemic areas? Based on the estimates of risk of malaria and adverse events, we propose that the prescription of chemoprophylaxis in areas with an endemicity level in the indigenous population below 10 cases per 1,000 population per year is not justified [7].

Should chemoprophylaxis be required, the proper selection of drugs and dosage for a given area can be found elsewhere [16–18].

5 Standby Emergency Treatment

Standby Emergency Treatment (SBET), where the traveller is prepared to self-treat if he or she has symptoms suggestive of malaria and is out of reach of medical attention, is recommended by many authorities as a possible strategy for short-term travellers to low-risk destinations [18]. Central European countries (Switzerland, Germany, Austria) are recommending increasingly this strategy for low-to-moderate risk areas except for sub-Saharan Africa and some high-risk areas in Indonesia and Papua New Guinea. SBET is, however, not indicated to travellers on trips shorter than the incubation period for malaria, which is a minimum of 6 days for *P. falciparum* malaria. The medications recommended vary by country depending on the registration status of medications and the national ideology.

6 Prophylaxis for Long-Term Visitors and Frequent Visitors

The risk of malaria cumulates over time, but the increased risk cannot just be attributed to longer exposure alone [10]. Long-term travellers, defined as persons staying permanently in an area for 6 months or longer and frequent travellers to endemic regions, behave differently from short-term visitors. Long-term travellers frequently discontinue chemoprophylaxis prematurely because they perceive the risk to be lower than expected. They also believe that they can effectively manage an infection and they are worried about side effects from long-term use of malaria chemoprophylaxis [19, 20]. Use of counterfeit drugs (*i.e.* fake drugs containing no or sub-therapeutic doses of active compounds) is particularly an issue for long-term travellers who often buy local supplies of dubious quality because they are cheaper and expatriates should be encouraged to bring adequate quantities of antimalarial medications with them [20]. The consequences of travellers using counterfeit drugs are far-reaching; untreated *P. falciparum* has a high mortality rate, sub-therapeutic dosages lead to inadequate prophylactic doses, a risk of increased adverse events due to excessive dosage or potentially toxic contaminants and finally a loss of faith in genuine medicines.

The health personnel advising long-term travellers should understand these aspects and accept that these are important issues, which must be addressed.

The key to managing malaria prevention in long-term and frequent travellers is to provide the travellers with knowledge and understanding of malaria so that they can take more responsibility for their own health compared to the short-term traveller.

Long-term travellers to high-risk areas should take malaria chemoprophylaxis even if this is necessary over several years. Mefloquine is the best documented drug used by long-term travellers and, if well tolerated, can be used for prolonged periods (*i.e.* there is no upper time limits for the use of mefloquine) [21]. It has a simple weekly dosing schedule that encourages adherence [22] and toxic accumulation in the body does not occur during long-term use [23]. Doxycycline (a tetracycline compound) is used for treatment of skin infections for months, but side effects, especially vaginal candidiasis in women is a problem in long-term users. The more expensive monohydrate form of doxycycline is considered to have superior tolerability to the older hyclate form of the drug. A third option is the use of atovaquone/proguanil [24]. The CDC has no upper limit on the duration of intake of atovaquone/proguanil [16], and recent United Kingdom guidelines consider atovaquone/proguanil to be safe for continuous use of up to 12 months [25].

Overall, long-term travellers must have knowledge about malaria as a disease including key symptoms such as fever, adequate use of SBET, the problems of counterfeit drugs and the need to identify reliable health care facility in case of emergency.

Mosquito bite prophylaxis is even more relevant for the long-term traveller and comprehensive information about screening, impregnated bednets, coils, repellents, insecticides and the biting habits of mosquitoes is required. Systemic use of

Table 1 Guidelines for preventing malaria in long-term travellers and travellers with frequent visits to malaria-endemic areas

Inform about the disease; symptoms, diagnosis and need for rapid treatment
Discuss access to qualified medical assistance at destination and the need to identify qualified medical staff before the traveller becomes ill
Inform about alternatives to continuous chemoprophylaxis
Provide detailed advice on methods to prevent mosquito bites
Inform about the use of standby emergency treatment, SBET, and emphasise the need for medical assessment despite the use of SBET
Inform about the problems with counterfeit drugs in many malaria-endemic countries, and that drugs for both chemoprophylaxis and SBET should be purchased before arriving at the destination
Rapid diagnostic tests can be recommended for selected travellers but require comprehensive pre-travel instruction

impregnated bednets can reduce the risk of malaria by 50% [26]. In selected cases, the long-term traveller may be trained to use the rapid malaria diagnostic kits, but this option needs to be restricted to persons staying in isolated places and thorough pre-travel instruction is essential [19, 27].

Health personnel advising travellers must recognise that this group is unlikely to take chemoprophylaxis continuously for years, and it will increase the credibility of health care workers if travel health advisors acknowledge this and try to prepare realistically this group for their long-term travel.

Suggestions for advice to long-term travellers and frequent visitors are summarised in Table 1. The drug of choice for SBET depends on the use of prophylaxis, if any, and the expected drug susceptibility pattern at the destination and the guidelines of the traveller's country of origin, and as a rule, the drug used for treatment should not be the same as that used for prophylaxis. Malaria breakthrough while compliant and on prophylaxis could be due to resistance of the *P. falciparum* parasite to the chemoprophylactic drug, which is why the drug used for prophylaxis should not be re-used for treatment. There is also the possibility of reaching potentially toxic drug levels if the prophylaxis and treatment drug are the same.

7 Prophylaxis Against *P. vivax* and *P. ovale*

The main goal of malaria chemoprophylaxis is to prevent *P. falciparum*, which is primarily responsible for malaria fatalities. However, *P. vivax*, *P. ovale P.malariae* and *P. knowlesi* can cause serious febrile illness in non-immune individuals. The clinical presentation of *P. vivax* and *P. ovale* cannot be distinguished from *P. falciparum* malaria [28]. The burden of infection is considerable in indigenous populations, and *P. vivax* is the most prevalent malaria type in Southeast Asia and

South America and is also found in East Africa [29]. *P. vivax* is susceptible to most antimalarial drugs although isolates with reduced sensitivity to chloroquine and primaquine are found in parts of Indonesia, Papua New Guinea, Iraq and Afghanistan [1, 30, 31]. There is a decreased susceptibility of *P. vivax* in Thailand to sulfadoxine/pyrimethamine [32].

A study from Europe of 518 imported cases of *P. vivax* found that 60% were admitted to hospital in an average of 4 days after the start of symptoms, and seven had severe complications: hepato-splenomegaly (3 patients), spleen-rupture (1 patient), pancytopenia (1 patient), macrohaematuria (1 patient) and psychosis (1 patient) [33].

P. vivax and *P. ovale* develop hypnozoites when the host is infected, which may relapse later and cause malaria symptoms long after the traveller has returned home. The hypnozoites are only susceptible to primaquine. One study found that the first *P. vivax* attack was seen approximately 3 months after leaving the malarious area regardless of whether the traveller had taken prophylaxis or not [34]. Prevention of relapsing *P. vivax* can only be achieved by treating presumptively post travel with a course of primaquine or by using primaquine as a chemoprophylaxis during travel. So far, neither of these options has been extensively used and primaquine is not registered in most countries as a primary prophylaxis. Terminating a stay in a *P. vivax* endemic area with a 2-week course of primaquine without knowing whether the traveller is infected is not attractive for practitioners or patients, as primaquine has some side effects (primarily causing methaemoglobinemia). Use of primaquine requires that the individuals are tested for G6PD deficiency. The recommended adult dose for "anti-relapse treatment" based on clinical trials and expert opinion is 30 mg given daily for 14 days, starting on return from a malarious region and taken together with a blood schizonticide. This is based on evidence from the 1950s showing that primaquine's activity against hypnozoites is enhanced when given with chloroquine. The adult dose for primary prophylaxis is 30 mg daily starting 1 day before travel and continuing for 7 days after return [35].

One study used primaquine as prophylaxis alone in an area with a high risk of *P. falciparum* infections and found breakthroughs in four users out of 106 [36]. Primaquine has only limited activity against blood stage *P. falciparum* and if parasites emerge from the liver into the blood, primaquine alone will be insufficient as chemoprophylaxis.

Using primaquine as the drug of choice for *P. vivax* in endemic areas raises the concern of selecting for primaquine resistance, which so far has been found only in Irian Jaya, Papua New Guinea and a single report from Iraq and Afghanistan [31].

As long as the absolute risk of *P. vivax* and *P. ovale* infection is not known, the best strategy is still to inform travellers of the risk of a late onset attack of either parasite after return and cessation of chemoprophylaxis.

8 Malaria Prevention for Pregnant and Breastfeeding Women

A significant proportion of travellers are women of childbearing potential who need evidence-based advice on the use of antimalarials in the peri-conception period, during pregnancy and breastfeeding. Malaria during pregnancy is hazardous for the mother, the foetus and the neonate and is an important cause of maternal and child morbidity and mortality [37]. *P. falciparum* is responsible for the main burden of malarial disease in pregnant women [38]. Other malaria species do not parasitize placental blood to the same extent and hence have less impact [39]. The clinical features of *P. falciparum* malaria in pregnancy depend to a large extent on the immune status of the woman, which in turn is determined by her prior exposure to malaria. Non-immune travellers have little or no immunity and are prone to episodes of severe malaria leading to stillbirths, spontaneous abortions or even maternal death. Mosquito bite protection is essential and research shows that *N*,*N*-diethyl-3-methylbenzamide (DEET) is effective, safe and has a low risk of accumulation in the foetus [38]. Pyrethroid insecticide-treated nets are safe and have been shown to substantially reduce the risk of placental malaria [33, 39]. Bednets are particularly indicated in rooms with no air-conditioning.

