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Targeting the Liver Stage of Malaria Parasites: A Yet Unmet Goal

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INTRODUCTION: AN OVERVIEW OF LIVER-STAGE MALARIA

Malaria remains one of the world's most prevalent tropical diseases because of its high mortality and morbidity burden, as well as its economic and social impacts on the development of malaria-endemic countries. The emergence and spread of chloroquine-resistant *Plasmodium falciparum*, the causative agent of the most lethal form of human malaria, are major obstacles in the control of the disease.[1,2] More recently, concern has been raised as to whether there is evidence of resistance to artemisinins, which could endanger the artemisinin combination therapies (ACTs) now widely adopted. Although continued attempts to develop a vaccine for malaria are ongoing, drugs continue to be the only treatment option,.[3,4] The most currently used antimalarials are potent blood schizontocidals; i.e., they act rapidly against the parasite forms that invade erythrocytes and cause the well-described malaria symptoms.[2] However, before the onset of clinical symptoms, the clinically silent pre-erythrocytic life cycle stages, transmitted by *Anopheles* mosquitoes, invade and develop in the liver (Figure 1). The sporogonic stage develops within a parasite oocyst in the mosquito midgut from which sporozoites are released and invade the salivary glands. Sporozoites deposited in the mammalian host's circulation during the mosquito's blood meal reach the liver and infect hepatocytes. *Plasmodium* liver stages (LS) grow and undergo nuclear replication within a parasitophorous vacuole, culminating in the release of tens of thousands of merozoites into the bloodstream. This intrahepatic developmental process takes approximately 2 days in the case of rodent *Plasmodium* parasites and 7−14 days for human *Plasmodium* spp., depending on parasite species.[5] Free merozoites rapidly adhere to and invade erythrocytes, replicate, and generate further infectious merozoites. Blood parasitemia then develops, leading to clinical symptoms.[6,7]

The LS of *Plasmodium* spp. obligatorily precedes blood stages and therefore represents a potential drug target.[5,8] Full inhibition of LS parasite development would lead to true causal prophylaxis.[1] Transmission would also be interrupted because it depends on gametocytes that mature in red blood cells, following completion of the liver stage of infection. Furthermore, the low number of hepatic forms substantially reduces the likelihood of emergence of drug-resistant parasites.[8] The difficulties in developing a drug with true causal prophylactic activity against malaria are mostly related to the biology of *Plasmodium* spp. LS and the inherent technical difficulties in studying them.[9] Recently, a proteome and transcriptome analysis revealed a set of proteins specific to the LS of malaria, including those of the highly active type II fatty acid synthesis (FAS-II) pathway. The redox metabolism, tricarboxylic acid cycle, electron transport system, and protein metabolism account for other over-represented pathways.[10] Also, infectiousness of sporozoites is variable among batches and in vivo studies are often carried out in mice models infected with *P. berghei* or *P. yoelii*.[8] This represents a major limitation, since the rodent malaria parasites do not form latent liver forms (hypnozoites) which are present in *P. vivax* and *P. ovale* and can cause relapses of malaria long after initial bloodstream infections have been cleared.

Most of the known LS inhibitors have resulted from traditional medicinal chemistry programs, which started over 70 years ago. However, those studies mostly explored 8-aminoquinolines, and thus, there is a relative lack of chemical diversity among potent inhibitors. More recently, efforts toward finding novel leads have also been supported by computational or natural product chemistry, as well as the discovery of a possible new application for known drugs/molecules.[11] Herein we provide an overview of inhibitors targeting the LS; special emphasis will be given to structure−activity relationships (SARs) and to recent developments in the field.

ASSESSMENT OF COMPOUND ACTIVITY AGAINST *PLASMODIUM* LIVER STAGES

The efficacy of a compound against *Plasmodium* LS can be assessed through in vitro or in vivo approaches. In vitro assessment of the antiplasmodial activity of compounds targeting the liver stage of *Plasmodium*'s life cycle is based on the measurement of parasite loads of cell lines or primary hepatocytes, infected in the presence of the desired compounds. Several parasite-hepatic cell combinations have been established as suitable models for *Plasmodium* infection. Most commonly, rodent-infective model *Plasmodium* species are employed to infect hepatoma cell lines or primary hepatocytes. In all cases, cells are infected with sporozoites obtained from freshly dissected salivary glands of *Anopheles* mosquitoes and infection load is determined at selected time points. In vivo, compounds are assayed by administration to animal models previously infected by injection of isolated sporozoites or by mosquito bite. Efficacy can be assessed by determining liver parasite loads or by monitoring the appearance of blood-stage parasites.

LS parasite quantification can be accomplished by a variety of established methods. Quantitative real-time polymerase chain reaction (qRT-PCR) is the gold standard method for measurement of hepatic parasite loads in infected cells in culture[12] or animal model livers.[13] Primers are used to amplify a *Plasmodium*-specific target cDNA and parasite copy numbers are normalized

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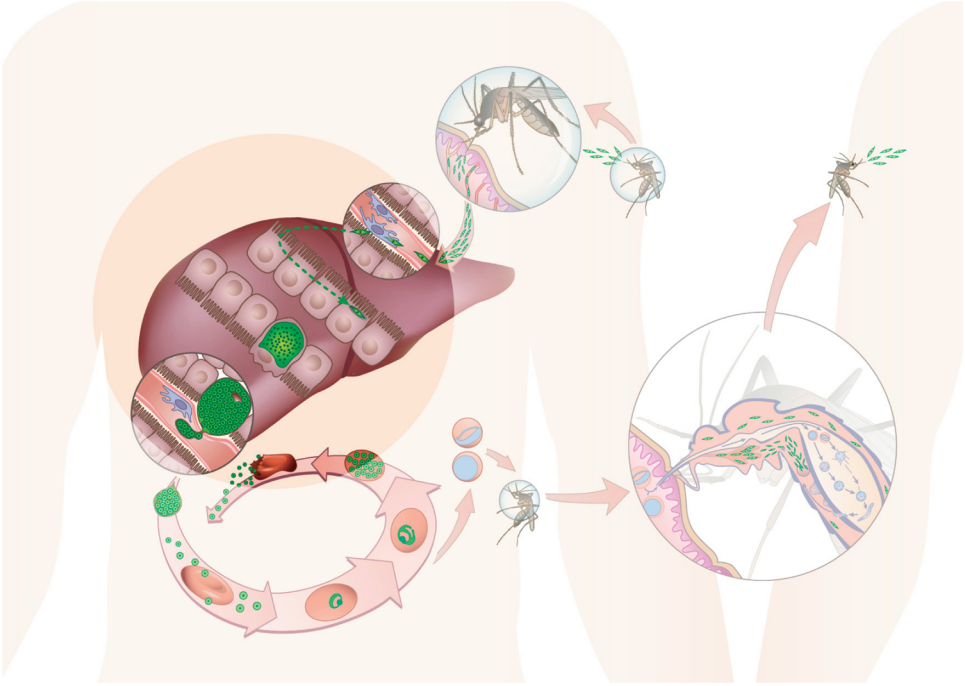

Figure 1. *Plasmodium* life cycle. When an infected female *Anopheles* mosquito bites a vertebrate host, sporozoites are deposited under the host skin. Once in the skin, sporozoites glide through dermis until they find a blood vessel and enter the circulatory system that allows reaching the liver. During the liver stage infection (highlighted), sporozoites migrate through several hepatocytes before invading a final one where each sporozoite generates thousands of merozoites. Merozoites are released into the bloodstream where they invade and multiply inside red blood cells. Some merozoites differentiate into female and male gametocytes that can be taken up by the mosquito during a posterior blood meal. Within the mosquito midgut, gametocytes develop into gametes and fertilize each other, forming ookinetes, a motile zygote. Ookinetes leave the mosquito midgut forming an oocyst in which sporozoites are formed. Once sporozoites are mature, they migrate to salivary glands and are able to infect another host during a subsequent mosquito bite.

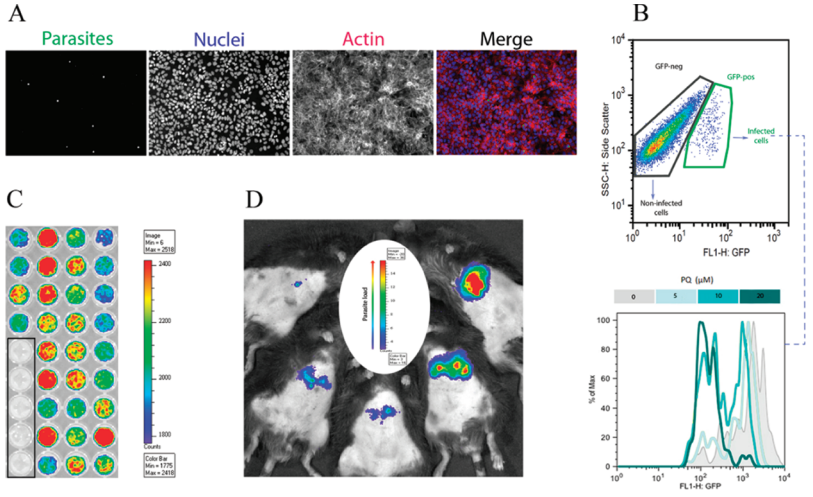

Figure 2. Quantification of parasite loads by different methods. (A) Immunoflurescence microscopy. The panels show, from left to right, acquisition channels for parasites, nuclei, actin, and a composite merged image of the three. (B) Flow cytometry. The top panel shows cells analyzed at 2 h after sporozoite addition. The percentage of GFP-positive cells at this time point indicates cell invasion rates. The bottom panel shows histograms of GFP intensity of GFP-positive cells analyzed 48 h after sporozoite addition in the presence of increasing amounts of primaquine. The geometric mean of GFP intensities for each cell population is a measure of the extent of parasite development. (C) Luminescence in vitro. Lysates of cells infected with different amounts of luciferase-expressing sporozoites following reaction with the luciferin substrate. Signal intensity corresponds to the parasite load in the cells. Wells indicated by the black box contain noninfected cells lysates. (D) Luminescence in vivo. Mice infected with different amounts of luciferase-expressing sporozoites imaged following luciferin injection 44 h after infection. The intensity of the bioluminescent signal is proportional to the liver parasite burdens.

