BMCL Digest

Recent advances in malaria drug discovery

Marco A. Biamonte a,*, Jutta Wanner b, Karine G. Le Roch c

a Drug Discovery for Tropical Diseases, Suite 230, San Diego, CA 92121, USA
b Hoffmann-La Roche Inc., pRED, Pharma Research & Early Development, Small Molecules Research, Discovery Chemistry, 340 Kingsland Street, Nutley, NJ 07110, USA
c Institute for Integrative Genome Biology, Center for Disease Vector Research, Department of Cell Biology and Neuroscience, University of California at Riverside, CA 92521, USA

A R T I C L E   I N F O

Article history:
Received 15 January 2013
Revised 11 March 2013
Accepted 20 March 2013
Available online 27 March 2013

Keywords:
Malaria
Antimalarial
Plasmodium
Drugs
Review

A B S T R A C T

This digest covers some of the most relevant progress in malaria drug discovery published between 2010 and 2012. There is an urgent need to develop new antimalarial drugs. Such drugs can target the blood stage of the disease to alleviate the symptoms, the liver stage to prevent relapses, and the transmission stage to protect other humans. The pipeline for the blood stage is becoming robust, but this should not be a source of complacency, as the current therapies set a high standard. Drug discovery efforts directed towards the liver and transmission stages are in their infancy but are receiving increasing attention as targeting these stages could be instrumental in eradicating malaria.

© 2013 Elsevier Ltd. Open access under CC BY-NC-ND license.

Malaria remains one of the most prevalent and deadly infectious diseases across Africa, Asia, and the Americas. The World Health Organization (WHO) estimates 154–289 million malaria cases in 2010, with 660,000 associated deaths. An independent study suggests that the mortality is twice as high when including cases of malaria that are undiagnosed or untreated. Eighty percent of the estimated cases occur in sub-Saharan Africa and 86% of deaths occur in children less than 5 years of age. In Africa, the economic burden is estimated at $12 billion/year, but the sales of antimalarial drugs are orders of magnitude lower. Advances in malaria research are often reviewed and a recent monograph will prove useful to the medicinal chemist. This digest covers some of the most relevant progress in malaria drug discovery from 2010 to 2012, and limits itself to compounds with EC values <100 nM in a parasite proliferation assay. We cover the blood stage, the liver stage, and the transmission stage of the disease. Each section contains several scaffolds, and within each scaffold the compounds are arranged, when possible, with the marketed drugs first and the research compounds last.

Several species of Plasmodium cause malaria in humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and the simian Plasmodium knowlesi. The most lethal species is P. falciparum, found predominantly in Africa. If left untreated, P. falciparum causes organ failures (severe malaria) and accumulates in the brain capillaries (cerebral malaria), leading to coma and eventually death. Furthermore, there is growing evidence that the lethality of P. vivax has been underestimated.

The parasite has a complex life cycle and in order to eradicate the disease, every stage should be considered for treatment (Scheme 1):

1. Liver stage. Once the mosquito inoculates the parasites (sporozoites) into the bloodstream, the parasites invade the liver within 30 min and start replicating there (schizonts). In addition, P. vivax and P. ovale can remain dormant in the liver (hypnozoites, not shown in Scheme 1) and cause relapses years after the initial infection. Drugs that target the liver stages are important to prevent the disease from developing (prophylactic treatment) and to provide what is known as a “radical cure” for P. vivax and P. ovale.

2. Blood stage. After approximately 5–10 days, the liver cells burst and merozoites invade the red blood cells where they rapidly proliferate, causing the symptomatic high fevers and the pathology. In their intraerythrocytic phase, the merozoites go through various forms (rings, trophozoites, schizonts) to form an average of 20 daughter merozoites that are released into the bloodstream and infect new red blood cells. Drugs that target the blood stages are important to control the symptoms of the disease and associated mortality.

3. Transmission stage. After several cycles of asexual reproduction, some parasites further differentiate into male and female gametocytes, which contain only a half set of chromosomes.

4. Mosquito stage. When ingested by mosquitoes, the male and female gametocytes fuse in the midgut to form a zygote that further develops into new sporozoites ready for the next human host.
are important to prevent the infection of other humans, and would benefit an eradication agenda.

Artemisinin-based combination therapies (ACTs) are the current standard of care for uncomplicated malaria. Artemisinin (1, Scheme 2) and its derivatives (2–4) have a fast onset of action but are cleared rapidly (human $t_{1/2} \approx 1$ h), and are therefore combined with slow-clearing drugs to kill residual parasites. Typical partner drugs include lumefantrine (5, human $t_{1/2} = 3–4$ days) and piperaquine (6, human $t_{1/2} = 8–16$ days). The most popular combination consists of tablets containing artemether (3, 20 mg) and lumefantrine (5, 120 mg) sold as Coartem™ (Novartis). Adults take four tablets twice a day for 3 days, but compliance to this six-dose regimen is variable. In 2011, the European Medicines Agency (EMA) approved the combination of dihydroartemisinin (2) and piperaquine (6) which is taken once a day for 3 days (Eurartesim™, Sigma-Tau). The ACTs have supplanted the previously recommended sulfadoxine–pyrimethamine (7/8, Fansidar™, Roche), which in turn replaced chloroquine (9). Parenteral artesunate (4) is the drug of choice for severe malaria.