Malaria chemoprophylaxis in pregnancy is complex. Due to ethical and safety restrictions, few antimalarials have been evaluated for pregnant travellers, and there is also a dearth of information on drug disposition in the pregnant woman (Table 2). Chloroquine can be used but widespread resistance limits this option. Doxycycline and primaquine are contra-indicated. Due to insufficient data, atovaquone/proguanil is not recommended, although proguanil is considered safe in pregnancy and no teratogenicity has been observed in animal studies using atovaquone [40]. Mefloquine is an option for pregnant women who cannot defer travel and who need chemoprophylaxis for chloroquine-resistant malaria-endemic areas. Some authorities now allow the use of mefloquine in all trimesters, others advise against using the drug in the first trimester apart from exceptional circumstances. A recent trial of chloroquine prophylaxis for *P. vivax* malaria in pregnant women in Thailand found no effect on maternal anaemia or birth weight [41]. However, in areas with predominantly *P. vivax* malaria, infection during pregnancy contributes to maternal morbidity and mortality [42].

With regard to breastfeeding, chloroquine, hydroxychloroquine and mefloquine are considered compatible with breastfeeding and atovaquone/proguanil can be used if the breastfed infant weighs more than 5 kg. Proguanil is excreted into human milk in small quantities. Infants who are breastfed do not receive adequate concentrations of any antimalarials and require their own chemoprophylaxis [13].

Table 2 Antimalarials for chemoprophylaxis and stand-by emergency treatment in pregnancy

Antimalarial	Chemoprophylaxis in pregnancy	Emergency self-treatment in pregnancy	Comments
Atovaquone/proguanil	No data, should not be used	No data, should only be used if no other options are available	Not recommended due to lack of safety data. Inadvertent use in pregnancy probably safe but few data available
Chloroquine (hydroxychloroquine)	Can be used	Can be used	Regarded as safe but resistance is widespread
Proguanil	Can be used	Not used for treatment	Supplement with folic acid is recommended. Should be used only in combination with chloroquine
Doxycycline	Not recommended	Not recommended	May cause bone malformation and discoloured teeth
Mefloquine	Can be used after the first trimester. Some authorities (WHO, CDC) allow the use of mefloquine in the first trimester if the risk of malaria is high and travel cannot be deferred	Can be used after 16th gestational week or if no other options are available	Regarded as safe after 16th gestational week based on post-marketing surveillance. Inadvertent use in the peri-conception period or during pregnancy is not considered an indication for a termination
Artemisinins	Not used for prophylaxis	Few data. Can only be used if no other options are available	One small study found no adverse impact on the pregnant mother or the foetus
Quinine	Not used for prophylaxis	Can be used	Drug of choice in *P. falciparum* malaria. Combination with clindamycin is recommended
Primaquine	Contra-indicated	Treatment of *P. vivax* hypnozoites should be deferred until after the pregnancy	Use of primaquine in pregnancy would necessitate G6PD testing for mother and foetus

9 Malaria Prevention for Small Children

Imported malaria case numbers in children are increasing with the rise in travel among children and changing profiles of immigrants, particularly settled immigrants VFR in malaria-endemic countries. The use of DEET containing insect repellents is recommended for children older than 2 months and an alternative repellent, picaridin, can be recommended for children older than 2 years. Chloroquine is safe for children of all ages and weights but the use of this option is limited by widespread resistance to the drug. Mefloquine can be used for children >5 kg and atovaquone/proguanil prophylaxis (as paediatric tablets) can be used for children >5 kg according to new CDC guidelines [16]. The manufacturer, the World Health Organisation [18] and some European authorities sanction the use of this combination only for children weighing more than 11 kg. Coartem®, an artemisinin combination, in dispersible tablet form with cherry flavour, has been available since 2009 in Switzerland and some African countries for the treatment of infants (personal communication Novartis, Switzerland), but cannot be used for prophylaxis. Doxycycline is for children aged >8 years (in the UK, allowed for children aged over 12 years). Table 3 shows the currently recommended doses for malaria prophylaxis in children.

When possible, deferral of travel is recommended for pregnant and breastfeeding women and also for young children.

Table 3 Antimalarial chemoprophylaxis for children

Antimalarial	Chemoprophylaxis	Dosing	Comments
Atovaquone/proguanil	[a]>5 kg CDC >11 kg manufacturer some European countries	–*Daily* –Paediatric tablets[**]	–Palatable –Expensive
Chloroquine (hydroxychloroquine)	All ages and weights	5 mg base/kg *weekly*	Limited use due to resistance
Proguanil	All ages and weights	3 mg/kg per day	Only in combination with chloroquine
Doxycycline	Children > 8 years	1.5 mg salt/kg *daily*	Contra-indicated for children under 12 years
Mefloquine	>5 kg	5 mg/kg *weekly*	Bitter taste
Primaquine	Children > 4 years WHO CDC specifies no lower age limit	0.5 mg/kg base *daily*	–G6PD testing essential – Last choice

[a]New 2007

[**]Specified by the manufacturer

References

1. Baird JK, Purnomo BH, Bangs MJ, Subianto B, Patchen LC, Hoffman SL (1991) Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. Am J Trop Med Hyg 44:547–552
2. Chen LH, Keystone JS (2005) New strategies for the prevention of malaria in travelers. Infect Dis Clin North Am 19:185–210
3. Schlagenhauf P (ed) (2008) Traveler's malaria, 2nd edn. BC Decker, Hamilton, ON
4. Jelinek T, Behrens R, Bisoffi Z, Bjorkmann A, Gascon J, Hellgren U, Petersen E, Zoller T, Andersen RH, Blaxhult A for the TropNetEurop (2007) Recent cases of falciparum malaria imported to Europe from Goa, India, December 2006-January 2007. Euro Surveill 12: E070111.1
5. Schmid S, Chiodini P, Legros F, D'Amato S, Schöneberg I, Liu C, Janzon R, Schlagenhauf P (2009) The risk of malaria in travelers to India. J Travel Med 16:194–199
6. Behrens RH, Carroll B, Hellgren U, Visser LG, Siikamaki H, Vestergaard LS, Calleri G, Janisch T, Myrvang B, Gascon J, Hatz C (2010) The incidence of malaria in travellers to South-East Asia: is local malaria transmission a useful risk indicator? Malar J 9:266
7. Schlagenhauf P, Petersen E (2008) Malaria chemoprophylaxis: strategies for risk groups. Clin Microbiol Rev 21:466–472
8. Schlagenhauf P (2010) Cochrane Review highlights the need for more targeted research on the tolerability of malaria chemoprophylaxis in travellers. Evid Based Med 15:25–26
9. Askling HH, Nilsson J, Tegnell A, Janzon R, Ekdahl K (2005) Malaria risk in travelers. Emerg Infect Dis 11:436–441
10. Phillips-Howard PA, Radalowicz A, Mitchell J, Bradley DJ (1990) Risk of malaria in British residents returning from malarious areas. Br Med J 300:499–503
11. Kofoed K, Petersen E (2003) The efficacy of chemophrophylaxis against malaria with chloroquine plus proguanil, mefloquine and atovaquone plus proguanil in travellers from Denmark. J Travel Med 10:150–154
12. Behrens RH, Carroll B, Beran J, Bouchaud O, Hellgren U, Hatz C, Jelinek T, Legros F, Mühlberger N, Myrvang B, Siikamäki H, Visser L for TropnetEurop (2007) The low and declining risk of malaria in travellers to Latin America: is there still an indication for chemoprophylaxis? Malar J 6:114
13. Schlagenhauf P, Tschopp A, Johnson R, Nothdurft HD, Beck B, Schwartz E, Herold M, Krebs B, Veit O, Allwinn R, Steffen R (2003) Tolerability of malaria chemoprophylaxis in non-immune travellers to sub-Saharan Africa: multicentre, randomised, double blind, four arm study. Br Med J 327:1078–1085
14. Chen LH, Wilson ME, Schlagenhauf P (2007) Controversies and misconceptions in malaria chemoprophylaxis for travelers. JAMA 297:2251–2263
15. Arguin P, Schlagenhauf P (2008) Malaria deaths in travelers. In: Schlagenhauf P (ed) Travelers malaria, 2nd edn. BC Decker, London
16. CDC (2011) Travellers health, Yellow Book 2011. (http://www.cdc.gov)
17. Schlagenhauf P (2007) Pregnant? Breastfeeding? Traveling with infants? Malaria prevention challenges for mothers. Abstract PL03.03 P34. In: Proceedings of the 10th conference of the international society of travel medicine, Vancouver, Canada, 20–24 May 2007
18. WHO (2011) International travel and health. (http://www.who.int/ITH)
19. Chen LH, Wilson ME, Schlagenhauf P (2006) Prevention of malaria in long-term travelers. JAMA 296:2234–2244
20. Toovey S, Moerman F, van Gompel A (2007) Special infectious disease risks of expatriates and long-term travelers in tropical countries. Part I: Malaria. J Travel Med 14:42–49
21. Sonmez A, Harlak A, Kilic S, Polat Z, Hayat L, Keskin O, Dogru T, Yilmaz MI, Acikel CH, Kocar IH (2005) The efficacy and tolerability of doxycycline and mefloquine in malaria prophylaxis of the ISAF troops in Afghanistan. J Infect 51:253–258