against a housekeeping gene. Although this method is costly and time-consuming, it can provide very accurate results. Immunofluorescence microscopy techniques enable direct visualization of the parasites and have been widely used to assess infection, both in cultured cells[12,14−16] and in liver slices.[17] Infection quantifications are achieved by determining the number and the area of imaged parasite LS and normalizing the data against cell numbers or confluence (Figure 2A). The method is relatively

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time-consuming, although automated image acquisition and treatment protocols can be used, particularly in high-throughput approaches, to facilitate data collection and analysis.[12,16] Flow cytometry enables measuring infection in cell lines infected with fluorescent transgenic parasites.[18] Furthermore, this technique can be used to distinguish cell invasion from parasite development[19] (Figure 2B). Invasion rates can be measured by determining the percentage of fluorescence-positive cells at early time points after sporozoite addition.[12,16,19−21] At later time points, fluorescence intensity is an effective measure of parasite copy numbers in cells, i.e., development.[12,15,19−22] Fast sample acquisition enables using flow cytometry for medium-to-high throughput analyses. Finally, luminescence provides a valuable alternative for hepatic infection measurements.[23,24] Luciferase-expressing parasites can be used to assess infection both in vitro and in vivo. In vitro, addition of luciferase's substrate, luciferin, to cell lysates results in the emission of an amount of light that is proportional to the parasite load in the lysate[23] (Figure 2C). A dye such as AlamarBlue can be employed to assess cell confluency prior to lysis. The speed and simplicity of luciferase-based infection measurement make it well-suited for high-throughput studies where large numbers of samples need to be analyzed. In vivo, infection can be quantified by injecting luciferin into anesthetized, infected mice and imaging the bioluminescent signal emitted by the parasites in the liver[23,24] (Figure 2D).

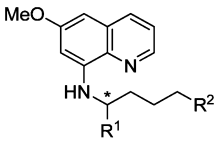
One important technical challenge faced when measuring infection is the low infectivity levels that can be obtained experimentally. In fact, in vitro invasion rates of cells by sporozoites are low, rarely above 4% and often below 1%. An increase in signal-to-noise ratio as a result of the parasite's intracellular multiplication facilitates detection and quantification of infection. Nevertheless, infection can be measured by a variety of established methods with high sensitivity. Microscopy and flow cytometry techniques, for example, enable quantification of invasion by single parasites.[19,25]

The array of methods outlined above provides several alternatives to assess *Plasmodium* LS infection. While each has advantages and drawbacks, they can all provide valuable information about compound efficacy and guide researchers in the development of novel, efficient compounds to act on this crucial stage of the parasite's life cycle.[26]

SMALL MOLECULES TARGETING LIVER-STAGE OF MALARIA

8-Aminoquinolines. In the 1930s, a series of experiments by Shute and co-workers with pamaquine, **1**, demonstrated that administration of 60 mg/day po (per os) of this compound acted as a true causal prophylactic for *P. falciparum* and *P. vivax* infections,[27] in contrast with later reports showing that pamaquine only delayed the development of the exoerythrocytic forms that cause relapses.[28,29] Methemoglobinemia was regularly found in treated patients, which hampered the clinical use of pamaquine.[28] On the other hand, Alving et al. showed in 1948 that pentaquine, **2**, had plasmodicidal activity identical to that of pamaquine at doses that produced only mild reactions to individuals. Also, it was 80- to 128-fold more potent than quinine, suppressing fever and clearing parasitemia in a maximum of 6 days when administered alone in a 60 mg/day oral regimen. This dosage of pentaquine, coadministrated with quinine in moderately infected individuals, resulted in radical cure in 97% of the cases, significantly higher than when individuals were only treated with suppressive drugs (33%).[30] Higher doses of pentaquine were required for severely infected individuals, but they also resulted in serious toxic effects, which did not warrant safety in prophylaxis or prolonged treatment of malaria.[30]

From 1947 until 1951, both the synthesis of new 8-aminoquinolines and their evaluation in infected human volunteers were guided by results of assessments of curative and prophylactic activities in monkeys either actively infected or recently challenged with sporozoites of *P. cynomolgi* and by appraisal of subacute toxicity in noninfected monkeys. This process led to the identification of primaquine (PQ), **3**. The exact mechanism by which PQ effectively eliminates *Plasmodium* hypnozoites and gametocytes is still unknown, but it is thought that the mitochondrial metabolism is impaired by interference with the electron transport chain.[31−33] Presently, PQ is still the only available active drug against both gametocytes, which are responsible for the transmission of parasites between human and mosquito, and all the exoerythrocytic forms of *Plasmodium*, including the latent hypnozoites of *P. ovale* and *P. vivax*. A total of 200 mg of PQ leads to radical cure, and the usually adopted regimen of 15 mg/day po over 14 days is generally well-tolerated.[34] However, the use of PQ has been curtailed by two factors: (i) low oral bioavailability due to rapid oxidative deamination to carboxyprimaquine;[35,36] (ii) hemolytic anemia after induction of methemoglobinemia, which is highest in individuals with deficiency in glucose 6-phosphate dehydrogenase.[34,37,38]



1: R^1= Me, R^2=NEt_2
2: R^1= H, R^2=$CH_2NHCHMe_2$
3: R^1= Me, R^2=NH_2

In the years following the discovery of PQ, hundreds of 8-aminoquinolines were assayed for their antimalarial activity in Rhesus monkeys and mouse models.[39,40] In 1983, Schmidt disclosed 34 compounds found to display activity equal to or higher than PQ, and several SARs were drawn. Like PQ, all the active compounds contained the 6-OMe substituent, 24 of them had an additional substitution on the quinoline ring, and 10 had two substitutions. Methyl substitutions at C2 or C4 had a favorable impact on activity and resulted in compounds that were less toxic than PQ. Alkoxy, fluoro, and meta- or para-substituted phenoxy groups at C5 also improved activity. Regarding the substituents at C8 most of the 34 compounds carried a branched alkyl side chain near the 8-amino group and had four or five carbons in length.[39] The structures of those compounds can be found in Table 1. From analysis of Table 1 it is also noteworthy that there is the apparent disconnection between the high potency in vivo of 8-aminoquinolines and their modest in vitro activity, e.g. ED_{50} of 0.38 mg/kg in vivo and IC_{50} of 2 μM in vitro for PQ.[21] Biotransformation of 8-aminoquinolines seems to be necessary for their toxicity as well as efficacy,[34] a reason why compounds from this class have consistently displayed higher activities in vivo rather than in vitro.

On the other hand, aromatic substituents at C2 generally decreased activity and branching at the C8 side chain had greater impact on activity for terminal secondary/tertiary amines. Further SAR analysis by Schmidt includes the detrimental effect on activity of hydroxyl groups and cyclic substituents, either aromatic or aliphatic, at the C8 side chain.[39] Moreover, Davidson et al. showed that 6- and 7-aminoquinolines were devoid of

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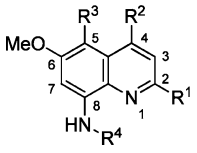
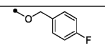
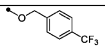
Table 1. Structures of 8-Aminoquinolines with Activity Equal or Superior to Primaquine in Monkeys[39]



Compd.	R^1	R^2	R^3	R^4	ED_{50} / mg•kg^{-1} body weight (95% confidence interval)
3	H	H	H		0.38 (0.33-0.43)
4	H	Me	H		0.11 (0.087-0.14)
5	H	Me	H		0.12 (0.099-0.14)
6	H	Me	H		0.074 (0.05-0.011)
7	H	Me	OMe		0.098 (0.088-0.11)
8	H	Me	OMe		0.12 (0.098-0-15)
9	H	Me			0.11
10	Me	H	H		0.21 (0.16-0.27)
11	H	Me	H		0.20 (0.17-0.24)
12	H	Me	H		0.23 (0.13-0.42)
13	H	H	OEt		0.18 (0.11-0.29)
14	H	H			0.23 (0.18-0.30)
15	H	H			0.25 (0.16-0.33)
16	Me	H	F		0.21 (0.14-0.31)
17	Me	H			0.19 (0.12-0-28)
18	Me	H			0.22
19	Me	H	H		0.38 (0.29-0.49)
20		H	H		0.35
21		H	H		0.50
22	NH_2	H	H		0.36
23	H	Me	H		0.48
24	H	Me	H		0.50
25	H	Me	H		0.32
26	H	Me	H		0.35
27	H	Me	H		0.51 (0.41-0.64)
28	H	Me	H		0.35
29	H	Et	H		0.45 (0.32-0.64)
30	H	$CH=CH_2$	H		0.50
31	H	H	OMe		0.44
32	H	H			0.50
33	H	H	$COCF_3$		0.44
34	Me	Me	H		0.36
35	Me	Me	H		0.36
36	Me	H			0.36
37	H	Me	F		0.36

significant curative activity.[40] The quest to develop safer and more active PQ analogues has been a mainstay in malaria-related medicinal chemistry. One such example is enamine CDRI 80/53 or bulaquine (elubaquine), **38**, which was approved in India and is more active than PQ and is efficacious against *P. vivax* and also significantly less toxic than the parent compound.[41−44]

Other compounds with the PQ core include the imidazoquines recently described by Gomes and co-workers which have shown interesting anti-LS activity in the micromolar range.[21] For this series, it appeared that the size of the substituent at R^2 was essential for efficient inhibition (Table 2).