For the liver stages, primaquine (10, Scheme 3) is the only drug approved to eliminate hypnozoites. As for prophylactic treatment, atovaquone–proguanil (11/12) (Malarone, GlaxoSmithKline) is usually preferred because it is well tolerated, but is expensive. Incidentally, proguanil is a pro-drug, of which cycloguanil (13) is the active metabolite. For the transmission stages, primaquine (10) is the only registered drug active against the mature gametocyte. Resistance against the many existing antimalarials is well documented, and especially troubling is the emerging resistance to artesminins. Combining drugs can limit the emergence of resistance, but this technique is not infallible. For instance, in parts of Cambodia, the proportion of patients who were still parasitemic after 3 days of treatment with the dihydroartemisinin–piperaquine combination increased from 26% in 2008 to 45% in 2010. The problem of drug resistance requires new drugs. The challenge is that drug resistance is not the only feature. New, innovative drugs should also (i) be fast acting, (ii) be safe for children and pregnant women, and (iii) ideally be amenable to a single-dose administration. An example of how difficult it is to combine all these features is seen in melfloquine. It is the only registered drug effective in a single dose (14, Scheme 4, human $t_{1/2} = 2–4$ weeks, adult dose = 1250 mg), however, drug resistance is problematic. Similarly, the only marketed antimalarial drug combination effective as a single dose is sulfadoxine–pyrimethamine, but it also suffers from drug resistance.

Artemisinin is commercially produced by extraction from sweet wormwood (Artemisia annua) at a cost of $400–1100/kg. A recent alternative production method involves a yeast fermentation process that delivers the biosynthetic precursor artemisinic acid (15, Scheme 5, Amyris). The latter is converted to artemisinin in 62% yield using a photochemical oxidation process being implemented by Sanofi. An independent group adapted the process to a continuous flow reactor, better suited for conducting photochemistry at an industrial scale, thus potentially reducing production costs.

In addition, Zhu and Cook published a remarkably concise synthesis of (+)-artemisinin, where cyclohexenone is converted in only
five pots to the desired product, thus challenging the paradigm that total synthesis is not amenable to an industrially viable process.

The combination of an artemisinin derivative with slow-clearing drugs can cure malaria in a single dose. The combination of artemisinin (1000 mg) and naphthoquine (17, Scheme 6, 400 mg) introduced by the Chinese army, appears to be effective as a single dose of eight tablets (ARCO, Phase III). Artemisone (18), a drug in Phase II trials, is 10 times more potent than artesunate (4) in vitro and 4–10 times more potent in mice. It provides a single-dose cure in Aotus monkeys infected with *P. falciparum* at 10 mg/kg when combined with mefloquine (5 mg/kg). Artemisone is also active in a murine model of cerebral malaria. Combining artemisinin derivatives 19 or 20 (6 or 30 mg/kg, respectively) with the longer-acting mefloquine hydrochloride (18 mg/kg) was found to be curative in a single dose.

The antimalarial action of artemisinin is thought to involve the cleavage of the peroxide bond by Fe(II) found in heme proteins, thus generating toxic oxygen radicals. Synthetic peroxides are proving to be useful substitues for artemisinin. The first-generation peroxide OZ439 (22, Scheme 7) is now in Phase IIa trials. It features an 8'-aryl group. This seemingly inconspicuous modification has far-reaching consequences. The stability of the O–O bond towards Fe(II) increases by 50-fold, presumably because of steric reasons. This in turn translates into a much longer half-life in both rats (1/2 = 20 h for OZ439 vs. 1 h for OZ277) and humans (1/2 = 25–30 h for OZ439). The improved pharmacokinetic profile renders OZ439 capable of completely curing mice of malaria in a single dose of 20 mg/kg, a feat that artemisinin derivatives cannot achieve without the addition of a second drug. Furthermore, OZ439 has significant prophylactic activity, and a single 30 mg/kg oral dose of OZ439 administered 48 h prior to parasite inoculation is protective. In rat, multiple doses of OZ439 are tolerated up to 300 mg/kg. These oxonides are synthesized from an oxime and a ketone in the presence of ozone.
exerts its antimalarial action by interfering with the formation of hemozoin within the parasite's digestive vacuole. Hemozoin is a crystalline derivative of heme that the parasite makes as a way of disposing of toxic heme released upon hemoglobin digestion. Resistance to chloroquine is now found in all areas of the world, and involves multiple mutations in the \( P. falciparum \) chloroquine resistance transporter, PfCRT. These mutations result in an increased efflux of chloroquine from the acidic digestive vacuole to the cytosol of the parasite. Ferroquine (33, Scheme 10) was found to be active against chloroquine-resistant strains, and is currently undergoing Phase II clinical trials. Ferroquine, unlike chloroquine, accumulates in the digestive vacuole of the chloroquine-resistant parasites. This was demonstrated by X-ray fluorescence microscopy, a technique that allows visualizing the chlorine atom of both drugs.\(^77\) It was also shown that replacing the expensive ferrocene moiety of ferroquine with a simple and inexpensive benzene ring as in 34 and 35 retains activity against chloroquine-resistant strains (K1, W2).\(^78\) Because of its basicity, 35 is expected to accumulate like other 4-aminoquinolines in the acidic (pH = 5) environment of the food vacuole. In this organelle, the concentration of 35 is calculated to reach 370 \( \mu \)M at pharmacologically relevant doses, thus enabling PfCRT inhibition (IC\(_{50} = 69 \mu M\)) to become an operational second mode of action.\(^79\) The fused 'dimeric quinoline' 36 is active in vitro against drug-resistant strains, and in mouse when administered orally at 80 mg/kg.\(^80\)