22. Wells TS, Smith TC, Smith B, Wang LZ, Hansen CJ, Reed RJ, Goldfinger WE, Corbeil TE, Spooner CN, Ryan MA (2006) Mefloquine use and hospitalizations among US service members, 2002–2004. Am J Trop Med Hyg 74:744–749
23. Hellgren U, Angel VH, Bergqvist Y, Forero-Gomez JS, Rombo L (1991) Plasma concentrations of sulfadoxine-pyrimethamine, mefloquine and its main metabolite after regular malaria prophylaxis for two years. Trans R Soc Trop Med Hyg 85:356–357
24. Genderen PJ, van Koene HR, Spong K, Overbosch D (2007) The safety and tolerance of atovaquone/proguanil for the long-term prophylaxis of *Plasmodium falciparum* malaria in non-immune travelers and expatriates. J Travel Med 14:92–95
25. Swales CA, Chiodini PL, Bannister BA for the Health Protection Agency Advisory Committee on Malaria Prevention in UK Travellers (2007) New guidelines on malaria prevention: a summary. J Infect 54:107–110
26. Marbiah NT, Petersen E, David K, Lines J, Magbity E, Bradley DB (1998) Control of clinical malaria due to *Plasmodium falciparum* in children in an area of rural Sierra Leone with perennial transmission: a community-wide trial of lambdacyhalothrin-impregnated mosquito nets alone and/or with fortnightly dapsone/pyrimethamine/placebo chemoprophylaxis. Am J Trop Med Hyg 58:1–6
27. Schlagenhauf P, Adamcova M, Regep L, Schaerer MT, Rhein HG (2010) The position of mefloquine as a 21st century malaria chemoprophylaxis. Malar J 9:357
28. Bottieau E, Clerinx J, Van Den Enden E, Van Esbroeck M, Colebunders R, Van Gompel A, Van Den Ende J (2006) Imported non-*Plasmodium falciparum* malaria: a five-year prospective study in a European referral center. Am J Trop Med Hyg 75:133–138
29. Mendis K, Sina BJ, Marchesini P, Carter R (2001) The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg 64(1–2 Suppl):97–106
30. Schwartz IK, Lackritz EM, Patchen LC (1991) Chloroquine-resistant *Plasmodium vivax* from Indonesia. N Engl J Med 324:927
31. Spudick JM, Garcia LS, Graham DM, Haake DA (2005) Diagnostic and therapeutic pitfalls associated with primaquine-tolerant *Plasmodium vivax*. J Clin Microbiol 43:978–981
32. Pukrittayakamee S, Imwong M, Looareesuwan S, White NJ (2004) Therapeutic responses to antimalarial and antibacterial drugs in vivax malaria. Acta Trop 89:351–356
33. Mühlberger N, Jelinek T, Gascon J, Probst M, Zoller T, Schunk M (2004) Epidemiology and clinical features of vivax malaria imported to Europe: sentinel surveillance data from TropNetEurop. Malar J 3:5
34. Elliott JH, O'Brien D, Leder K, Kitchener S, Schwartz E, Weld L, Brown GV, Kain KC, Torresi J for the GeoSentinel Surveillance Network (2004) Imported Plasmodium vivax malaria: demographic and clinical features in nonimmune travelers. J Travel Med 11:213–217
35. Hill DR, Baird JK, Parise ME, Lewis LS, Ryan ET, Magill AJ (2006) Primaquine: report from CDC expert meeting on malaria chemoprophylaxis I. Am J Trop Med Hyg 75:402–415
36. Schwartz E, Regev-Yochay G (1999) Primaquine as prophylaxis for malaria for nonimmune travelers: a comparison with mefloquine and doxycycline. Clin Infect Dis 29:1502–1506
37. Desai M, ter Kuile FO, Nosten F, McGready R, Asamoa K, Brabin B, Newman RD (2007) Epidemiology and burden of malaria in pregnancy. Lancet Infect Dis 7:93–104
38. McGready R, Ashley EA, Nosten F (2004) Malaria and the pregnant traveller. Travel Med Infect Dis 2:127–142
39. Lengeler C (2004) Insecticide-treated bed nets and curtains for preventing malaria. Cochrane Database Syst Rev 2:CD000363
40. Pasternak B, Hviid A (2011) Atovaquone-proguanil use in early pregnancy and the risk of birth defects. Arch Intern Med 171:259–260
41. Villegas L, McGready R, Htway M, Paw MK, Pimanpanarak M, Arunjerdja R, Viladpai-Nguen SJ, Greenwood B, White NJ, Nosten F (2007) Chloroquine prophylaxis against vivax malaria in pregnancy: a randomized, double-blind, placebo-controlled trial. Trop Med Int Health 12:209–218
42. Rodriguez-Morales AJ, Sanchez E, Vargas M, Piccolo C, Colina R, Arria M, Franco-Paredes C (2006) Pregnancy outcomes associated with *Plasmodium vivax* malaria in northeastern Venezuela. Am J Trop Med Hyg 74:755–757

Malaria Diagnostics: Lighting the Path

David Bell and Mark D. Perkins

Abstract Drugs cure malaria, while diagnostics reduce the use of drugs. Thus, they not only impose a risk, demanding strong health service structures, but also deliver clear advantages in drug targeting and evidence-based management of fever. Often in the past, the costs of delivering accurate diagnostic results were seen as a reason for remaining blind, contributing to a stagnation in malaria and febrile disease management. The advent of new diagnostic technologies and demands of declining transmission rates have brought diagnosis to center stage in malaria management, and promise to catalyze reform in the management of febrile illness and wider health service delivery.

On an October day in 1880, Laveran first identified a microscopic parasite in the blood of a febrile patient in Algeria. In doing so, he opened our eyes to the possibility of addressing malaria as a specific disease with a specific cause, as many of his contemporaries were doing with other human ailments. Malaria is almost uniquely distinguished in that many eyes were later closed again; across the swathes of the malaria-endemic world, the ability to distinguish malaria from other causes of tropical fever syndrome was deliberately ignored. In sub-Saharan Africa in particular, despite the lack of pathognomonic symptoms, building on Laveran's discovery required a level of effort deemed unmanageable or unnecessary. Essentially, all malaria-like fevers were to be managed as one; a legacy that is still influencing African health systems today.

D. Bell • M.D. Perkins (✉)
Foundation for Innovative New Diagnostics, Avenue de Budé 16, Geneva 1202, Switzerland
e-mail: mark.perkins@finddiagnostics.org

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2_15,

1 Evolution of Diagnostics in Malaria Control

Malaria shares symptoms with several other common diseases that exact significant mortality and/or morbidity, such as the early stages of acute respiratory tract infection, meningitis, typhoid, and typhus [1–3]. Algorithms to distinguish malaria reliably on clinical grounds lack accuracy [4–8]. Managing each case of suspected malaria on an evidence base requires a significant degree of sophistication in health systems that until recently was considered unreachable, at least with the resources available. As a result, malaria diagnosis was largely guesswork, and many antimalarial drugs went to patients with other diseases. Not only was this practice wasteful of antimalarial drugs and dangerous for those misdiagnosed, but it also ensured continued ignorance about the epidemiology of malaria and the effectiveness of control efforts: malaria prevalence is highly heterogeneous but fever is quite uniformly common. The potential value of *excluding* malaria opened up by Laveran, thereby allowing a more focused treatment approach to other diseases, was largely lost.

Across much of Asia and South America, and the endemic areas of Europe and North America from which malaria is now largely eliminated, health systems did sometimes rise to the challenge of creating much of the diagnostic infrastructure necessary to allow targeted use of the slowly evolving stable of antimalarial drugs. Vertical structures to support microscopy were often associated with campaigns that were successful in eliminating malaria in Europe and North America, and in greatly reducing transmission in Asia and South America [9–12]. However, it is only in the past few years that guidelines for malaria management have fully embraced an evidence-based treatment policy such as is standard practice for most other infectious diseases, and in doing so opened the possibility of addressing a plethora of other diseases often exacting a similar or greater mortality in the same populations [13]. The recent increased use of parasite-based diagnosis is in part responsible for the steady reduction in reported incidence of malaria in areas where it was long considered the major public health problem; fever no longer equals malaria [14]. A review of published clinical studies, which tend to be carried out in high transmission areas, shows that malaria as a cause of fever has substantially declined across sub-Saharan Africa (Fig. 1) in the last 20 years: the median fraction of all patients with presumptive malaria found to be parasitemic was 22% in studies published since the year 2000 [15]. In many areas of sub-Saharan Africa previously thought to support high malaria transmission, incidence and prevalence are now known to be low [14]. In some areas, especially where effective control measures have been put in place, the decline in reported malaria that accompanies the introduction of diagnostics is dramatic. Data such as that reported from Livingstone district in Zambia (Fig. 2) demonstrate the impossibility of measuring the effectiveness of control measures in the absence of parasite-based diagnostics. As the proportion of febrile disease caused by malaria declines, the imperative for parasite-based diagnosis to target the use of antimalarial drugs increases further.

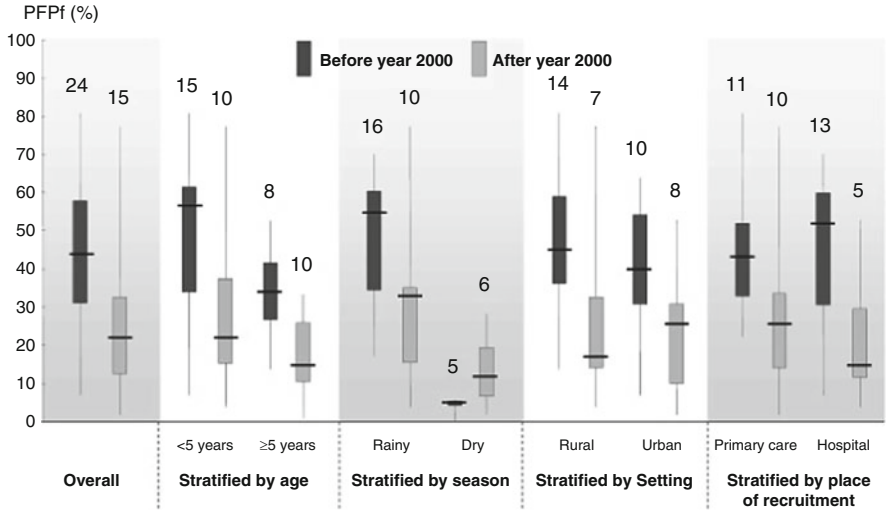


Fig. 1 Comparison between the proportions of fevers associated with *Plasmodium falciparum* parasitaemia (PFPf) in years ≤2000 and >2000, stratified by baseline characteristics (*numbers above plots* corresponds to the number of studies involved). Taken from [15]

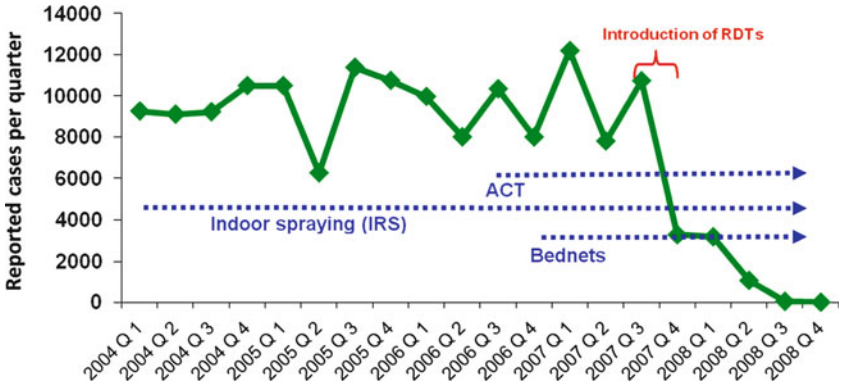


Fig. 2 Reported incidence of malaria in Livingstone district, Zambia, 2004–2008. Until late 2007, malaria was largely diagnosed clinically, masking the true incidence of disease and the effectiveness of control measures. Courtesy of the Zambia National Malaria Control Program