Table 2. Structures of Imidazoquines and Activity against LS Forms of *P. berghei*[21]

Compound	R^1	R^2	IC_{50} / μM
39	H	H	22
40	H	Me	10
41	H	$CHMe_2$	17
42	H	CH_2CHMe_2	24
43	H	CH(Me)Et	13
44	H	$(CH_2)_2SMe$	31
45	H	CH_2Ph	21
46	Me	H	21
PQ			2

Tafenoquine, **47**, formerly known as WR 238605, is a safer and more effective derivative of PQ that possibly inhibits cytochrome bc_1 in *Plasmodium* spp.[45−50]It displays a longer half-life, making it more suitable for long-term prophylaxis. The efficacy and safety of tafenoquine treatment for radical cure of *P. vivax* malaria are still under evaluation. Moreover, Strube and co-workers also showed the better tissue schizonticidal activity of **48** (NPC1161C) in infected primates compared to PQ,[51] which was confirmed recently by Nanayakkara et al. with a 100% cure rate at 1 mg/kg po for 3 days and no toxic effects at 64 mg/kg in the same period of time.[52] It was also found that (−)-NPC1161B was several-fold more active than (+)-NPC1161A in murine models. The (−)-enantiomer was markedly less toxic in mice than its (+)-counterpart and reduced hemotoxicity in a dog model of methemoglobinemia.[52] The success of tafenoquine prompted recent studies probing its nearest chemical space. Jain and co-workers showed the curative activity of 2-*tert*-butyl analogue **49** in a dose of 25 mg kg^{-1} day^{-1} po over 4 days in mice infected with *P. berghei*. In this case bulkier substituents at C4, e.g., methyl and ethyl groups, were detrimental for in vivo activity.[53] In another study by Lin and co-workers, the C5 biphenyl analogues, e.g., **50**, did not present significant causal prophylactic activity in infected mice.[54] Finally, our laboratory has disclosed recently PQ-artemisinin hybrids with promising results both in vitro and in vivo. Compound **52** presented an IC_{50} of 155 nM against hepatic *Plasmodium berghei* in vitro. It also decreased parasite loads in the livers of infected

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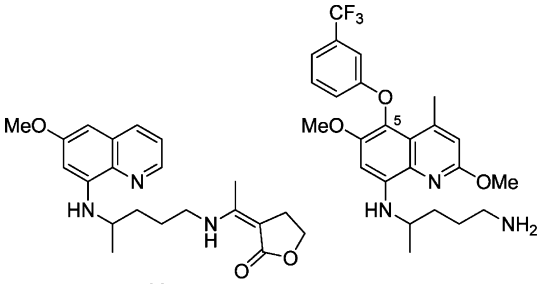
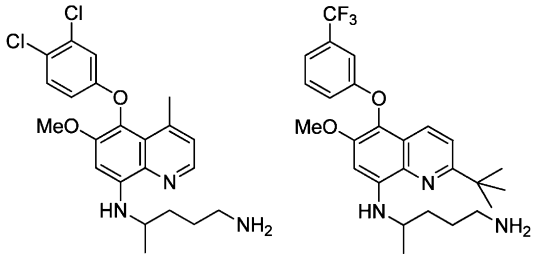
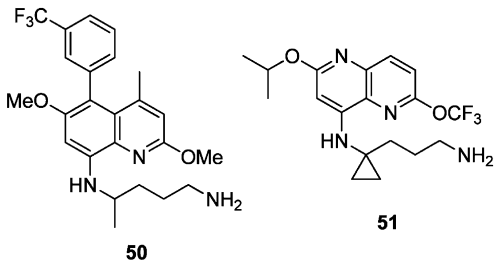
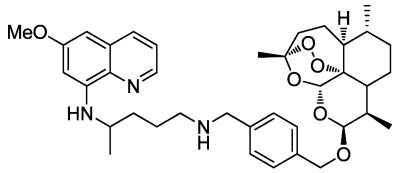




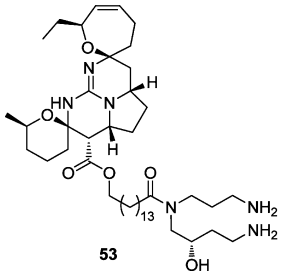


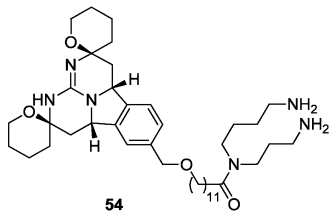
mice with a single intraperitoneal (ip) injection of 26 μmol/kg delivered after 3 h of infection and nearly abrogated liver infection after oral administration.[55]



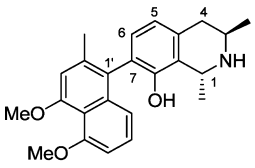
Alkaloids. Natural products have been a source for new starting points for the discovery of novel drugs against *Plasmodium*.[56] Alkaloids, in particular, have been shown to present interesting anti-LS activity. For example, cambrescidin 800, **53**, which displays potent blood stage activity, was taken by Diquet and co-workers as a lead for the synthesis of simpler analogues with the guanidine scaffold.[57] Compound **54** displayed potent activity (IC_{50} = 9.2 μM) against *P. yoelii yoelii* LS. However, it did not significantly prolong median survival time after a single subcutaneous administration of 80 mg/kg in *P. berghei*-infected mice.[57]

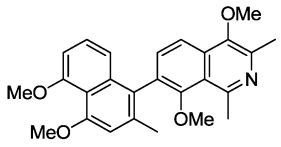
The alkaloids dioncophylline A, **55**, dioncophyllacine A, **56**, and ancistrobarterine A, **57**, have also displayed excellent anti-LS activity of 67.5%, 41.9%, and 51.6% *P. berghei* growth inhibition, respectively, at 10 μg/mL. In an attempt to elucidate the SARs of **55** a selection of structurally related alkaloids was examined by François et al. Besides **55** and **57**, only 5′-*O*-demethyl-8-*O*-methyl-7-*epi*-dioncophylline A, **58**, exerted significant inhibitory activity, 48.8% at 10 μg/mL, but was also significantly toxic. Also, two dioncophylline C derivatives,

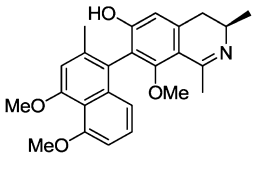


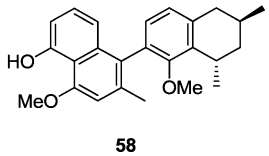


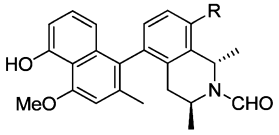
N-formyl-8-*O*-methyldioncophylline C, **59**, and *N*-formyl-8-*O*-benzoyldioncophylline C, **60**, inhibited the growth of parasitic LS in 98.3% and 52.7%, respectively, at 10 μg/mL.[58] These studies indicated that a dioncophylline A-like biaryl coupling, i.e., with the axis between C-7 and C-1′, would favorably influence activity.[58]







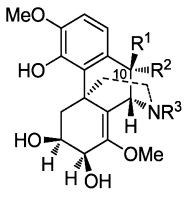




59: R = Me
60: R = COPh

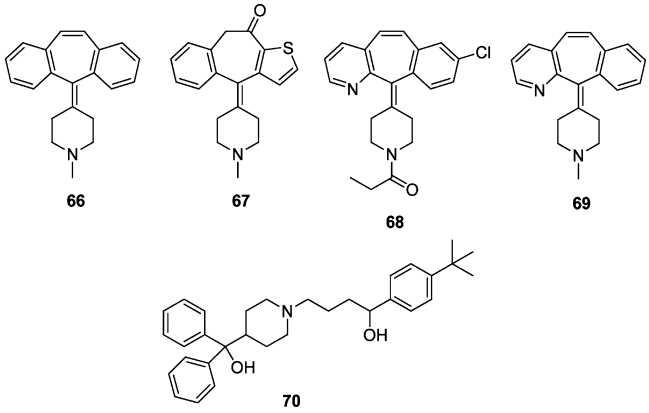
Other alkaloids of interest include the morphinan derivatives tazopsine, **61**, sinococuline, **62**, and 10-*epi*-tazopsine, **63**, which were obtained from the bioassay-guided fractionation of *Strychnopsis thouarsii* stem bark extracts.[59] Compounds **61**−**63** exhibited significant and selective inhibitory activity (SI > 13.8) against *P. yoelii* LS. Tazopsine and sinococulide showed IC_{50} values of 3.1 and 4.5 μM, respectively, while 10-*epi*-tazopsine was 5-fold less active and less toxic than **61**. Tazopsine displayed an IC_{50} of 4.2 μM against *P. falciparum*.[60] Mazier and co-workers suggested that substitution at C10 was not necessary for the antimalarial activity. Furthermore, the presence of a free hydroxyl group with (*R*)-stereochemistry enhanced the inhibitory effect against *P. yoelii* LS, while the presence of (*S*)-stereochemistry significantly decreased the toxicity on mouse primary hepatocytes. In contrast, the corresponding β-glucoside, 10-*epi*-tazosine, **64**, was inactive, a problem ascribed to the steric bulk. Total inhibition was observed for **61** at 7.1 μM, whereas *P. yoelii* parasites could still be observed in cultures treated with high concentrations of PQ; 38.6 μM only inhibited 80% of parasites.[59] Finally, NCP-tazopsine, **65**, presented similar activity to **61** but was significantly less toxic. In this case, oral administration of NCP-tazopsine completely protected mice from a sporozoite challenge and, unlike the parent molecule, the derivative was uniquely active against *Plasmodium* LS.[60]