Amodiaquine (37, Scheme 11) is also active against most chloroquine-resistant strains; however, hepatitis, myelotoxicity and agranulocytosis restrict its use to treating acute malaria. Amodiaquine is rapidly absorbed after oral administration in human, and rapidly metabolized, mostly, via N-deethylation. In addition, two reactive metabolites are formed, namely imine 38 and aldehyde 39, and are the likely cause of the hepatotoxicity and agranulocytosis, respectively.

\( N \)-tert-Butyl isouquinoline (GSK369796, 40, Scheme 11) was designed to avoid the formation of quinone imines, and entered Phase I studies. It is potent in vitro, including in the chloroquine-resistant strain K1 (IC\(_{50} = 13 \mu M\)) and is active in vivo with an ED\(_{50} = 3.8 \)mg/kg/day, thus being comparable to amodiaquine.\(^38,81,82\) In spite of the excellent exposures and near quantitative oral bioavailabilities in animal models, its development was discontinued due to exposures insufficient to demonstrate drug safety superior to chloroquine.\(^17\)

Analog exemplified by 41 (Scheme 11) were also designed to prevent the formation of quinone imines, and were found to retain activity against chloroquine-resistant strains.\(^9\)

Mefloquine (14, Scheme 12) has been widely used for malaria prevention due to a long half-life (2–4 weeks in human) that necessitates only a once-weekly dosing. Mefloquine is sold as a racemate (Lariam), and causes a relatively high incidence of depression, psychosis, and nightmares. While both enantiomers
are active, the (−)-enantiomer 42 is believed to cause the neurological side effects by binding the adenosine receptors in the brain.84 In an effort to select the next generation of quinoline methanol derivatives that could serve as a replacement for mefloquine, the Walter Reed Army Institute of Research screened for analogs with a lower brain penetration, and identified WR621308 (44).85,86 WR621308 has a substantially lower permeability across MDCK cell monolayers than mefloquine, suggesting lower brain exposures.

Some of the most important malaria drugs, cycloguanil (13) and pyrimethamine (8), are inhibitors of dihydrofolate reductase (DHFR). DHFR converts 7,8-dihydrofolate (45, Scheme 13) into tetrahydrofolate (46), a cofactor involved in one-carbon transfer reactions and in the biosynthesis of nucleic acids. Inhibition of DHFR therefore arrests DNA replication,87 but resistance is widespread due to mutations in the enzyme.

Structure-based drug design resulted in P218 (48, Scheme 13), a DHFR inhibitor active against all clinically relevant mutations.88 P218 combines the pyrimidine ring of pyrimethamine (8, Scheme 13), which brings potency, and the linker of the DHFR inhibitor WR99210 (47, Scheme 13), which tolerates mutations due to its flexibility. Furthermore, the terminal carboxylate group forms a salt bridge to the conserved Arg122 residue, thus mimicking the α-carboxylate of dihydrofolate (45). P218 is more potent than pyrimethamine against DHFR in the wild-type strain TM4 (IC50 = 4.6 and 58 nM, respectively) as well as in the quadruple mutant strain V1/S (IC50 = 56 and >100,000 nM, respectively). P218 is active against quadruple mutant P. falciparum in mice, with an ED50 = 0.3 mg/kg/day, orally. In rat, the oral bioavailability is 46%, and the oral t1/2 is 7.3 h.88

About 125 million pregnancies are at risk of malaria every year, and 10,000 women and 200,000 babies die as a result. An intermittent preventative treatment (IPT) is recommended for pregnant women, but drug-resistance to the currently adopted IPT (sulfadoxine–pyrimethamine) necessitates new and effective regimens. Both azithromycin (49, Scheme 14) and chloroquine have demonstrated safety in children and pregnant women over a number of years. Notably, the azithromycin–chloroquine combination is designed to be synergistic against chloroquine-resistant strains of P. falciparum,89 and was shown to be synergistic in the treatment of symptomatic malaria in clinical trials.88 By itself, azithromycin is
a slow-acting antimalarial, with a maximum antiparasitic effect occurring only after two cycles of intraerythrocytic development (one cycle of invasion, development, and egress lasts 42–48 h). Finding azithromycin analogs with improved activity in mouse models of malaria has been challenging.