The diagnostic infrastructure established in the health systems of many Asian and South American countries likely had a significant impact in improving overall health system capacity and drug delivery. The structures set up to train, supply, and quality assure microscopists in countries such as India, Thailand, and Peru established supply lines and levels of management invaluable for delivery of a range of services, and a cadre of personnel trained sufficiently to use them [12]. While microscopy coverage often remained patchy and confined mostly to the

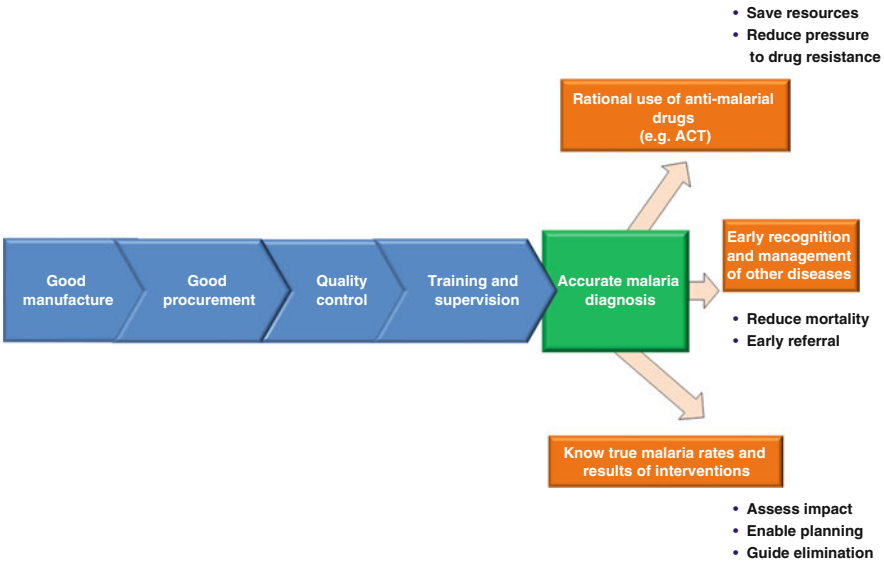


Fig. 3 Diagnostic effort and outcomes. Common to both microscopy and RDTs used at point of care, a well systematic quality control structure to ensure accuracy and provide confidence among users is expected to impart benefits beyond the management of malaria cases. Courtesy of the Foundation for Innovative New Diagnostics (FIND)

public health sector, the effort and infrastructure put in place to support diagnosis provided gains in malaria control well beyond case management (Fig. 3). It also strengthened advocacy for resources with some expectation that they would be directed to those who required them. The implementation of diagnostics required dedication of resources on the part of malaria control programs; antimalarial drugs, particularly chloroquine, and sulphadoxine–pyrimethamine, remained relatively cheap up to the last decade, and well below the costs of diagnosis.

Several factors have influenced the move, over the last decade, toward parasite-based diagnosis as an essential element in case management. First, the costs of antimalarial therapy rose dramatically with the introduction of artesunate-based combination therapies on a wide scale, particularly in Africa. As shown by several cost–benefit models and analyses, it was no longer economically viable to hand out antimalarial therapy to a large section of the population that had some other disease [16, 17]. Second, the advent of commercial lateral flow tests for malaria in the 1990s offered an alternative to microscopy that required significantly less infrastructure to support it. Evidence of the reproducible accuracy of some of the products in both laboratory and field settings has enabled confidence in their use [18–20]. Third, the rapid increase in funding available for malaria control programs over the past decade through mechanisms such as the Global Fund to Fight AIDS, Tuberculosis and Malaria has enabled Ministries of Health, especially in sub-Saharan Africa, to contemplate investments in malaria management not previously possible.

The rise of drug resistance, first to chloroquine and sulphadoxine–pyrimethamine, and more recently concern over the possible development of resistance to artemisinin-based combination therapies (ACTs), has further fuelled interest in diagnostic-based therapy. While an uninfected person will not add to pressure toward parasite resistance by inappropriately ingesting an antimalarial drug, the concern is that prolonged circulation of partner compounds, such as those accompanying artemisinin-based compounds in ACTs, could expose parasites from an infection arising after the one being treated to subtherapeutic doses and select for resistant strains.

In 2009, the World Health Organization (WHO), in response to increasing evidence of the safety and impact of diagnostics, updated its malaria control recommendations to include parasite-based diagnosis prior to the prescription of antimalarial drugs.

2 Utilization of Diagnostics

Although malaria parasites cause an acute, often recurring febrile illness, a significant number of individuals living in endemic areas may have asymptomatic parasitemia (Fig. 4). Such asymptomatic parasite carriage may occur as a result of clinical immunity to blood stage carriage, particularly of *Plasmodium falciparum* and *P. malariae*, or carriage of liver stages of *P. vivax* or *P. ovale*. For clinical purposes, laboratory methods for malaria diagnosis can therefore usefully be

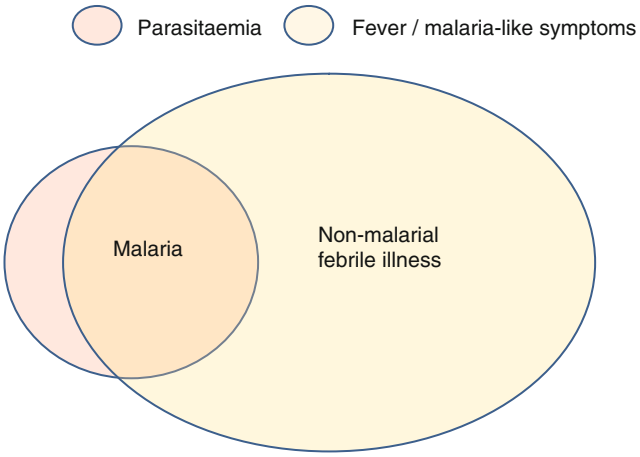


Fig. 4 The relationship between malaria parasitemia and malaria-like febrile illness in an endemic population. While most patients with malaria parasitemia are likely to have current or recent fever, a certain proportion can be asymptomatic and so do not present for diagnosis through fever case management. The clinical picture of febrile patients is indistinguishable for that of a number of other febrile diseases. Courtesy of the Foundation for Innovative New Diagnostics (FIND)

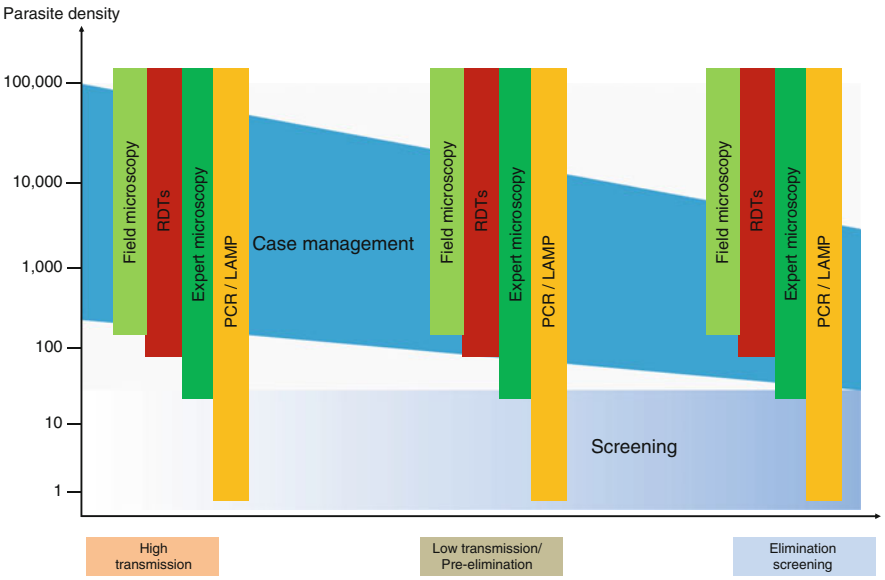


Fig. 5 The utility of different diagnostic methods will depend on the epidemiological setting of use, and on the indication – for case management or active screening. Courtesy of the Foundation for Innovative New Diagnostics (FIND)

divided into medical tests for case management – determining whether a febrile patient has malarial disease – and surveillance tools for determining the prevalence of parasites in a given population. Surveillance testing, especially in areas of low transmission and in elimination campaigns, needs to detect very low parasite densities (Fig. 5). Tests to detect other metabolic or pathological states relevant to malaria treatment are also required to varying extent to ensure safe case management; apart from routine biochemical monitoring to manage severe malaria, an example is the identification of glucose-6-phosphate dehydrogenase (G6PD) deficiency to allow safe use of 8-hydroxyquinolones in management of *P. vivax* and *P. ovale* (Box 1).

Box 1. *P. vivax*, Latent Parasites and Tafenoquine

An interesting case of the dependence of drug management on diagnostics, and the relative emphasis afforded the two areas of research and development, is seen in the currently rising interest in improved management and eventual elimination of *P. vivax*. In common with *P. ovale*, the latent liver-stage of *P. vivax* is not susceptible to drugs effective in clearing blood stages, which prevents acute treatment from clearing the parasite. Currently, the 8-aminoquinoline, primaquine, is the only available drug that can achieve radical cure, requiring a course conventionally of 14 days duration. Primaquine is further limited by the potential toxicity of the 8-aminoquinolines in cases of

glucose-6-phosphate dehydrogenase (G6PD) deficiency. A newer drug in the same class, tafenoquine, has a longer half-life than primaquine, which could greatly simplify dosing. However, concern over the toxicity of a long-acting drug in G6PD-deficient patients is a major obstacle to tafenoquine introduction. In this context, it is perhaps surprising the large investment over more than 20 years in the development and evaluation of the drug has not been matched by a far more modest investment in the development of field-ready G6PD screening tests to accompany its use.