dx.doi.org/10.1021/jm201095h | *J. Med. Chem.* 2012, 55, 995−1012

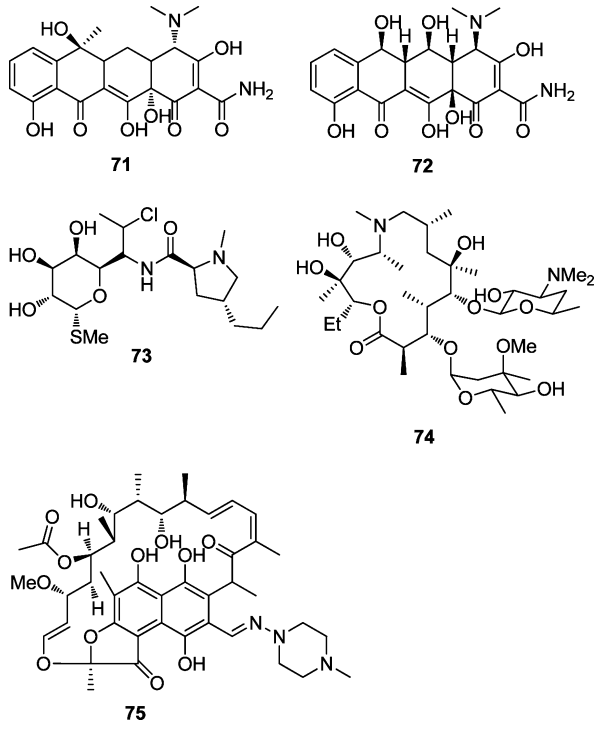


61: R^1 = OH, R^2 = R^3 = H
62: R^1 = R^2 = R^3 = H
63: R^1 = R^3 = H, R^2 = OH
64: R^1 = R^3 = H, R^2 = O-β-Glc
65: R^1 = OH, R^2 = H, R^3 = cyclopentyl

Antihistaminics. Cyproheptadine, **66**, ketotifen, **67**, loratadine, **68**, azatadine, **69**, and terfenadine, **70**, are histamine (H_1) inhibitors whose causal prophylactic activity against malaria was evaluated by Puri and Singh. Treatment of infected mice with cyproheptadine or ketotifen at 5 mg/kg and terfenadine at 50 mg/kg po for 3 days completely prevented the establishment of infection. Partial activity was recorded with lower doses of these drugs as well as with azatadine and loratadine at 10 mg/kg po as indicated by marginal delay in the development of infection after sporozoite challenge. Also, none of the compounds showed blood schizontocidal activity at doses found effective in the mouse causal prophylactic test.[61,62]



Antibiotics. Some currently used antibiotics have shown activity against blood stages of *P. vivax* and *P. falciparum*.[63,64] Tetracycline, **71**, doxycycline, **72**, clindamycin, **73**, and azithromycin, **74**, displayed modest antimalarial activity, but the use of **74** in combination with chloroquine or quinine showed a synergistic effect, highlighting the interest of using these molecules in endemic areas.[63] Moreover, **74** has long been known to be a causal prophylactic agent in malaria.[65,66] Andersen et al. showed that parenteral administration of 160 mg/kg **74** or **72** to mice was 100% effective while 40 mg/kg of **74** was 80% effective but 40 mg/kg of **72** was only 40% effective. When given orally, compound **74** was 100% effective at 160 mg/kg but was only 40% effective when given at a dose of 40 mg/kg.[65] Similar results were obtained recently by Singh and co-workers in a study that showed that **74** did not have activity against hypnozoites.[67] Additionally, **71**−**74** presented activity against the LS in individuals infected with *P. vivax*.[68,69] In a study by Pukrittayakamee et al., compounds **71**−**73** cured infections in 56−78% of the cases while **74** cured 58%, contrasting with the results in murine models.[68] Rifampicin, **75**, is another antibiotic that targets the apicoplast, where several overrepresented pathways in the LS are localized.[10,70,71] The IC_{50} of **75** against the LS of *P. yoelii* was 22.2 μM, slightly lower than that of tetracycline. Therefore, **75** may also find some applicability in prophylaxis of malaria.[10]



Antifolates. Dihydrofolate reductase (DHFR) inhibitors, such as pyrimethamine, **76**, and proguanil, **77**, are used mainly as blood schizonticides. However, these drugs were shown to be even more active against the development of primary exoerythrocytic stages, but without apparent effect on hypnozoites.[72−74] Also, pyrimethamine produces necrotic changes in maturing exoerythrocytic schizonts of the simian malaria parasite *P. cynomolgi*.[75] Pyrimethamine had detectable schizontocidal activity against cultured hepatocytes infected with *P. cynomolgi*, at 0.001 μg/mL, with complete inhibition at 0.01 μg/mL (~40 nM).[46] This contrasts sharply with a recent study by Mazier and co-workers showing that pyrimethamine had an IC_{50} of 1.03 μM in maturing exoerythrocytic forms of the same species.[76] On the other hand, proguanil and its active metabolite cycloguanil, **78**, showed activity at 0.0001 μg/mL without causing cytotoxicity.[46]

DHFR inhibitors also showed improved activity against LS when used in combination. Proguanil plus sulfamethoxazole, **79**, has been shown to provide effective prophylactic protection in individuals infected with *P. vivax* or *P. falciparum*, with marked synergy between the components.[77,78] A significant potentiation in the activity of pyrimethamine could also be observed when administered in combination with azithromycin.[79] Likewise, trimethoprim, **80**, showed efficacy in association with sulfonamides.[80,81] Malarone, the association of proguanil with atovaquone, **81**, is the most commonly used drug combination, is highly efficacious in prophylaxis of malaria, and is well tolerated.[82−84]

Naphthoquinones. Atovaquone, **81**, is a potent inhibitor of the bc_1 complex of malaria parasites that collapses the mitochondrial membrane potential.[32,85,86] It presents potent causal prophylactic activity, inhibiting the LS of *P. falciparum* with an IC_{50} of 3.95 nM,[87,88] which is in good agreement with the original studies on the effects of this drug against the LS of *P. berghei* cultured in vitro; a concentration of 1.85 nM, added

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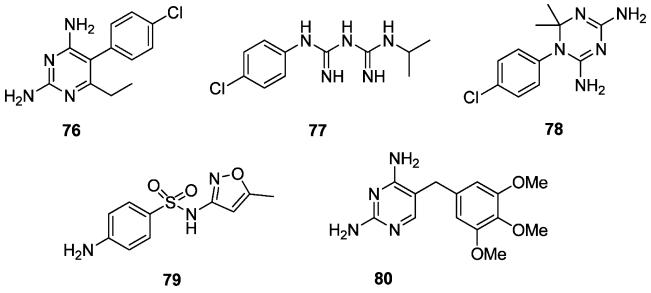
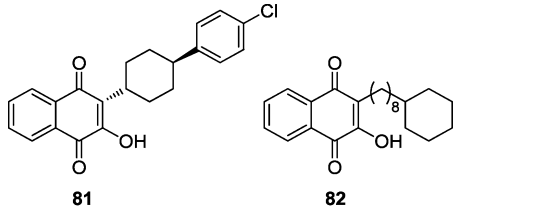
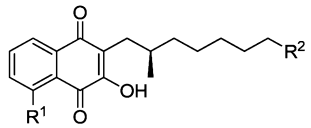


3 h after sporozoite invasion, reduced the numbers of exoerythrocytic forms visible at 48 h by 50%.[89,90] However, atovaquone does not prevent relapses of malaria by eliminating hypnozoites in vivo.[76] Presumably, the activity of **81** against LS can be extended to other naphthoquinones, since menoctone, **82** (a naphthoquinone developed before atovaquone), had already shown activity superior to that of PQ.[91] This is in line with a recent report from Treumpower and colleagues, where four other naphthoquinones presented activity against the LS of *P. berghei*. Compounds **83**–**86** displayed IC_{50} values of 96.2, 17.07, 246.5, and 4451.8 nM, respectively, with atovaquone exhibiting a value of 19.7 nM. Thus, a methyl group at R^1 appears to be detrimental for activity and the introduction of a CF_3 group at R^2, to improve metabolic stability, is beneficial,