The Novartis team screened its compound collection, identified spiroindolones as a novel chemotype, and optimized the series to deliver NITD-609 (Scheme 15), now in Phase II trials. The target was identified by genomics using clones with decreased susceptibility and was found to be the cation channel PfATPase4. In the medicinal chemistry route, the two enantiomers were separated by chiral chromatography. NITD-609 has an excellent potency, with EC50 = 0.7 nM (3D7) and is 100% orally bioavailable in mouse and rat. Its oral t1/2 is 10 h (mouse) and 27 h (rat). In mouse, NITD-609 has an ED50 = 1.2 mg/kg, and is thus more potent than artemesunate (ED50 = 6.2 mg/kg) or chloroquine (ED50 = 1.9 mg/kg). Three daily doses of 50 mg/kg, or a single dose of 100 mg/kg, afforded a complete cure. NITD-609 is also a potent inhibitor of gametocytes and blocks transmission to mosquitoes. The Medicines for Malaria Ventures (MMV) selected the spiroindolone project as the Project of the Year 2009.

Albitiazolium (Scheme 16, also known as T3 or SAR97276) is a drug that has reached Phase II clinical trials (CNRS/University of Montpellier/Sanofi). The understanding of its mechanism has recently been refined. Albitiazolium acts primarily by inhibiting the transport of choline into the parasite. The parasite requires choline to generate phosphatidylcholine, the main lipid of its cell membranes, as it replicates and forms new membranes. An important property of albitiazolium is that it accumulates irreversibly in the Plasmodium up to 1000-fold. Albitiazolium inhibits parasite growth (EC50 = 2 nM) and cures mice with an ED50 = 0.2 mg/kg/day (ip) without recrudescence. It is also active in severe conditions (ED50 ≤ 0.5 mg/kg ip at 5–10% parasitemia). Notably, a single injection is curative (ED50 = 1 mg/kg ip at 0.5–1% parasitemia). A single-dose cure is observed even at high parasitemia levels (ED50 = 2.5 mg/kg ip at 5–10% parasitemia).

Albitiazolium is also efficacious when given orally, but with a much lower ED50 = 13 mg/kg/day, suggesting an oral bioavailability of the order of 2% (mouse). Because of its low oral bioavail-
ability, the clinical trials have been conducted with intravenous or intramuscular injections. An oral version of albitiazolium would be highly desirable. The pro-drug TE3, reported in 2004–2005, has an oral ED50 = 5 mg/kg/day.108 This indicates a 2–3-fold improvement in mouse oral bioavailability; the rat bioavailability is 15%.100 In spite of numerous efforts made in the last few years,37,102–105 the bioavailability of these bis-cations has not been improved yet.

Unlike its human host, P. falciparum cannot salvage pyrimidines, and therefore depends on their de novo biosynthesis. Dihydroorotate dehydrogenase (DHODH) is the enzyme which catalyzes the rate-limiting step of the de novo pyrimidine biosynthetic pathway (56–59, Scheme 17). Factors that affect the human versus Plasmodium DHODH selectivity have been investigated by crystallography.106 The DHODH approach was awarded the MMV Project of the Year 2010.

A project coordinated by the MMV and involving the University of Texas Southwestern, the University of Washington, Monash University, and GlaxoSmithKline reported the preclinical candidate DSM265 (60, Scheme 17), expected to enter Phase I studies in 2013.107 DSM265 inhibits PfDHODH selectively over its human counterpart (IC50 = 33 nM and 2500 nM, respectively). It is orally bioavailable (rat: F = 57–68%, t1/2 = 12–28 h), and efficacious in vitro (EC50 = 43 nM (3D7)) and in mouse (ED50 = 2.8 mg/kg/day). A related effort identified triazolopyrimidine DSM190 (61, Scheme 17), which is less potent in vitro (IC50 = 190 nM, EC50 = 1.1 µM (3D7)) and in mouse (ED50 = 10 mg/kg/day), but has a bioavailability of 100% in rat.36

Genzyme reported DHODH inhibitor 62 (Scheme 17) as a potential drug development candidate.108 Benzimidazole 62 inhibits PfDHODH (IC50 = 40 nM) and parasite growth (EC50 = 7–10 nM, 3D7, Dd2). It is bioavailable in rat (49%) and dog (19%) and is eliminated with t1/2 = 0.85 h (rat) or 0.52 h (dog). In mouse, it is efficacious with an ED50 of 13 mg/kg/day.108

Most efforts use high-throughput phenotypic screens against the blood stage. GlaxoSmithKline,109 Novartis,110 and the St. Jude Children’s Research Hospital111 performed such screens and made 20,000 hits publically available. The MMV narrowed the list down to 400 compounds representing a diverse dataset with low toxicity. The resulting “Malaria Box” can be downloaded, and is available in plates from the MMV. The targets are not known and will require deconvolution.112,113 Additional screens are reported below and a recent review of the patent literature is also available.114