2.1 Diagnostics for Case Management

Light microscopy has remained essentially unchanged since the time of Laveran. Early improvements in staining, the advent of binocular microscopes, and light-emitting diode (LED) illumination have all made the technician's life easier and improved accuracy. However, despite notable exceptions, sometimes unsustained, in countries such as Thailand, India, Sri Lanka, and endemic areas of the Americas, in most countries microscopy has largely remained a tool limited to reference laboratories and hospitals. Because significant investment in human resources is required to maintain good malaria microscopy services, the extension of microscopy to lower levels of the health system has often resulted in a significant decline in performance standards [21–26].

From the 1970s to 1990s, a handful of new detection methods were developed to enhance or build on conventional light microscopy. Such methods, including fluorescent microscopy of acridine orange stained thick films or of centrifuged microhematocrit tubes [27–29], have thus far had limited impact. This is partially because they removed some advantages of light microscopy (including accurate species determination and utility of the equipment for a broad range of diseases), but more importantly because they failed to address the largest barrier to the success of microscopy in most malaria-endemic settings; the limited technical and human resources infrastructure available at the place where diagnostics are most needed – within walking distance of a sick patient.

Nucleic acid amplification [*e.g.*, polymerase chain reaction (PCR)] and enzyme linked immunoassays (ELISA) for plasmodial antigens added to the armamentarium of reference methods available at the top of national laboratory systems, but failed to offer a solution to the ease-of-use limitations of light microscopy for routine case management.

The development of lateral flow immunochromatographic test strips to detect plasmodial antigens in the early 1990s finally brought a challenge to the dominance of microscopy, and the potential to overcome many of its weaknesses in case management. Because of their simplicity, these immunochromatographic tests, commonly referred to as rapid diagnostic tests (RDTs) or "dipsticks," have been favored for field use over the other alternatives. Since the first RDT was evaluated

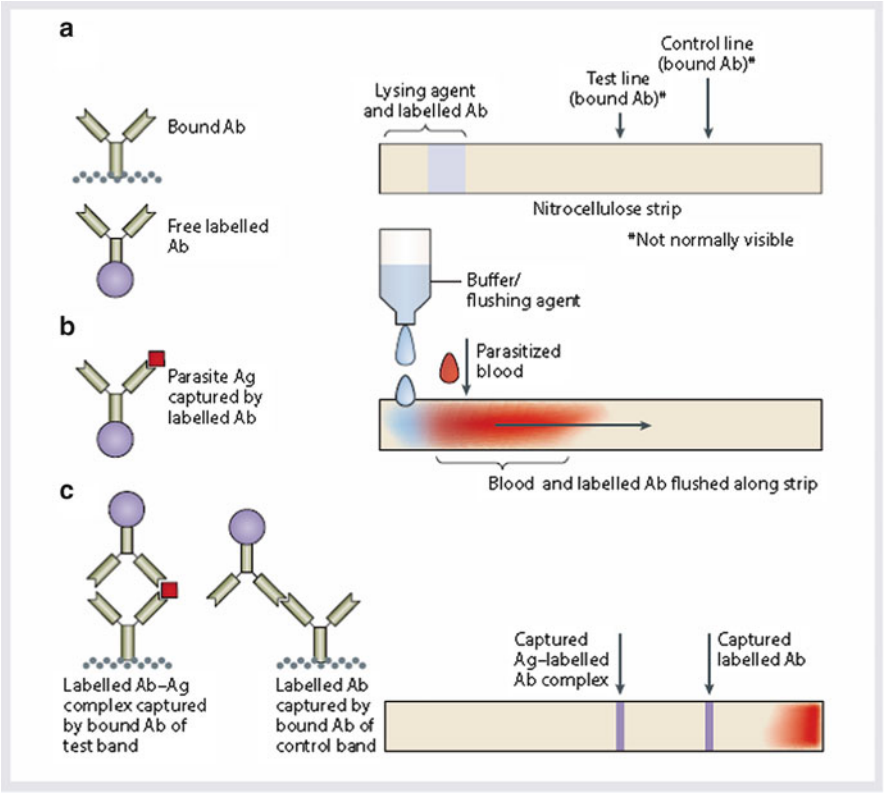


Fig. 6 Composition and detection mechanisms of lateral flow immunochromatographic tests for malaria. Courtesy of the WHO – Regional Office for the Western Pacific

in 1993, a steadily expanding number of malaria RDTs has appeared on the market, and others are under development [30]. All are based on the detection of parasite-derived protein in whole blood. Blood is lysed and drawn along a nitrocellulose test strip by a combination of capillary action and flushing by a buffer reagent. The protein is bound during testing by dye-labeled specific antibodies (colloidal gold is commonly used), and the labeled antigen–antibody complex is captured by a line of antibody bound to the strip (Fig. 6). Tests may contain multiple lines of antibody to detect species-differentiating *Plasmodium* proteins. A control line further along the strip, composed of anti-immunoglobulin antibody, also captures dye-labeled antibody and confirms that proper wicking has occurred.

Lateral flow tests are relatively simple to manufacture, given the availability of the main components including antibodies and nitrocellulose film. However, manufacturing in large quantities presents challenges in maintaining consistent quality. As a result, the number of available products mushroomed, but with a high variability in performance. More than 100 million RDTs are financed in the public sector in 2011 [31]. The diversity in quality, limited resources of the

manufacturers involved, and lack of reference standards necessitated the development of an independent quality assurance program to guide procurement and provide the confidence necessary for reliance on these tools [32, 33].

WHO and the Foundation for Innovative New Diagnostics (FIND) and partners have collaborated to establish a quality assurance system that includes product testing, to determine the comparative performance of commercial tests, and lot testing, to check the quality of specific manufactured lots before they are distributed. Manufacturers operating under appropriate ISO standards are invited annually to submit products for testing against a highly characterized panel of blood samples with clinically relevant concentrations of *P. falciparum* or *P. vivax*. To date, four annual rounds of product testing have been initiated on 168 products submitted by more than 30 manufacturers. The reports of this product testing show a wide variability in the capacity of different tests to detect low parasite densities and in thermal stability – the latter an essential requirement for remote area transport and storage [18, 19]. They also show that there are many tests that perform very well. Since product testing has begun, the quality of RDT manufacturing has increased, as evident from significant performance improvements in tests that have been resubmitted for product testing after manufacturing changes.

2.2 Risks of Parasite-Based Diagnosis for Case Management

Efficacious antimalarial drugs cure malaria, while diagnostics, when replacing symptom-based diagnosis, lead to withdrawal of drugs from those who would otherwise have received them. This puts a huge imperative on quality of result before routine parasite-based diagnosis is contemplated. When accurate, diagnosis leads to gains in drugs saved, quality of data for disease tracking, and the potential to reduce mortality through the improved early management of other diseases as malaria is excluded [13]. When inaccurate, diagnostic use may increase malaria mortality as a result of missed diagnoses.

Conversely, parasite-based diagnosis may lead to inappropriate assumptions that malaria parasitemia is a cause of illness. Although symptomatic malaria parasitemia always deserves to be treated, parasitemia may occur as a secondary cause of fever or as an incidental infection in a fever caused by other etiology. As with all diagnostic testing, test results should not be interpreted in the absence of clinical assessment.

All diagnostic tests have limits; malaria RDTs detect a certain threshold of parasite density, or more precisely an antigen concentration equivalent to this. This detection threshold must be low enough that clinically significant disease is not missed. The ratio of antigen concentration to parasite density varies widely, as does the severity of symptoms at any given parasite density vary, depending on the level of immunity. A threshold of approximately 200 parasite/μL is considered a reasonable cut-off that must be detected reliably to ensure safe management [34]

for the four common species of malaria infection humans. *P. knowlesi* may have a lower parasitemia threshold for causing symptoms.

Concern over the ability of tests to detect a sufficiently low threshold (adequate clinical sensitivity), together with the ingrained belief that "fever equals malaria," is probably largely responsible for the slow uptake of parasite-based diagnosis, whether by RDTs or microscopy. Evidence of poor adherence to diagnostic results is reported in antimalarial drug dispensing in several studies [35–37]. However, large reductions in drug dispensing are now being seen on a national scale, where RDT introduction has been systematically accompanied by programs to ensure both quality of the tests and training in their use [38, 39]. Moreover, studies on treatment withholding on negative test results have found that this is safe, even in young children, when the RDTs are confirmed to be working [40]. In order to establish this level of confidence, and safety, however, the structure of implementation and associated health systems development needs to be taken seriously and sufficiently resourced (Fig. 7).

The overall health benefits of parasite-based diagnosis and introduction of RDTs at a village or community level are difficult to quantify and are expected to vary in differing epidemiological situations, but may include improved adherence to antimalarial therapy promoted by surety of diagnosis, and better management of nonmalarial fever. Where early appropriate action is taken to identify and manage other etiologies of fever in patients with parasite-negative results, major gains in mortality reduction, especially for severely ill patients, may accrue [13, 16, 41], but the means and ability

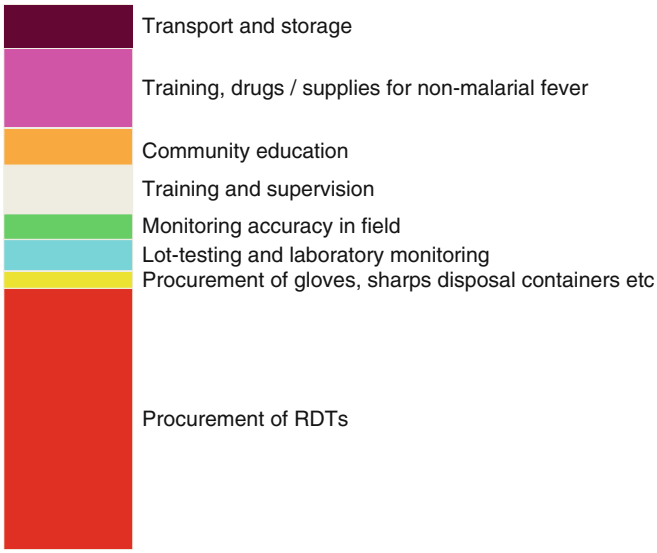


Fig. 7 The costs of implementation of malaria RDTs in a typical health system go well beyond the costs of RDT procurement. While the relative cost of the various areas of implementation will vary widely between programs, all must be adequately resourced. Courtesy of the WHO – Regional Office for the Western Pacific

to address these are often lacking. Together with exposing the need for the development of capacity for managing these nonmalarial febrile illnesses, RDT implementation, such as microscopy, requires considerable investment in health services to ensure adequate delivery and utilization of results. While this may slow implementation and impose new strains on resource-poor health systems, it also provides an opportunity to build structures to support community disease management that have spin-offs for other disease programs and other areas of health-care delivery.