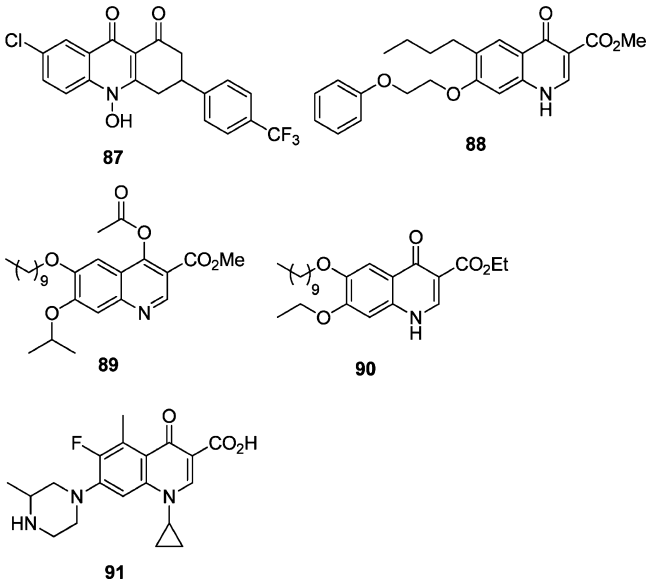




83: R^1 = H, R^2 = Me
84: R^1 = H, R^2 = CF_3
85: R^1 = R^2 = Me
86: R^1 = Me, R^2 = CF_3

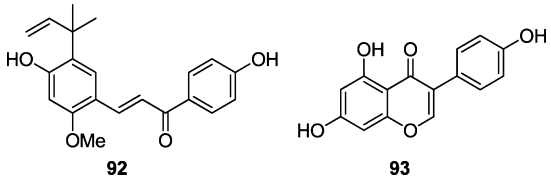
i.e., **84**. The opposite SAR could be observed for 8-methyl derivatives.[92]

Acridinediones, 4(1*H*)-Pyridones, and 4(1*H*)-Quinolones. Several inhibitors of the bc_1 complex have shown activity against *Plasmodium* LS. Floxacrine, **87**, is an acridinedione with potent activity against the parasite's blood stages through the inhibition of this critical pathway.[93] In addition, it has been shown to prevent and cure infections of *P. cynomolgi* sporozoites. Floxacrine had a high order of prophylactic activity, with a daily dose of 0.625 mg/kg po delivered throughout the incubation period, providing full protection against challenge of 10^6 infective sporozoites, and a dose of 0.156 mg/kg po fully protecting half of the test models. However, a single dose of 40.0 mg/kg po delivered 2 h before sporozoite challenge did not prevent development of infections, although it did prolong the incubation period significantly. Moreover, **87** was unable to cure established infections. The maximum tolerated daily dose (40.0 mg/kg po) effectively cleared blood schizonts but neither prevented nor delayed relapse, showing that floxacrine did not produce radical cure.[94] 4(1*H*)-Pyridones[95,96] and 4(1*H*)-quinolones[32,97] are other examples of mitochondrial electron transport chain inhibitors with activity against the LS of *Plasmodium*. Druilhe and co-workers showed that 4(1*H*)-pyridones displayed activity in the low nanomolar range in cultured parasites and efficacy at 10 mg/kg po in mice.[96] On the other hand, quinolones **88** (WR 197236) and **89** (WR 194905) exhibited antirelapse activity against sporozoite-induced *P. cynomolgi* infections in Rhesus monkeys. Both compounds were found to be curative when administered intramuscularly (im) in seven daily doses of 15 mg/kg, and no relapses were observed during an observation period of 120 days.[97] Very recently, Winzeler and co-workers showed that decoquinate, **90**, was active against *P. yoelii* in vitro with an IC_{50} of 177 pM. Furthermore, an oral dose of 50 mg/kg protected 80% of the infected mice.[98] Also, Mahmoudi et al. evaluated 25 quinolones and fluoroquinolones for their inhibitory potency against LS of *P. falciparum* and *P. yoelii yoelii*.[99] Among those, only grepafloxacin, **91**, had an IC_{50} in the 10–15 μM range. In addition, this compound presented potent activity against the blood stage of infection,



suggesting that it may find applicability in both treatment and prophylaxis of malaria.[99]

Other Inhibitors of the Mitochondrial Electron Transport Chain. Licochalcone A, **92**, is a potent bc_1 complex inhibitor,[11,100,101] and genistein, **93**, was also shown to interact with the mitochondrial electron transport chain and to present anti-LS activity.[14,102] Mahmoudi et al. recently found compound **92** active, through a molecular topology virtual screening, with an IC_{50} of 0.927 nM against *P. yoelli yoelli* (PQ IC_{50} = 75.7 nM).[11] In a different screen designed by Mota and co-workers, genistein presented an IC_{50} of ~20 μM against cultured *P. berghei* LS (PQ IC_{50} of ~13 μM). Furthermore, a single dose of 200 mg/kg **93** given orally resulted in 64% reduction of liver infection.[14]



Imidazolidinediones. The 2-guanidinoimidazolidinedione **94** (WR182393) is a mixture of two constitutional isomers (Figure 3) and presents both prophylactic and radical curative

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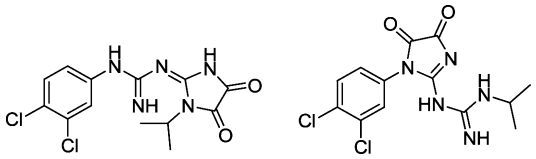
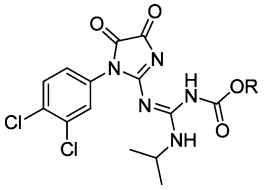


Figure 3. Constitutional isomers that make up WR182393, **94**.

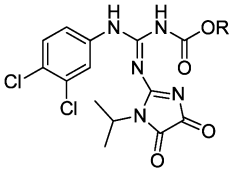
properties in the Rhesus monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* model. Milhous and colleagues showed the causal prophylactic activity of **94** in a regimen of 40 mg kg^{-1} day^{-1} im for 3 days beginning the day before intravenous challenge with *P. cynomolgi* sporozoites. Also, a regimen of 31 mg kg^{-1} day^{-1} im for 3 days maintained the monkeys parasite-free, and in combination with 10 mg kg^{-1} day^{-1} po of chloroquine for 7 days, resulted in radical cure. Relapses were observed for lower dosages of **94** but were significantly delayed, which highlighted valuable radical curative properties against *P. vivax*-like infections.[103]

Since it was difficult to isolate the isomers of **94**, some carbamate derivatives of **94** were subsequently synthesized to facilitate the separation through fractionated crystallization. Compounds **95** and **96** were also assayed for their prophylactic activity in sporozoite challenge assays. Monkeys treated with 30 mg/kg im of compound **95** were protected and stayed parasite-free 100 days after treatment, but the efficacy decreased at a dose of 10 mg/kg im. Compound **96** showed the best prophylactic efficacy, protecting all monkeys in doses as low as 10 mg/kg im. However, none of the compounds showed significant oral protective activity up to 60 mg/kg in Rhesus monkeys, but good oral activity was observed in mouse model.[104,105]

In mice, carbamates **96**–**98** showed 100% protection at 160 mg/kg po, **99** did not protect at 160 mg/kg po, and **100**–**102** protected only one out of five treated mice at the same dose. Whereas the isobutyl carbamate **95** protected 100% of the treated mice at a dosage as low as 5 mg/kg po, the minimum dose for 100% protection of isobutyl carbamate **96** was over 40 mg/kg po. Likewise, while *tert*-Boc **103** protected 4/5 mice at 5 mg/kg, *tert*-Boc **96** protected only 2/5 mice at a higher dose of 40 mg/kg po. Nevertheless, ethyl carbamates **98** and **104** displayed comparable causal prophylactic activity at all dose levels tested, showing 2/5 and 1/5 mice protection, respectively, at 10 mg/kg po.[105] In these studies, the mice model proved to be a weak predictor of activity in monkeys and, consequently, in humans. Lin and co-workers hypothesized that the low oral bioavailability of these carbamates in monkeys was due to rapid hydrolysis in acidic media. To circumvent this liability, the corresponding carboxamides of **94**–**103** were synthesized. In addition to a higher metabolic stability compared to their carbamate counterparts, these compounds also delayed patency of infection.[106] More recently, several *N*-alkyl- and *N*-alkoxyimidazolidinediones were described.[107,108] In general, these were metabolically stable and weakly active *in vitro* against *P. falciparum* D6 and W2 strains and in mice infected with *P. berghei* sporozoites. Compounds **105** and **106** showed good causal prophylactic activity in Rhesus monkeys treated with 30 mg kg^{-1} day^{-1} im for 3 consecutive days and delayed patency for 19–21 days and 54–86 days, respectively, compared to the untreated control. When administered orally, **106** showed only marginal activity in causal prophylactic and radical curative tests at 50 mg kg^{-1} day^{-1} × 3 and 30 mg kg^{-1} day^{-1} × 7 plus chloroquine 10 mg/kg po for 7 days, respectively.[107] A follow-up study by Lin and co-workers described a series of 2-guanidino-4-oxoimidazolines, which were metabolically unstable but



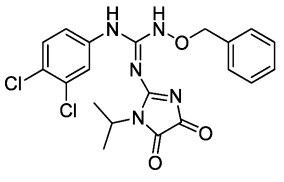
95: R = $CH_2CH(CH_3)_2$
103: R = $(CH_3)_3$
104: R = CH_2CH_3