Screening 70,000 compounds from the Broad Institute and the Harvard Medical School against the chloroquine resistant strain Dd2 led to Genzyme’s Genz-668764 (63, Scheme 18, single enantiomer, absolute configuration not published).115 Genz-668764 inhibits P. falciparum in vitro (EC50 = 28 and 65 nM, 3D7 and Dd2 strains, respectively) and is active in mouse at doses of the order of 100 mg/kg/day. Allometric scaling predicts a human dose of 6 mg/day qd for 3 days, which would maintain plasma trough lev-
els above the EC$_{50}$ against *P. falciparum* for at least 96 h after the last dose. The predicted human therapeutic index is approximately 3, on the basis of the exposure in rats at the no observable adverse effect level (NOAEL).

A similar screen of the 8000 compounds from the Broad Institute's diversity-oriented synthesis (DOS) library led to the discovery of the extremely potent ML238 (64, Scheme 18, EC$_{50}$ = 0.5 nM (Dd2)), which is also highly water soluble (120 μM) and not cytotoxic.116

Actelion reported ACT-213615 (65, Scheme 19).117,118 The compound is fast-acting against all asexual erythrocytic stages. Although 30 times less potent against the murine *Plasmodium berghei* than against *P. falciparum*, ACT-213615 completely cured *P. berghei*-infected mice with three consecutive oral daily doses of 750 mg/kg (ED$_{90}$ = 54 mg/kg/day). ACT-213615 was efficacious in the recently established SCID mouse *P. falciparum* model (ED$_{90}$ = 8.4 mg/kg/day) with potencies comparable to chloroquine (ED$_{90}$ = 8.4 mg/kg/day) with potencies comparable to chloroquine (ED$_{90}$ = 6.4 mg/kg/day). No acute toxicity was observed.

Anacor119 identified benzoxaborole (66, Scheme 19, EC$_{50}$ = 26–44 nM) as a promising starting point (MW = 206, low clogP, solubility: 750 μg/mL at pH 7, and low to no cytotoxicity).120,121 SAR studies suggested that an acidic side-chain is favored for high anti-malarial potency, and a number of compounds with EC$_{50}$ values <100 nM were discovered, such as 67. Further optimization is ongoing.

The screening network funded by the WHO Special Programme for Research and Training in Tropical Diseases (TDR) reported the results of a 10,000 compound screen against seven whole organism pathogens responsible for tropical diseases, including the intra-erythrocytic forms of *P. falciparum*.122 The most potent screening hit was TDR84420 (68, Scheme 19) with an EC$_{50}$ = 326 nM (K1). The publicly available screening data set from GSK has been analyzed to extract SAR trends123 and GSK identified 47 high quality starting points for further follow-up.124 Further manual filtering led to the selection of five series. One of them was deprioritized due to resistance issues. TCMDC-134142 (69, Scheme 19) represents one of the remaining series.125

Amongst the targeted approaches, kinase inhibitors have been evaluated but so far are weak inhibitors (EC$_{50}$ >100 nM)8,126 and are therefore outside of the scope of this digest.

GSK reported a series of highly potent 2-pyrimidincarbonitriles as inhibitors of falcipain-2 and falcipain-3, such as 70 (Scheme 20, EC$_{50}$ = 1 nM).127 Falcipains are cysteine proteases that hydrolyze the host hemoglobin to provide amino acids for parasite protein synthesis.

Harmin (71, Scheme 20) was reported to inhibit the *Plasmodium* heat shock protein 90 (Hsp90) and some selectivity over the human Hsp90 was observed. Its cellular activity is EC$_{50}$ = 50 nM (3D7).128 Hsp90 inhibitors have traditionally been pursued for cancer, and were found to have a limited therapeutic window.129

Epigenetic factors regulate the progression of the malaria parasite through its complex life cycle, and malarial histone acetyltransferase (HAT)130 and histone deacetylase (HDAC)131,132 inhibitors have been reported. Recently, the targeting of *P. falciparum* epigenetic factors was extended to include inhibitors of histone methyltransferases, such as BIX-01294133 and TM2-115 (72/73, Scheme 20).134 In an acute infection mouse model (highly virulent *P. berghei* ANKA strain), the compounds (dosed ip, 40 mg/kg) showed a rapid onset and a 2- and 18-fold parasite reduction.