2.3 Diagnostics for Screening and Surveillance

2.3.1 Nucleic Acid Detection

A nested PCR is the current gold standard for the detection of parasitemia, detecting less than one parasite per microliter [42, 43]. Quantitative PCR offers the potential to determine the concentration of circulating DNA, and therefore estimates of circulating parasite density and to a lesser extent parasite load. The applications of PCR-based methods are limited to well-equipped laboratories with specifically trained technicians, and are further limited by cost. Avoidance of contamination (leading to false-positive results) requires a high standard of laboratory practice. PCR capacity is limited in most malaria-endemic countries, and considerable resources would be required to establish and maintain this capacity. Restriction to well-equipped laboratories limits its utility for clinical care by preventing feedback timely enough for case management in most endemic areas, though the development of systems that automate and integrate sample processing, in place for some other diseases, may increase its accessibility [44].

Alternative molecular methods, which are inherently simpler than PCR, have been developed and applied to malaria. One such method is loop mediated isothermal amplification (LAMP), which operates at a single temperature and yields a turbid or fluorescent endpoint that can be detected by the naked eye. A sensitive malaria test using LAMP has been developed targeting mitochondrial DNA, and a report on a noncommercial early version of this assay published [45]. Such an approach has potential to reduce the training and infrastructure requirements of molecular diagnosis, making proximal implementation possible, where results could be rapidly available. This could be very useful for surveillance and active case finding for low-density parasitemia, and for monitoring parasite presence in drug efficacy monitoring and trials and vaccine trials. A LAMP assay that has sufficient throughput to be used for surveillance testing of large numbers of individuals has not yet been developed.

2.3.2 Serological Tests for Antibody Detection

Antibody detection, currently available in ELISA and RDT formats, can readily demonstrate infection with malaria. These tests are inappropriate for case

management because they cannot reliably differentiate between past and current infection, and because antibodies may not be detectable in blood stage infections of very recent onset. They do, however, have a potential role in tracking the epidemiology of malaria. Parasite prevalence data provide a snapshot in any given season of the epidemiology of disease, whereas antibody responses represent transmission intensity over several years, reducing seasonal or annual bias. Age-adjusted rates of immune responses may be used to estimate the force of infection [46, 47]. As humoral immune responses are very sensitive measures of infection, they may have potential, little used till now, to guide stratification of malaria risk, where transmission is very low.

Detection of antisporozoite antibodies has been suggested as a surrogate for detecting individuals with a high likelihood of carriage of *P. vivax* hypnozoites (evidence of infection), and could therefore be used to guide the use of 8-aminoquninolines for clearance of liver-stage *P. vivax* and *P. ovale*.

3 Conclusions

Parasite-based diagnosis has a large and growing role in malaria control and the case management of febrile illness, thanks to the development of simple and reliable assays and a mechanism to monitor their quality, and thanks to improved health funding and a clearer understanding of the benefits of parasite-based diagnosis. The expanding role of rapid testing in malaria control programs has in many areas transformed local understanding of the true prevalence of malaria and has, where properly supported by health-worker training, saved millions of courses of unwarranted malaria therapy. These benefits come with a cost, not just only for the commodities used, but also for the complexity that knowledge brings. Confronting a patient with an unknown cause of fever is much more complicated for health workers than simply dispensing, needed or not, antimalarial drugs. Having spent decades treating all fever in the tropics as malaria, health systems will have to adapt to maximize the advantages that knowing before treating can bring.

References

1. Cunha BA (2004) Osler on typhoid fever: differentiating typhoid from typhus and malaria. Infect Dis Clin North Am 18:111–125
2. Wright PW, Avery WG, Ardill WD, McLarty JW (1993) Initial clinical assessment of the comatose patient: cerebral malaria vs. meningitis. Pediatr Infect Dis J 12:37–41
3. Madhi SA, Klugman KP (2006) Acute respiratory infections. In: Jamison DT, Feachem RG, Makgoba MW et al (eds) Disease and mortality in Sub-Saharan Africa. World Bank, Washington, DC
4. Bassett MT, Taylor P, Bvirakare J, Chiteka F, Govere EJ (1991) Clinical diagnosis of malaria: can we improve? Trop Med Hyg 94:65–69

5. Redd SC, Kazembe PN, Luby SP, Nwanyanwu O, Hightower AW, Ziba C, Wirima JJ, Chitsulo L, Franco C, Olivar M (1996) Clinical algorithm for treatment of *Plasmodium falciparum* malaria in children. Lancet 347:223–227
6. Luxemburger C, Nosten F, Kyle DE, Kiricharoen L, Chongsuphafajaiidhi T, White NJ (1998) Clinical features cannot predict a diagnosis of malaria or differentiate the infecting species in children living in an area of low transmission. Trans R Soc Trop Med Hyg 92:45
7. Mwangi TW, Mohammed M, Dayo H, Snow RW, Marsh K (2005) Clinical algorithms for malaria diagnosis lack utility among people of different age groups. Trop Med Int Health 10:530–536
8. Quigley MA, Armstrong Schellenberg JR, Snow RW (1996) Algorithms for verbal autopsies: a validation study in Kenyan children. Bull World Health Organ 74:147–154
9. Hackett L (1937) Malaria in Europe: an ecological study. Oxford University Press, London
10. Ackerknecht EH (1945) Malaria in the Upper Mississippi Valley 1760–1900. Bull Hist Med (suppl) viii+142
11. Deane LM (1988) Malaria studies and control in Brazil. Am J Trop Med Hyg 38:223–230
12. Najera JA, Gonzalez-Silva M, Alonso PL (2011) Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). PLoS Med 8:e1000412
13. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P, Campbell H, Walker CF, Cibulskis R, Eisele T, Liu L, Mathers C; Child Health Epidemiology Reference Group of WHO, UNICEF (2010) Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet 375:1969–1987
14. WHO (2010) World Malaria Report. World Health Organization, Geneva
15. D'Acremont V, Lengeler C, Genton B (2010) Reduction in the proportion of fevers associated with *Plasmodium falciparum* parasitaemia in Africa: a systematic review. Malar J 9:240
16. Shillcutt S, Morel C, Goodman C, Coleman P, Bell D, Whitty CJ, Mills A (2008) Cost-effectiveness of malaria diagnostic methods in sub-Saharan Africa in an era of combination therapy. Bull World Health Organ 86:101–110
17. Lubell Y, Reyburn H, Mbakilwa H, Mwangi R, Chonya S, Whitty CJ, Mills A (2008) The impact of response to the results of diagnostic tests for malaria: cost-benefit analysis. Br Med J 336:202–205
18. WHO (2009) Malaria rapid diagnostic test performance, Round 1. World Health Organization, Geneva. ISBN 978 92 4 159807 1
19. WHO (2010) Malaria rapid diagnostic test performance, Round 2. World Health Organization, Geneva. ISBN 978 92 4 159946 7
20. Lot testing results. http://www.finddiagnostics.org/about/what_we_do/successes/malaria_rdt_lot_testing_results/index.html
21. Kachur SP, Nicolas E, Jean-François V, Benitez A, Bloland PB, Saint Jean Y, Mount DL, Ruebush TK 2nd, Nguyen-Dinh P (1998) Prevalence of malaria parasitemia and accuracy of microscopic diagnosis in Haiti, October 1995. Rev Panam Salud Publ 3:35–39
22. Durrheim DN, Becker PJ, Billinghurst K (1997) Diagnostic disagreement–the lessons learnt from malaria diagnosis in Mpumalanga. S Afr Med J 87:1016
23. Kain KC, Harrington MA, Tennyson S, Keystone JS (1998) Imported malaria: prospective analysis of problems in diagnosis and management. Clin Infect Dis 27:142–149
24. Coleman RE, Maneechai N, Rachaphaew N, Kumpitak C, Miller RS, Soyseng V, Thimasarn K, Sattabongkot J (2002) Comparison of field and expert laboratory microscopy for active surveillance for asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* in western Thailand. Am J Trop Med Hyg 67:141–144
25. O'Meara WP, McKenzie FE, Magill AJ, Forney JR, Permpanich B, Lucas C, Gasser RA Jr, Wongsrichanalai C (2005) Sources of variability in determining malaria parasite density by microscopy. Am J Trop Med Hyg 73:593–598
26. Kilian AH, Metzger WG, Mutschelknauss EJ, Kabagambe G, Langi P, Korte R, von Sonnenburg F (2000) Reliability of malaria microscopy in epidemiological studies: results of quality control. Trop Med Int Health 5:3–8