96: R = $(CH_3)_3$
97: R = $CH_2CH(CH_3)_2$
98: R = CH_2CH_3
99: R = CH_2Ph
100: R = $(CH_2)_5CH_3$
101: R = $CH_2CH_2CH{=}CH_2$
102: R = $(CH_2)_2OCH_2Ph$

presented good prophylactic activity. Isopropyl groups at either R^1 or R^2 yielded compounds with curative activity in prophylactic tests in mice. Compound **107**, with both R^1 and R^2 being the isopropyl group, showed the best activity among the compounds tested, with 5/5 and 4/5 mice protected at doses of 320 and 160 mg/kg ×3, respectively. Besides the isopropyl group, *tert*-Boc also appeared to be a favorable substituent at R^2. Additionally, compound **107** presented activity comparable to those of PQ and atovaquone/proguanil and delayed patency in 2–5 days after oral administration. Curative tests with monkeys showed that **108** was effective at 30 mg/kg × 3 by im, combined with 10 mg/kg of chloroquine.[109]

Ionophores. Ionophores such as monensin, **109**, and nigericin, **110**, intercalate into intracellular organelle membranes and exchange protons for K^+ or Na^+. Schrével, Vial, and their colleagues showed the antiplasmodial activities of **109** and **110** in the nanomolar and picomolar ranges, respectively, in blood stages of *P. falciparum*[110,111] but also strong inhibitory activities against *P. berghei* and *P. yoelii* sporozoites. Incubation of *P. berghei*-infected hepatocytes with monensin at 1 nM partially inhibited infection, whereas 10 nM and higher concentrations resulted in complete inhibition.[112] These results are in line with a study by Mahmoudi et al., although in this case the minimal dose of monensin and nigericin required for inhibition of



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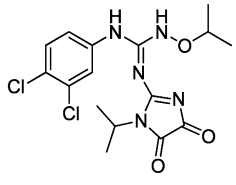

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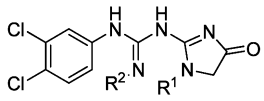

107: $R^1 = R^2 = CH(CH_3)_2$
108: $R^1 = CH(CH_3)_2$, $R^2 = CO_2C(CH_3)_3$

P. yoelii sporozoite infection was below 10^{-3} nM. However, the different times of incubation of the cultures with the drug and the use of primary cultures used by Mahmoudi et al. versus the cell line used by Leitao et al. could account for this difference.[11,112]

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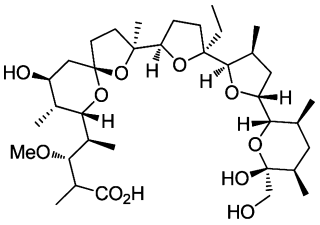

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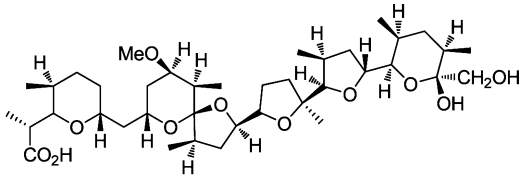

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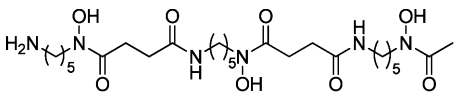
Iron-Chelating Agents. Iron is required for a number of essential enzyme systems, including dihydroorotate dehydrogenase, involved in the synthesis of pyrimidines, and cytochrome oxidase, an essential component of the mitochondrial electron transport system.[113,114] Several iron-chelating agents are capable of inhibiting the growth of *P. falciparum*. Desferrioxamine, **111**, and desferrithiocin, **112**, are two iron chelators with activity against the LS of *P. yoelii* and *P. falciparum* in rodent and the human hepatocytes in vitro. Stahel et al. found that **111** and **112** inhibited the liver schizogony of both *Plasmodium* species at concentrations achievable in vivo. At maximum drug exposure time (96 h), desferrioxamine exhibited an IC_{50} of 20 μM against LS of *P. yoelii* and 3.4 μM in the case of *P. falciparum*. On the other hand, desferrithiocin displayed IC_{50} values of 10 and 2.9 μM against the LS of *P. yoelii* and *P. falciparum*, respectively.[115] Another iron chelator with anti-LS activity is dexrazoxane, **113**, which inhibited the development of *P. yoelii* sporozoites in concentrations ranging from 50 to 200 μM.[116]

Protease Inhibitors. HIV protease inhibitors have also demonstrated activity against the LS of *P. berghei* in vitro. Treatment of cultured parasites with 10 μM saquinavir, **114**, reduced the number of exoerythrocytic forms in 55%, while the same concentration of lopinavir, **115**, resulted in 38% reduction. In mice infected with *P. yoelii*, Hoobs et al. showed that lopinavir/ritonavir, **116**, combination exerted a dose-dependent effect in



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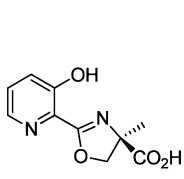

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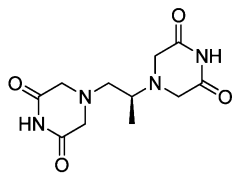
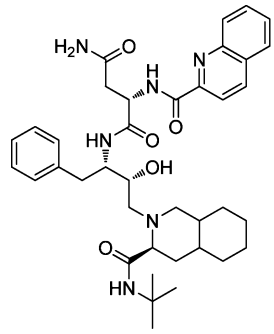


113

reducing liver parasite burden. When 25 mg/kg lopinavir with 12.5 mg/kg ritonavir or 100 mg/kg lopinavir with 50 mg/kg ritonavir were administered by gavage to mice 6 h before infection and then again 18 h after sporozoite inoculation, parasite liver burden was reduced by 45% and 93%, respectively.[117] Previous work by Mahmoudi et al. had already shown potent activity of **114** and **116** in the nanomolar range (~30 nM) and, to a lesser extent, of indinavir, **117** (5 μM).[11] HIV protease



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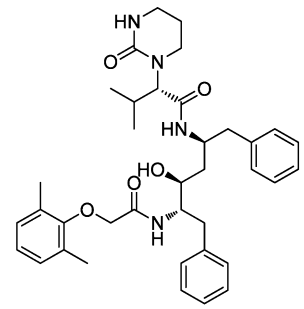

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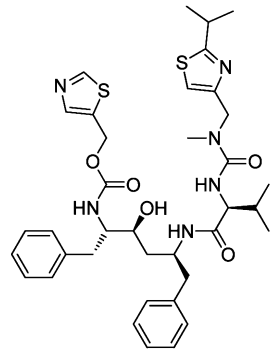

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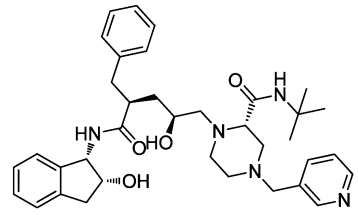

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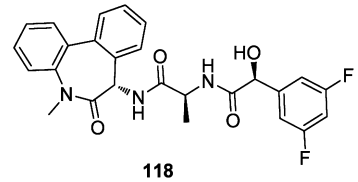

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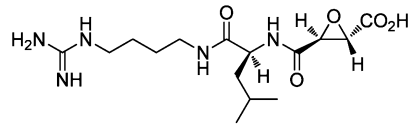

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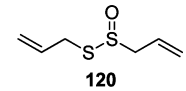

120

inhibitors target aspartyl proteases,[118] and *Plasmodium* expresses a number of these enzymes, called plasmepsins. Plasmepsins are also expressed during the *P. yoelii* preerythrocytic stages;[10] therefore, these remain possible targets for HIV protease inhibitors during this stage of the parasite's life cycle. Other aspartyl proteases may be targeted in *Plasmodium* LS. Compound **118** (LY411,575) is an inhibitor of γ-secretase and signal peptide protease with potent activity in vitro against *P. berghei* sporozoite-infected hepatocytes (IC_{50} = 80 nM), without affecting invasion by the parasite. Additionally, Mota and co-workers showed that **118** decreased the parasite load in the liver in vivo and increased the resistance of mice to cerebral malaria by 55%.[15]

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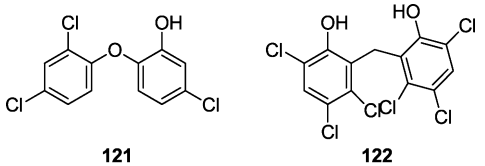
Inhibitors of other proteases, such as falcipain, have also shown activity against LS. E-64, **119**, is a highly specific cysteine protease inhibitor that completely blocked sporozoite infectivity in vitro at 10 μM and in vivo at 50 mg/kg ip.[119] Allicin, **120**, is an example of another compound that displayed results similar to those of E-64, regarding the inhibition of hepatocyte infection. In this case, 10 μM **120** resulted in 37% inhibition, but 50 μM yielded results comparable to those of **119** at 10 μM. Also, 8 mg/kg intravenous (iv) **120** significantly protected mice from developing infection.[120]