Febrifugine (74, Scheme 21) is the active component of the Chinese herb Chang Shan (Dichroa febrifuga), and is an appealing antimalarial because of its rapid effect and availability. However, strong liver toxicity has precluded its use as a clinical drug. Radix Pharmaceuticals discovered febrifugine analog 75 with a much improved therapeutic index in mouse (TI = 132 vs 3), thus exceeding the therapeutic index of artemisin (TI = 37).135 The therapeutic index was defined as the ratio between the maximum tolerated dose (MTD), and the minimum clearance dose (MCD). Compound 75 is effective in mouse (EC$_{50}$ = 0.3 mg/kg/day) and in *Aotus* monkeys (EC$_{50}$ = 2 mg/kg/day).135

Several additional active compounds were identified by various approaches (Scheme 22), including the potent marine natural product salinosporamide A (74136), SSJ-183 (75),137,138 prodiginine 74,139 tsitsikammamine C (77),140 the berberine analog 78,141 quin...
dolone, propafenone, sulfonamide, 2,4-diamine, and iThemba’s iridoid. Currently, most approved malaria drugs target only the blood stages of the disease. The two exceptions are the combination of atovaquone/proguanil which is also effective in clearing parasites from the liver, and primaquine. The latter clears not only liver schizonts but also hypnozoites, the dormant liver-stage parasites in P. vivax and P. ovale infections, thus providing what is known as a radical cure. Hypnozoites are long-lasting reservoirs responsible for recurring malaria episodes in the absence of mosquito bites, and are a major health concern, especially in the case of P. vivax.

The search for liver stage drugs has been severely hampered by the lack of culture techniques and by cumbersome primate animal models. It has been suggested that for prophylactic treatment, compounds without blood stage activity might be preferred in order to minimize the risk of the emergence of drug-resistant parasites.

Primaquine is a drug that acts slowly, and is therefore given together with other drugs, for example, chloroquine. Its mechanism of action is unclear, but is believed to be mediated by reactive metabolites which destroy the mitochondrial structure of the parasite. Primaquine, however, causes hemolytic anemia in people with glucose-6-phosphate dehydrogenase (G6PD) deficiencies, which occur in ~10% of the population, and are particularly prevalent in malaria endemic countries. In fact, the spatial extent of P. vivax malaria overlaps widely with that of G6PD deficiency. Additionally, compliance with the primaquine 14-day treatment regimen is difficult.

The primaquine analog tafenoquine is currently in Phase II/III clinical trials and has proven activity against hypnozoites. Tafenoquine has the same G6PD deficiency liability as primaquine, but has the advantage of being a single-dose treatment.

Recent drug discovery efforts have focused specifically on targeting the asymptomatic liver stage sporozoites and/or hypnozoites (possibly in addition to the blood stages) in order to provide novel, non-8-aminoquinoline drugs that are not affected by the G6PD liability. A new imaging technique of Plasmodium liver stages, described by The Scripps Research Institute and Novartis, constitutes a breakthrough, and was applied to prioritize 4000 compounds already possessing blood-stage activity. Imidazolopiperazines emerged as a hit series, exemplified by GNF-Pf-5069, which was then optimized to provide GNF179 and GNF156. The latter is currently in Phase I clinical trials.

Most data was initially reported for the nonclinical compound GNF179, which inhibits both the blood stages of P. falciparum (EC50 = 6 nM) and the liver stages of the murine Plasmodium yoelii. (EC50 = 5 nM). GNF179 is orally bioavailable in mouse (F = 58%, I50 = 8.9 h), and reduces P. berghei parasitemia in mice by 99.7% at 100 mg/kg. Importantly, a single dose of 15 mg/kg of
GNF179 was shown to be completely protective in mice challenged with *P. berghei* sporozoites.

GNF156 was recently shown to be equally as potent as GNF179 against *P. falciparum* (EC$_{50}$ = 6 nM (3D7)). The potency against *P. yoelii* liver stages has not been disclosed. GNF156 is orally bioavailable in mouse ($F = 72\%$, $t_{1/2} = 2.2$ h) and rat ($F = 20\%–57\%$, $t_{1/2} = 4.7–8.4$ h). It is noteworthy that GNF156 not only inhibits the liver stages, but also transmission.

It is not yet known whether these compounds are active against the hypnozoites of *P. vivax* and *P. ovale* and would thus be able to provide a radical cure. The mechanism of action of the imidazolopiperazines remains unknown.

Atovaquone (11) targets the electron transport chain (ECT) of the mitochondrial, and specifically the cytochrome $b_{c_1}$ complex. Additional quinones have been reported, without obvious advantage, and most progress was made with pyridones.

GSK reported a back-up to pyridone GW844520 (89, Scheme 25), a molecule targeting cytochrome $b_{c_1}$; hydroxymethyl derivative 90 remarkably improved the mouse oral bioavailability (50%) as compared to GW844520 (20%).