27. Anthony RL, Bangs MJ, Anthony JM, Purnomo (1992) On-site diagnosis of *Plasmodium falciparum*, *P. vivax*, and *P. malariae* by using the Quantitative Buffy Coat system. J Parasitol 78:994–998
28. Parija SC, Dhodapkar R, Elangovan S, Chaya DR (2009) A comparative study of blood smear, QBC and antigen detection for diagnosis of malaria. Indian J Pathol Microbiol 52:200–202
29. Adeoye GO, Nga IC (2007) Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa-stained Thick Film (GTF) for diagnosis of malaria. Parasitol Int 56:308–312
30. Shiff CJ, Premji Z, Minjas JN (1993) The rapid manual ParaSight-F test. A new diagnostic tool for *Plasmodium falciparum* infection. Trans R Soc Trop Med Hyg 87:646–648
31. RBM, MMV: Tracking progress in scaling-up diagnosis and treatment for malaria. Geneva, Roll back Malaria Partnership, Medicines for Malaria Venture. 2009. http://www.rbm.who.int/partnership/wg/wg_procurementsupply/docs/ACT_RDTscaleUpAssessment_MMVRBMaugust2010.pdf
32. WHO-TDR-FIND (2010) Methods manual for laboratory quality control testing of malaria rapid diagnostic tests, Version Six. 2010. World Health Organization, Geneva
33. WHO-FIND-TDR-USCDC (2010) Methods manual for product testing of malaria rapid diagnostic tests (version 3). World Health Organization, Geneva
34. WHO (2009) Parasitological confirmation of malaria diagnosis. Report of a WHO technical consultation, Geneva, 6–8 Oct 2009. ISBN 978 92 4 159941 2
35. Bisoffi Z, Sirima BS, Angheben A, Lodesani C, Gobbi F, Tinto H, Van den Ende J (2009) Rapid malaria diagnostic tests vs. clinical management of malaria in rural Burkina Faso: safety and effect on clinical decisions. A randomized trial. Trop Med Int Health 14:491–498
36. Zurovac D, Njogu J, Akhwale W, Hamer DH, Larson BA, Snow RW (2008) Effects of revised diagnostic recommendations on malaria treatment practices across age groups in Kenya. Trop Med Int Health 13:784–787
37. Chandler CI, Chonya S, Boniface G, Juma K, Reyburn H, Whitty CJ (2008) The importance of context in malaria diagnosis and treatment decisions – a quantitative analysis of observed clinical encounters in Tanzania. Trop Med Int Health 13:1131–1142
38. D'Acremont V, Kahama-Maro J, Swai N, Mtasiwa D, Genton B, Lengeler C (2011) Reduction of anti-malarial consumption after rapid diagnostic tests implementation in Dar es Salaam: a before-after and cluster randomized controlled study. Malar J 10:107
39. Thiam S, Thior M, Faye B, Ndiop M, Diouf ML, Diouf MB, Diallo I, Fall FB, Ndiaye JL, Albertini A et al (2011) Major reduction in anti-malarial drug consumption in Senegal after nation-wide introduction of malaria rapid diagnostic tests. PLoS One 6:e18419
40. D'Acremont V, Malila A, Swai N, Tillya R, Kahama-Maro J, Lengeler C, Genton B (2010) Withholding antimalarials in febrile children who have a negative result for a rapid diagnostic test. Clin Infect Dis 51:506–511
41. Msellem MI, Martensson A, Rotllant G, Bhattarai A, Stromberg J, Kahigwa E, Garcia M, Petzold M, Olumese P, Ali A et al (2009) Influence of rapid malaria diagnostic tests on treatment and health outcome in fever patients, Zanzibar: a crossover validation study. PLoS Med 6:e1000070
42. Snounou GS, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN (1993) High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 61:315–320
43. Mixson-Hayden T, Lucchi NW, Udhayakumar V (2010) Evaluation of three PCR-based diagnostic assays for detecting mixed *Plasmodium* infection. BMC Res Notes 3:88
44. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, Allen J, Tahirli R, Blakemore R, Rustomjee R et al (2010) Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med 363:1005–1015
45. Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, Chiodini PL, Sutherland CJ (2010) Mitochondrial DNA targets increase sensitivity of malaria detection using loop mediated isothermal amplification. J Clin Microbiol 48:2866–2871

46. Snow RW, Molyneux CS, Warn PA, Omumbo J, Nevill CG, Gupta S, Marsh K (1996) Infant parasite rates and immunoglobulin M seroprevalence as a measure of exposure to *Plasmodium falciparum* during a randomized controlled trial of insecticide-treated bed nets on the Kenyan coast. Am J Trop Med Hyg 55:144–149
47. Drakeley CJ, Corran PH, Coleman PG, Tongren JE, McDonald SL, Carneiro I, Malima R, Lusingu J, Manjurano A, Nkya WM et al (2005) Estimating medium- and long-term trends in malaria transmission by using serological markers of malaria exposure. Proc Natl Acad Sci USA 102:5108–5113

Index

A
ACT. *See* Artemisinin combination therapy (ACT)
4-Aminoquinolines
  AQ modification, toxicity reduction
    CQ and AQ metabolism, 30–31
    metabolic structural alerts modification, 31–34
  CQ/AQ combinations, 34–37
  CQ improvement, modification
    amodiaquine, 30
    AQ–13, 26–27
    ferroquine, 27
    piperaquine, 28–29
    trioxaquine SAR116242, 29–30
  CQ resistance development
    CQ recycling, 24–25
    parasite-resistance mechanisms, 23–24
  history and development, 20
  quinoline antimalarials
    acidic food vacuole, CQ accumulation, 22
    haeme–CQ drug complexes, 21
8-Aminoquinolines
  primaquine
    antirelapse therapy and radical cure, 71–72
    chemistry, 70
    chemoprophylaxis, 71
    clinical use, 70–71
    drug combinations, 72–73
    mechanism of action, 74–75
    pharmacokinetics and metabolism, 75–76
    resistance, 77
    safety and tolerability, 76–77
    transmission blocking, 73–74
  tafenoquine
    chemistry, 79
    clinical use, 82–87
    development, 77–79
    mechanism of action and resistance development, 80
    pharmacokinetics and metabolism, 80–81
    safety and tolerability, 81–82
AQ (*See* 4-Aminoquinolines), 219
Amyl alcohols. *See* 4-Methanolquinolines
Antibiotics
  molecular markers, *Plasmodium* resistance
    clindamycin, 265
    doxycycline, 264
    erythromycin derivative, 265
    fosmidomycin, 265
    fosmidomycin-resistant parasites, 266
    strain classification, 264
    structural models, 265, 266
Antibody detection, 303–304
Antifolates
  anticancer drugs, for malaria, 119–120
  with chlorproguanil, 117
  folate biochemistry, 113–114
  folate salvage inhibition, 119
  molecular markers, *Plasmodium* resistance
    artemisinin-based combination therapy, 262
    dihydrofolate reductase (DHFR), 260–261
    dihydropteroate synthase (DHPS), 260–262

H.M. Staines and S. Krishna (eds.), *Treatment and Prevention of Malaria*,
DOI 10.1007/978-3-0346-0480-2,

efficacy, 260
P.falciparum resistance, 260
pyrimethamine-resistant, 261
quadruple mutant, 261, 262
sulfadoxinepyrimethamine, 261
with proguanil
atovaquone, non-sulpha-based drug, 114–116
dapsone, 114
with pyrimethamine
intermittent preventive treatment, 116–117
malaria treatment, 116
resistance
combination pyrimethamine/sulphadoxine, 117–118
parasites, origin, 118–119
Antimalarial drugs. *See also* 8-Aminoquinolines; Molecular markers, *Plasmodium* resistance; Quinine
medicines, malaria, 232–239
naphthoquinones, DHODH inhibitors, 136
targeting mitochondrial functions (*see* Naphthoquinones)
Arbor febrifuga, 214
Artemether. *See* Artemisinins
Artemisia annua, 158
Artemisinin combination therapy (ACT). *See also* Artemisinins, combination therapy
applications, 230–231
artemisinin derivatives, 240
clinical trials, 231
dihydroartemisinin-piperaquine, 231
history, 161
medicines, malaria, 232–239
mefloquine-artesunate, 240
pyronaridine-artesunate, 231
Artemisinins
ACT and antimalarial drug resistance, 217–218
causes, 221–222
chemistry, 161–163
clinical uses
parenteral treatment of severe malaria, 178–179
rectal administration, 179–181
uncomplicated malarial treatment, 176–178
coformulation, 217
combination therapy, 215–216
cross-resistance, 218
definition, 220–221
derivatives, pharmacology
absorption and bioavailability, 170–172
antimalarial activity, 173
disadvantages, 191–192
metabolism and elimination, 172
pharmacokinetic–pharmacodynamic relationships, 172
safety and tolerability, 173–175
history, 213–214
ACT, 161
antimalarial properties, 158–159
clinical efficacy, 160
dihydroartemisinin, 159
medicinal uses, 158
qinghao, 158
rodent malaria, 159
therapeutic compounds, 160
mechanism of action
bioactivation, 164
haem pathway, 164
mechanistic hypotheses, 163–164
mitochondrial function, 166
PfATP6 inhibition, 165
protein alkylation, 165
safty and efficacy, 163
structure–efficacy relationships, 163
medicinal development, 241–242
molecular markers, *Plasmodium* resistance
genetic approach, 263
linkage group selection, 263
monotherapy treatment, 262
pcubp1, 263
PfATP6, 262
S769N mutation, 263
translationally controlled tumor protein homolog (TCTP), 264
ubiquitin-specific protease–1 (UBP–1), 263
partner drugs, 219
pharmacokinetic mismatch and compliance, 216
pharmacokinetic properties, 215
resistance
clinical isolates monitoring, 168
drug transport, 166
P.falciparum, 167
rodent model, 167
strategies, 170
treatment efficacy, 169
synthetic peroxides, 214–215
in Thai–Cambodian border, 220

Artemisone
accumulation and co-factors, 198–199
drug–drug interaction, 193–194
efficacy of, 195
Fe(II) interaction and peroxide bond, 196–198
preclinical studies, 195
primary targets, 198
Artesunate. *See* Artemisinins
Asymptomatic parasitemia, 297
Atovaquone, 267–268
molecular markers, *Plasmodium* resistance, 267–268
naphthoquinones
atovaquone-proguanil combination therapy, 131, 134–135
resistance, 130–131
Azithromycin, 265, 266

C
Chemoprophylaxis. *See* Prevention, malaria
Children, malarial prevention, 290
Chloroquine resistance (CQR), 250–255
Chloroquine (CQ) *See* 4-Aminoquinolines
Chlorproguanil, antifolates, 117
Cinchona alkaloids. *See* Quinine
Cinchona spp., 214
Clindamycin, 265
Coarsucam, 231
Coartem-D, 231
Control and elimination, malaria. *See* Antimalarial drugs