FAS-II Inhibitors. FAS-II has been shown to be essential only during the LS of the life cycle of *P. berghei* and is one of the most active pathways at that stage.[10,121] Therefore, compounds inhibiting enzymes from this pathway are likely to be active against LS. Surolia and co-workers showed that triclosan, **120**, displayed an IC_{50} of 6.8 μM against *P. berghei* for treatment before invasion of hepatocytes and an IC_{50} of 39.4 μM for treatment after invasion.[122] The IC_{50} against *P. yoelii* was reported to be around 40 μM, whereas hexachlorophene, **122**, was shown to be significantly more active (IC_{50} of 4–7 μM).[10,123] Furthermore, Tasdemir et al. revealed that 2-hexadecynoic acid, **123**, displayed an IC_{50} of ~61 μM against the same species.[124] Recently, Singh et al. reported potent activities against *Plasmodium* LS and high (>100) therapeutic indexes for a new generation of bisphosphonates designed as anticancer agents that block protein prenylation. In that study, the commercially available pamidronate, **124**, was inactive up to 200 μM, but risedronate, **125**, displayed an IC_{50} of 16 μM. On the other hand, the more lipophilic bisphosphonates **126** (BPH-715), **127** (BPH-942), and **128** (BPH-943) had IC_{50} values in the range of 8–10 μM. In vivo studies with *P. berghei* showed that compound **126** completely protected mice in doses as low as 1.5 mg/kg ip.[125]

Miscellaneous. Several other molecules have been reported to present activity against LS of various malaria models. However, those studies are scattered and no further attempts to optimize hit molecules could be found in the literature. Compound **129**, is one such example. It is known that 25 mg/kg po prevented the development and/or maturation of exoerythrocytic stages in *P. cynomolgi*, thus showing causal prophylaxis.[126,127] Schmidt et al. reported that even at 6.25 mg/kg the efficacy did not decrease significantly. Nevertheless, as a result of limited activity against blood stages by **129**, coadministration of chloroquine is required for radical cure.[128]



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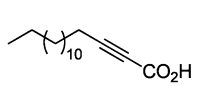

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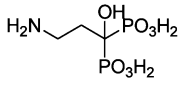

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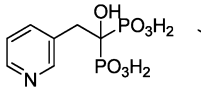

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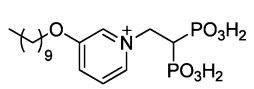

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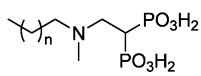
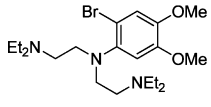


127: n = 6
128: n = 8



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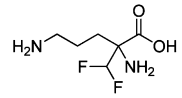

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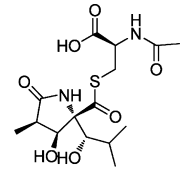

131

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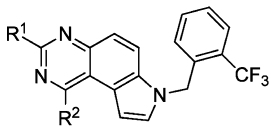
DL-α-Difluoromethylornithine, **130**, is a specific irreversible inhibitor of ornithine decarboxylase, a key enzyme in the biosynthesis of polyamines. Compound **130** administered at 1% in drinking water showed prophylactic activity in mice inoculated with *P. berghei* sporozoites, through the inhibition of early stage exoerythrocytic schizogony.[129–131] On the other hand, Sinnis and co-workers showed that the irreversible inhibitor of 20S proteasome lactacystin, **131**, inhibited the LS forms of *P. berghei* both in vitro and in vivo in nanomolar concentrations but had no effect regarding the infectivity of sporozoites.[132]

More recently, a few studies have also disclosed the anti-LS activity for some series of structurally diverse compounds. Guan et al. demonstrated that pyrrolo[3,2-*f*]quinazolines **132** and **133** cleared *P. falciparum* parasitemia in *Aotus* monkeys, by day 3, at an oral dose of 1 mg/kg, and no recrudescence was observed for over 100 days. Compound **133** at 1 and 3 mg kg^{-1} day^{-1} po for 3 days cured *Aotus* monkeys from *P. vivax* infection. Compound **132** was, however, less active at the same doses, with only 1/2 monkeys cured at 3 mg/kg po and none cured at 0.5 and 1 mg/kg po. Compound **133** completely prevented the development of parasitemia at doses ranging from 0.65 to 40 mg/kg for 3 days po in *P. yoelii* sporozoite-challenged mice. In this test, tafenoquine was used as a positive control. Full protection was observed for mice treated with compound **133** at a dosage as low as 1.29 μmol/kg po compared to a dosage of 10.8 μmol/kg po for tafenoquine in the same test.[133] At the same time, Ghichard and co-workers disclosed 1,3,5-triazepan-2,6-diones **134** and **135** and reported their modest anti-LS activity against *P. yoelii*. Despite the IC_{50} values being higher than 200 μM, these could serve as leads for future exploratory work.[134] Conversely, compound **136** significantly inhibited the infection of hepatocytes by *P. yoelli* sporozoites. In vitro, this inhibition was dose dependent and observed at 2 μM, within the first 3 h of sporozoite infection. While sporozoite motility was partially inhibited by the trisubstituted pyrrole, sporozoite invasion of hepatocytes could be completely blocked in a dose-dependent manner and with an IC_{50} below 1 μM. In vivo, administration of a single dose (50 mg/kg ip) of **136** caused a significant reduction in the LS burden of mice infected with *P. yoelii* sporozoites and led to partial protection against the appearance of blood-stage parasites. A higher dose of **136** (50 mg/kg ip × 3) completely prevented the appearance of blood-stage parasites in a sporozoite-initiated infection.[135]

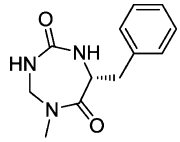
The topological-based virtual screening of Mahmoudi et al. in 2008 led to the identification of known drugs with potent activity in *P. yoelii yoelii* LS.[11] Their structures and activities can be found in Table 3.

Finally, Mazier and co-workers reported that polysphorin, **153**, and analogues had potent antiplasmodial activity against both *P. yoelii* and *P. falciparum* in vitro (Table 4).[136] In general, compounds with a cyclic dioxo side chain, i.e., **143–146**, were less active than their trimethoxy counterparts, i.e., **147–152**.

dx.doi.org/10.1021/jm201095h | J. Med. Chem. 2012, 55, 995–1012



132: $R^1 = R^2 = (EtOCO)NH$
133: $R^1 = R^2 = (MeCO)_2N$



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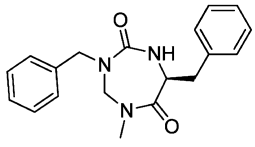

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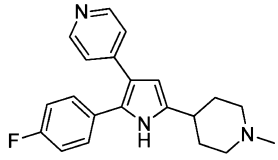


136

It was also noted that the stereochemistry of the compounds was very important for anti-LS activity. Whereas mixtures **143** and **144** displayed similar IC_{50} values, increasing the *syn* isomer proportion of **145** to **146** resulted in a 3-fold increase in potency. The improved potency of *syn* isomers was more marked for compounds **148** (vs **147**) and **150** (vs **149**). While the *anti* isomers were moderately active, compounds **148** and **150** presented IC_{50} values of 2.52 μM and 4.5 nM against *P. yoelii*, respectively. Further exploratory work on the surrounding chemical space showed that substitution of the methyl group at R^1 for a phenyl group increased the potency of *anti*-configured compounds, i.e., **149** vs **151**, but the opposite trend was observed for *syn* compounds, i.e., **150** vs **151**, against *P. yoelii*.[136]

CONCLUSIONS AND FUTURE PROSPECTS ON LIVER STAGE ANTIMALARIALS

There can be no doubt that new and efficient drugs against malaria are necessary. While past and present research on antimalarial drug development has focused almost exclusively on blood stage parasites, the liver stage, whose study is technically challenging, offers important advantages for prophylactic/therapeutic intervention. Not only is the number of parasites present during this stage relatively low but also intervention at this stage acts before the onset of symptoms and provides a prophylactic strategy (reviewed in ref 5). Besides, the liver stage is particularly important for any eradication campaign because *P. vivax* (as well as *P. ovale*) infections can generate cryptic forms called hypnozoites that persist in the liver for long periods of time. These dormant forms cause relapsing malaria, which may occur without new infection taking place, after latent periods of months or even years. As previously mentioned, the only drug with known efficacy against both the replicating and dormant liver stages, primaquine, has several liabilities, and its ability to cause hemolytic anemia in patients with glucose 6-phosphate dehydrogenase deficiency has severely restricted its use.

The liver stage also presents the best opportunity for developing drugs that hit new targets, which is quite relevant in the context of the actual drug resistance map. Indeed, several recent reports revealed a remarkable number of genes and proteins that are expressed only during the liver stage.[137−139] While this marked difference between blood and liver stages is unsurprising, as the different parasite forms infect very different host cells and reach replication rates that differ by 3 orders of magnitude, they likely represent stage-specific drug targets.