The paucity of compounds with anti-relapse properties led to the reinvestigation of the quinolone ICI 56,780 (91, Scheme 25). The latter was discovered in the 1960s and a 7-day regimen of 91 prevents relapses for 120 days in rhesus monkeys infected with the hypnozoites of *P. yoelii* against *P. falciparum* (EC$_{50}$ = 6 nM (3D7)). The potency against *P. berghei* liver stage (estimated EC$_{50}$ of 2.6 nM) as well as the asexual (EC$_{50}$ = 10 nM) and sexual (EC$_{50}$ = 36 nM) *P. falciparum* blood stages. The decoquinate-induced reduction of mosquito transmission has not been reported. Decoquinate (administered p.o., 10 mg/kg) was found to completely protect *P. berghei* infected mice from developing disease when treated 24 h after the infection. The molecular target is the parasitic cytochrome $b_{c_1}$ complex (IC$_{50}$ = 2 nM, >5000-fold selectivity over its human counterpart). Unfortunately, decoquinate has so far only been tested in animals, which makes the repurposing of this drug challenging.

The O’Neill group, at the University of Liverpool, searched for compounds that would inhibit another enzyme involved in the ECT, namely NADH:ubiquinone reductase (PNDH2). A succession of in silico screens, HTS, and medicinal chemistry activities led to CK-2-25 (95, Scheme 25), which is specific for PNDH2 over $b_{c_1}$, and is potent in mouse, with an ED$_{50}$ = 1.8 mg/kg.

**Scheme 24.** Imidazolopiperazines. EC$_{50}$ values are reported for the drug-sensitive *P. falciparum* strain 3D7, and the hepatic stages of the murine *P. yoelii*.

**Scheme 25.** Pyridones with activity against liver stages. EC$_{50}$ values are reported for the drug-sensitive strain 3D7 and multi-drug resistant strain W2.

**Plasmodium cynomolgi,** suggesting potency against hypnozoites. It was abandoned because resistance was obtained after only a single passage in *P. berghei* infected mice. The group of Manetsch at the University of South Florida, reported compound 92 with an EC$_{50}$ = 28 nM against the W2 strain, and of 31 nM against the atovaquone-resistant TM90-C2B strain (chloroquine-, mefloquine-, pyrimethamine-, and atovaquone-resistant). The Guy group at St. Jude Children’s Research Hospital, Memphis, reported compound 93 with an EC$_{50}$ = 83 nM, which has suppression activity in mice comparable to that of amodiaquine (57% suppression of parasitemia at 30 mg/kg) but is not curative. It remains to be investigated if activity against hypnozoites is maintained, and if resistance against 92 and 93 develops as fast as with ICI 56,780.

In a screen including 1037 compounds which have reached at least Phase I clinical trials (veterinary and/or human), decoquinate (94, Scheme 25), an approved veterinary drug, was identified to potentially inhibit the *P. yoelii* liver stage (estimated EC$_{50}$ of 2.6 nM) as well as the asexual (EC$_{50}$ = 10 nM) and sexual (EC$_{50}$ = 36 nM) *P. falciparum* blood stages. The decoquinate-induced reduction of mosquito transmission has not been reported. Decoquinate (administered p.o., 10 mg/kg) was found to completely protect *P. berghei* infected mice from developing disease when treated 24 h after the infection. The molecular target is the parasitic cytochrome $b_{c_1}$ complex (IC$_{50}$ = 2 nM, >5000-fold selectivity over its human counterpart). Unfortunately, decoquinate has so far only been tested in animals, which makes the repurposing of this drug challenging.

The O’Neill group, at the University of Liverpool, searched for compounds that would inhibit another enzyme involved in the ECT, namely NADH:ubiquinone reductase (PNDH2). A succession of in silico screens, HTS, and medicinal chemistry activities led to CK-2-25 (95, Scheme 25), which is specific for PNDH2 over $b_{c_1}$, and is potent in mouse, with an ED$_{50}$ = 1.8 mg/kg.
The first screening examples have been reported, such as the imidazolopiperazines 86–88 described above. Additionally, a set of ~5300 biologically active compounds, which included 640 FDA-approved drugs, was screened and 37 structurally diverse compounds with varied known biological functions were identified to also inhibit the malarial liver stages. Screening a natural product library highlighted the secondary fungal metabolite cladosporin (96, Scheme 26), which potently inhibits the blood and liver stages in drug sensitive and multiple drug-resistant cell lines (IC50 ~40–90 nM). The molecular target was identified to be cyto- 

plasmic lysyl-tRNA synthetase. (PKrs1) Cladosporin is selective over human Krs1 (IC50 >20 μM).

In addition, there is a growing interest in signal peptide peptides (SPP) such as NITD-731 (97, Scheme 26) which inhibits P. yoelii liver stages with EC50 = 7.8 nM. Since many different proteins are expressed during the liver and blood stages of the parasite’s life cycle and since the necessary medium- to high-throughput liver stages assays are continuously being developed and refined including assays which allow the assessment of hypnozoitocidal activities,171–174 it is reasonable to believe that in the near future many new chemotypes and biological targets will emerge from these efforts.

Drugs that can reduce the formation of gametocytes (gametocytogenesis), or can kill them (gametocytocides), are highly desirable but have been underexplored because of the lack of quantitative high throughput assays. These transmission-blocking drugs could target endpoints such as:

1. The effective and complete killing of mature gametocytes once they are formed in the human host.
2. The inhibition of the onward development of gametocytes into ookinetes and ultimately into sporozoites in the mosquito. This assumes that enough drug from the blood sample reaches the gut of the mosquito.