D
Dapsone, antifolates, 114
Diagnosis, malaria
clinical history, 293
evolution, malaria control
case management, 296
drug resistance, 297
effectiveness measurement, 294, 295
epidemiology, 294
influencing factors, 296
Plasmodium falciparum parasitaemia, 295
symptoms, 294
screening and surveillance
antibody detection, 303–304
nucleic acid detection, 303
utilization
asymptomatic parasitemia, 297
case management, 298–301
parasite-based diagnosis, 301–303
P.vivax, latent parasites and tafenoquine, 298–299
Dihydroartemisinin. *See* Artemisinins
Dihydroartemisinin-piperaquine, 231
Dihydrofolate reductase (DHFR), 114, 260–261
Dihydropteroate synthase (DHPS), 114, 261–262
Doxycycline, 145
long-term visitors and frequent visitors prevention, 285
resistance molecular markers, 264

F
Fixed dose artemisinin combination therapy (FACT)
applications, 230–231
characteristics, 229
clinical trials, 231
dihydroartemisinin-piperaquine, 231
mefloquine-artesunate, 240
pyronaridine-artesunate, 231
Fluorescent microscopy, 299
Folate. *See* Antifolates
Fosmidomycin, 265–266
Foundation for Innovative New Diagnostics (FIND), 296–298, 301

G
Gametocytes eradication, 244–245
Glucose 6-phosphate 1-dehydrogenase (G6PD), 69, 76, 82, 244
Griesbaum co-ozonolysis, 200

H
Haem pathway, artemisinins, 164
Halofantrine
structure, action and resistance, 102
tolerability, 103
Huanghuahao, 158
Hypnozoites eradication, 244–245

I
Immunochromatographic tests, 299, 300

L
Light microscopy, 299
Lumefantrine
artemisinins, 219

Lumefantrine (*cont.*)
pharmacokinetics, 105–106
resistance, 106
structure and action, 104–105

M
malaria
Anopheles, 2
control and elimination, 5–12
etiology, 1–2
lifecycle of, 3
morbidity and mortality, 2
sporozoite form, 3
transmission prevention, 4–5
Malaria lifecycle fingerprinting (MMV), 245
Medicinal development, malaria
artemisinin combination therapy
applications, 230–231
artemisinin derivatives, 240
clinical trials, 231
dihydroartemisinin-piperaquine, 231
medicines, used in, 232–239
mefloquine-artesunate, 240
pyronaridine-artesunate, 231
artemisinins, 241–242
drug discovery and development, 227–228
gametocytes and hypnozoites eradication, 244–245
genomes and screens, 242–243
Mefloquine
artesunate, 240
hydrochloride
clinical use, 97–98
pharmacokinetics, 97
resistance, 98
structure and action, 96–97
tolerability, 98–99
long-term visitors and frequent visitors prevention, 285
partner drugs resistance, 219
4-Methanolquinolines
halofantrine
structure, action and resistance, 102
tolerability, 103
lumefantrine
pharmacokinetics, 105–106
resistance, 106
structure and action, 104–105
mefloquine
clinical use, 97–98
pharmacokinetics, 97
resistance, 98
structure and action, 96–97
tolerability, 98–99
piperaquine
pharmacokinetics, 104
resistance, 104
structure and action, 103–104
pyronaridine
pharmacokinetics, 101
resistance, 101
structure and action, 100–101
Mitochondrial electron transport chain (mtETC)
naphthoquinones
blood stage *P. falciparum*, 132–133
description, 127
P.falciparum viability, 132
selective inhibition, 128–130
yDHODH-transgenic parasites, proguanil, 133–134
Molecular markers, *Plasmodium* resistance
antibiotics
azithromycin, 265, 266
clindamycin, 265
doxycycline, 264
erythromycin derivative, 265
fosmidomycin, 265–266
fosmidomycin-resistant parasites, 266
strain classification, 264
structural models, 265, 266
antifolates
artemisinin-based combination therapy, 262
dihydrofolate reductase, 260–261
dihydropteroate synthase, 261–262
efficacy, 260
P.falciparum resistance, 260
pyrimethamine-resistant, 261
quadruple mutant, 261, 262
sulfadoxinepyrimethamine, 261
artemisinins
genetic approach, 263
linkage group, 263
linkage group selection, 263
monotherapy treatment, 262
pcubp1, 263
PfATP6, 262
S769N mutation, 263
translationally controlled tumor protein homolog, 264
ubiquitin-specific protease–1 (UBP–1), 263

atovaquone, 267–268
determination, 250
drug resistance, *P. vivax*
pfcrt and *pfmdr1*, 268
pyrimethamine binding, 268, 269
P.vivax, 268–270
quinoline-based drugs
Na^+/H^+ exchanger 1, 259–260
P.falciparum chloroquine resistance transporter, 250–255
P.falciparum multidrug resistance protein 1, 255–258
P.falciparum Na+/H+ exchanger 1, 259–260
PfCRT, 250, 252–255
*pfmdr*1, 255–258
PfMRP, 259
transporters, 259

N
Naphthoquinones
atovaquone
proguanil combination therapy, 131, 134–135
resistance, 130–131
DHODH inhibitors, antimalarials, 136
mitochondrial electron transport chain (mtETC)
blood stage *P. falciparum*, 132–133
description, 127
P.falciparum viability, 132
selective inhibition, 128–130
yDHODH-transgenic parasites, proguanil, 133–134
tricarboxylic acid (TCA) metabolism, 136
yDHODH-transgenic parasites, decipher mode, 135–136
Nucleic acid detection, 303

O
Ozonides (OZ). *See* 1,2,4-trioxolanes (ozonides)

P
Parasite-based diagnosis, 301–303
Parasite reduction ratio (PRR), 173
Parasites, artemisinins activity, 163–164
Phentathrenes. *See* 4-Methanolquinolines
Piperaquine, 219
pharmacokinetics, 104
resistance, 104
structure and action, 103–104
Plasmodium falciparum
artemisinin combination therapy, 217
drug resistance, 167–168
Plasmodium falciparum chloroquine resistance transporter *(pfcrt)*
allelic exchange experiments, 252
chloroquine resistance (CQR), 250
genetic cross, 250, 252
identification, 251
K76T, 252, 253
mutations, 255
polymorphisms, 254
quantitative trait loci (QTL) analysis, 252, 253
Plasmodium falciparum multidrug resistance-associated protein *(PfMRP)*, 259
Y184F, 257
Plasmodium falciparum multidrug resistance protein 1 (PfMRP1)
allelic exchange, 258
amplification, 256
ATP-binding cassette (ABC), 255
CQ-resistant lines, 256
disruption, 259
genetic disruption, 256
identification, 256
mutations, 257, 258
P-glycoprotein homologue 1 (Pgh–1), 256
polymorphisms, 257, 258
Y184F, 257
Plasmodium falciparum Na^+/H^+ exchanger 1 *(pfnhe–1)*, 259–260
Plasmodium falciparum parasitaemia (PFPf), 295
Plasmodium ovale
prevention, 286–287
Plasmodium resistance. *See* Molecular markers, *Plasmodium* resistance
Plasmodium vivax, 9
diagnostics
latent parasites and tafenoquine, 298–299
molecular markers, plasmodium resistance, 268–270
prevention, 286–287
Pregnant and breastfeeding women, malarial prevention, 288–289
Prevention, malaria
adverse events risk, 283–284

Prevention (*cont.*)
chemoprophylaxis, 282
children, 290
long-term visitors and frequent visitors
doxycycline, 285
guidelines, 286
mefloquine, 285
mosquito bite prophylaxis, 285–286
low risk, malaria infection, 282–283
pregnant and breastfeeding women, 288–289
P.vivax and *P.ovale*, 286–287
short-term visitors, 284
standby emergency treatment, 284
Primaquine
8-aminoquinolines
antirelapse therapy and radical cure, 71–72
chemistry, 70
chemoprophylaxis, 71
clinical use, 70–71
drug combinations, 72–73
mechanism of action, 74–75
pharmacokinetics and metabolism, 75–76
resistance, 77
safety and tolerability, 76–77
transmission blocking, 73–74
Plasmodium vivax, 9
prophylaxis, *P. vivax* and *P. ovale*, 287
Proguanil. *See* Antifolates
Pyrimethamine. *See* Antifolates
Pyronaridine
artesunate, 231
pharmacokinetics, 101
resistance, 101
structure and action, 100–101

Q
Qinghao, 158, 159, 214
Quinine
combination therapy, 214
drug failure, 50–51
extraction, 46–48
history of, 45–46
mechanism of action, 49–50
molecular-resistance mechanisms, 54–57
pharmacokinetics, 59–60
resistance
avian malaria *P. relictum*, 54
chinin, 52
IC_{50} values, 53
prophylaxis, 51
in *P. vivax* and *P. malariae*, 53
race, malaria parasite, 51
in severe malaria, 60
structure activity, 50
synthesis, 48
toxicity, 58–59
Quinoline-based drugs
P.falciparum chloroquine resistance transporter, 250–255
P.falciparum multidrug resistance protein 1, 255–258
P.falciparum Na^+/H^+ exchanger 1, 259–260
transporters, 259

R
Rapid diagnostic test (RDT), 299–300

S
Sarco/endoplasmic reticulum membrane calcium ATPase (SERCA), 165
Second-generation peroxides
artemisinin derivatives, 191–192
artemisone
accumulation and co-factors, 198–199
drug–drug interaction, 193–194
efficacy of, 195
Fe(II) interaction and peroxide bond, 196–198
preclinical studies, 195
primary targets, 198
1,2,4-trioxolanes (ozonides)
OZ209, OZ277 and OZ339, 200–205
second generation of, 205–207
Standby emergency treatment (SBET), 284
Sulphadoxine. *See* Antifolates

T
Tafenoquine, 8-aminoquinolines
antirelapse therapy and radical cure, 85–87
chemistry, 79
development, 77–79
mechanism of action and resistance development, 80
P. falciparum vs. P. vivax, chemoprophylaxis, 82–84
pharmacokinetics and metabolism, 80–81
safety and tolerability, 81–82

1,2,4-trioxolanes (ozonides)
advantage, 200
antimalarial efficacy, 202
comparative data, 203
integrated pharmacophores, 205
metabolic stability, 202
microsomal metabolites, 201
OZ209, OZ277 and OZ339, 200–205
second generation of, 205–207
stereochemistry, 204, 205
structures, 204
synthesis, 200

U
Ubiquitin-specific protease–1 (UBP–1), 263

W
World Health Organization (WHO), 228

X
Xenopus laevis, 262