But how to find compounds that hit these new targets? Drug discovery screens can be either target-based or phenotypic. Target-based screens are often operationally simpler, as they can use pure proteins in a biochemical assay, but they require selecting the target prior to screening, and they will only generate candidate molecules that modulate the chosen target. Several target-based screens for suitable therapeutic agents have been developed in the past 10 years. Histone deacetylases (HDAC),[140,141] dihydroorotate dehydrogenase (DHODH),[142,143]

Table 3. Structures of Compounds Active in Vitro against *P. yoelii yoelii*, Predicted by Molecular Topology[11]

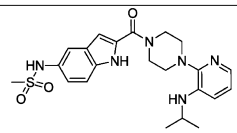
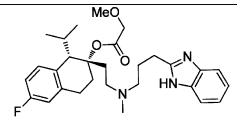
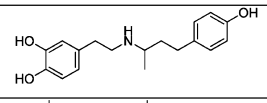
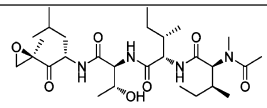
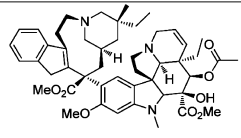
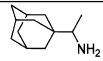
Compound	Name	Structure	IC_{50} / nM
137	Delavirdine		0.846
138	Mibefradil		0.873
139	Dobutamine		3.7
140	Epoxomicin		3,950
141	Vinblastine		7,950
142	Rimantadine		35.6
3	PQ		75.7

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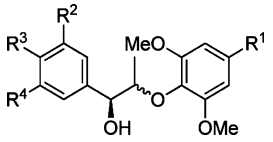
Table 4. Structures of Compounds Active against *P. yoelii* and *P. falciparum* LS[136]



Compound	R^1	R^2	R^3	R^4	*P. yoelii* IC_{50} / μM	*P. falciparum* IC_{50} / μM
143 (1*S*,2*R*)/(1*S*,2*S*) = 5/95	H	H			20.13	ND
144 (1*S*,2*R*)/(1*S*,2*S*)= 54/46	H	H			20.19	ND
145 (1*S*,2*R*)/(1*S*,2*S*)= 2/98	Me	H			19	ND
146 (1*S*,2*R*)/(1*S*,2*S*)= 51/49	Me	H			5.62	ND
147 (1*S*,2*R*)/(1*S*,2*S*)= 0/100	H	OMe	OMe	OMe	28.61	ND
148 (1*S*,2*R*)/(1*S*,2*S*) = 100/0	H	OMe	OMe	OMe	2.52	ND
149 (1*S*,2*R*)/(1*S*,2*S*) = 0/100	Me	OMe	OMe	OMe	50.1	ND
150 (1*S*,2*R*)/(1*S*,2*S*) = 100/0	Me	OMe	OMe	OMe	0.0045 ± 0.005	0.38
151 (1*S*,2*R*)/(1*S*,2*S*) = 0/100	Ph	OMe	OMe	OMe	3.54 ± 4.9	0.02
152 (1*S*,2*R*)/(1*S*,2*S*) = 100/0	Ph	OMe	OMe	OMe	3.61 ± 4	0.007
PQ					0.075	ND
153 (1*S*,2*R*) (1*S*,2*S*)	$CHCHCH_3$	OMe	OMe	OMe	9.54 9.53	2.72 3.65

dihydrofolate reductase (DHFR),[144] heat shock protein 90 (Hsp90),[145] and enzymes involved in fatty acid biosynthesis[146,147] have been among the most promising. While these assays have been designed to identify compounds that inhibit *Plasmodium* growth within red blood cells, some have been tested later against liver stage activity and showed some promising results.[10] However, the lack of species-specificity between the *Plasmodium* and human enzymes has limited drug development. Several recent reports have analyzed both transcriptome and proteome expression levels of malaria parasites in different life stages[137−139] and revealed a number of genes and proteins that are expressed during the liver stage, which may represent drug targets. These include several different *Plasmodium* proteases as well as enzymes involved in the redox metabolism and fatty acid synthesis, including the FAS-II pathway found in plants, prokaryotes, and *Archaea*. The bacterial origin of this pathway and the absence of FAS-II in the human host make it an attractive target for antimalarial drugs.

Still, most differentially expressed liver stage genes encode hypothetical proteins of unknown function, a finding that while suggesting that they could reveal many new targets, complicates selection new targets. Thus, in our view, a phenotypic liver stage screen, involving infection of host cells by the parasite, would provide the most efficient path to discovering new drugs

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that would hit new targets. Not surprisingly, this type of screen has the drawback of being more complicated to undertake. As such, the only widely used phenotypic malaria screens so far have involved blood-stage parasites,[148−151] and there has not been yet a phenotypic screen for *Plasmodium* liver stages. Nevertheless, infection reporting methods are arising and a quantitative high-throughput liver stage screen using the rodent malaria strains, *P. yoelii* and *P. berghei*, is already possible.

Unfortunately, less progress has been made toward reliable in vitro models for hynozoite assays. To date, the only widely used model to screen for antihypnozoite activity is via the infection of Rhesus monkeys with *P. cynomolgi* sporozoites (reviewed in ref 9). Recently, slow growing *P. cynomolgi* hepatic forms were characterized after sporozoite infection of *Macaca fascicularis* primary hepatocytes.[76] Similarly, small parasite forms that may be hypnozoites have also been reported after infection of hepatoma cells with either fresh or cyropreserved *P. vivax* sporozoites.[152−155] These reports are undoubtedly important first steps to establishing in vitro hypnozoite models, but further validation of the systems is needed before screening efforts can begin. Thus, the establishment of culture systems that allow long periods of infection are indeed critical.

Achieving a new generation of liver stage inhibitors in the near future is a highly desirable goal. Bringing together specialists on *Plasmodium*, liver, and hepatocyte biology as well as on medicinal chemistry will be essential to generate an interesting pipeline of drugs that would hit both the replicative and the dormant forms of the *Plasmodium* liver stage. Malaria's LS presents an attractive pathway for developing novel drugs, given that malarial symptoms can be prevented from its effective blocking. Since World War II, 8-aminoquinolines have been by far the most studied class of compounds for prophylactic use. Hence, the discovery of PQ constituted a landmark in the fight against malaria, but its side effects have curtailed its clinical use. Consequently, most discovery efforts have focused on ablating the hemotoxicity of PQ, although with limited success. Bulaquine, **38**, the only additional 8-aminoquinoline marketed for prevention of relapse in *P. vivax* malaria, provides a good example of a prodrug approach to overcome toxicity and efficacy issues.

Only recently the scientific community has turned its attention to the discovery of other classes of compounds, but only seldom have good leads been followed up by medicinal chemists. Importantly, some FDA-approved drugs (Table 5) have shown anti-LS activity in malaria and could present good opportunities for medicinal chemistry programs. Also, some cases of druglike compounds presenting IC_{50} values in the nanomolar range have already been reported. This is the case of pyridines and quinolones (e.g., **90**), which provide excellent platforms for further optimization against LS, provided metabolism and bioavailability issues are solved. However, it should be noted that the major challenge is the discovery of unique scaffolds displaying anti-LS activity and preferentially without any other biological activity annotation. An example is the imidazolidinedione scaffold (e.g., **106**), which represents a completely new entry in the armamentarium against the LS infection with the potential for antirelapse therapy. Still in preclinical evaluation, existing imidazolidinediones may become interesting starting points to develop orally active analogues.

The scarcity of literature reporting active molecules against LS, compared to reports of blood stage antiplasmodials, can also be partly ascribed to the technical difficulties in testing anti-LS candidates. These difficulties are patent from the short number of laboratories carrying out such studies. Furthermore, the IC_{50} values of positive controls are rarely comparable between them. In times when few drug options are available, the discovery of efficient drugs that could prevent the onset of clinical symptoms is urgently needed. Once the drawbacks of in vitro assays are overcome, medicinal chemistry of anti-LS antimalarials may become a central pillar in the overall discovery efforts.

Table 5. FDA-Approved Drugs with Anti-LS Malaria Activity

primaquine
cyproheptadine
ketotifen
loratadine
azatadine
tetracycline
doxycycline
clindamycin
azithromycin
pyrimethamine
proguanil
atovaquone
saquinavir
lopinavir
ritonavir
indinavir
pamidronate
delavirdine
dobutamine
vinblastine
rimantadine

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Biographies

Tiago Rodrigues received his M.Sc. (2006) from the Faculty of Pharmacy, University of Lisbon, Portugal, and his Ph.D. in Medicinal Chemistry (2010) from the University of Lisbon, under the supervision of Dr. Francisca Lopes. He recently joined the group of Prof. Gisbert Schneider at ETH Zürich, Switzerland, as a postdoctoral researcher. His research interests are in the field of medicinal chemistry, including the discovery and optimization of novel antimalarial leads, with particular focus on cytochrome bc_1 inhibitors.

Miguel Prudêncio received his Ph.D. in Biochemistry from the University of East Anglia, U.K. (2000), and then became a researcher at the Leiden University, The Netherlands (2000−2004). He became a researcher at the Host-Plasmodium Interactions Group of the Instituto Gulbenkian de Ciência, Portugal (2004), before moving to the Malaria Unit (UMA) of the Instituto de Medicina Molecular (IMM), Portugal (2005). In 2008 he was appointed Staff Scientist of the IMM's UMA, of which he is now a Principal Investigator. His present research interests concern biomedical relevant basic and applied research on malaria. His current research focuses on understanding host−parasite interactions during the liver stage of malaria. His ultimate goal is to exploit this knowledge to develop infection prevention strategies.

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Francisca Lopes received her Ph.D. in Pharmaceutical Sciences from the University of Lisbon, Portugal (2000), and then became Assistant Professor at the Faculty of Pharmacy, University of Lisbon. Her current scientific interests lie in the area of medicinal and bioorganic chemistry applied to the design and synthesis of antimalarial agents targeting the liver and blood stages of malaria parasites. She has also been involved in prodrug chemistry, including the study of structure–reactivity and structure–metabolism relationships as tools to improve chemical and metabolic stability.

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■ ABBREVIATIONS USED

ACT, artemisinin combination therapy; LS, liver stages; qRT-PCR, quantitative real-time polymerase chain reaction; GFP, green fluorescent protein; PQ, primaquine; NCP, *N*-cyclopentyl; HIV, human immunodeficiency virus; DHFR, dihydrofolate reductase; HDAC, histone deacetylase; DHODH, dihydroorotate dehydrogenase

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