Gametocyte development goes through five stages of maturation, with stage V being the only form which can infect mosquitoes (Scheme 1). For P. falciparum, these mature gametocytes start to be present ~12 days after disease symptoms, circulate on average for 2.5–6.5 days, and persist for up to 22 days. Thus, circulating gametocytes can sustain malaria transmission well after drug treatment has caused disease symptoms to disappear. For the other Plasmodium parasites, mature gametocytes appear much earlier, closer to 1 day after disease symptoms appear. This difference in biology represents an additional challenge when optimizing dosing regimens of transmission-blocking drugs in the clinic.

Most currently approved anti-malarial drugs, including ACTs, are only effective against blood stages and young gametocytes up to stage III and possibly stage IV of gametocyte maturation (stage III can be observed at day 4–6 and IV is observed at day 7–9; Scheme 1). This unfortunately does not result in complete clearance of mature gametocytes. To make matters worse, some drug treatments, for example, chloroquine and sulfadoxine-pyrimethamine, were found to induce gametocytogenesis, thus potentially contributing to increased numbers of transmissions and increased rates of new infections.

The transmission-blocking potential of approved and clinical antimalarials has been reviewed. Currently, the only fully effective gametocytocidal drug is primaquine (10, Scheme 3), which acts against gametocytes of all malaria species and represents the WHO recommended treatment option against P. falciparum gametocytes. Until recently, the WHO recommendation was a single primaquine dose of 0.75 mg/kg, provided that the risk for acute hemolytic anemia (G6PD deficiency) had been evaluated prior to treatment. In 2012, the WHO recommended that the dose be lowered to 0.25 mg/kg, which is still effective at lowering transmission while being unlikely to cause serious toxicity in subjects with G6PD variants. Thus, a single dose of primaquine (0.25 mg base/kg) should be given to all patients with parasitologically-confirmed P. falciparum malaria on the first day of treatment in addition to an ACT, except for pregnant women and infants <1 year of age.

Because of the risks associated with primaquine, novel transmission-blocking drugs are being sought for achieving the goal of global malaria eradication. Large strides have recently been made in understanding the specifics of transmission-stage biology, and in developing in vitro assays focused on late-stage gametocyte development, lethality of mature gametocytes and the gamocyte–ookinete/sporozoite transition.

Tafenoquine (84, NITD609 (52), and GNF156 (88) were shown to have transmission-blocking activities in vitro. Tafeno-quine was also found to delay sporozoite formation in P. vivax. Interestingly, a recently developed gametocyte drug screening assay identified methylene blue (97, Scheme 27), as a potent inhibitor of gametocyte development across all stages. Methylene blue is an approved Injectable monoamine oxidase inhibitor for methemoglobinemia, which almost fully abolishes P. falciparum transmission to mosquitoes at concentrations readily achievable in humans, highlighting the potential of this chemical class to reduce the spread of malaria. Incidentally, methylene blue was the first antimalarial to be tested in man (1891), based on Ehrlich’s observation that it could stain the malaria parasite.

Additional examples of compounds with transmission-blocking activities include, amongst others, trioxyquin DU1302 (98, Scheme 27), epoxomicin (99, nonselective over human cells), HIV protease inhibitors such as tipranavir (100), kinase inhibitor BKI1 (101), ketotifen (102), thioestrepton (103), and cycloheximide (104).

Anti-malarial strategies are ideally a balanced use of mosquito control, anti-Plasmodium treatments, and a general improvement of sanitation and awareness. This is how malaria was eradicated from developed countries. Vaccines would also be extremely useful. Nonetheless, there is an urgent need for developing new anti-malarial drugs. The new drugs can target the blood stage of the disease to alleviate the symptoms, the liver stage to prevent relapses, and the transmission stage to protect other humans.

The pipeline for the blood stage is arguably the best in history, but still needs to be expanded. The last few years have seen an explosion of potent new chemotypes, and the new challenge is to assess the potential of these chemotypes. Ideally, the new drug should: (i) address drug-resistance issues, (ii) have a rapid onset of action, (iii) be safe, especially in children and pregnant women, and (iv) cure malaria in a single dose. The challenge is to find a drug that addresses all of these features. It is our hope that with the rich variety of new chemical entities, such a drug will be discovered. Nevertheless, drug discovery efforts should continue, as the artemisinins set a high standard of efficacy and safety.
Drugs that target the liver and transmission stages have the potential to be transformational, but research efforts have been hampered by the absence of high-throughput screens. New imaging techniques are beginning to solve this problem and open up novel avenues, with an innovative clinical compound having liver stage activity. The field of transmission-blocking agents is in its infancy, but may be the most transformative of all in achieving the ultimate goal of eradicating malaria.

Acknowledgements

KLR is supported by the National Institutes of Health (Grant R01 AI85077-01A1). The authors thank S. Cervantes for generating Scheme 1.

References and notes
