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Brief Article

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Quinolin-4(1*H*)-imines are Potent Antiplasmodial Drugs Targeting the Liver Stage of Malaria

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Supporting Information

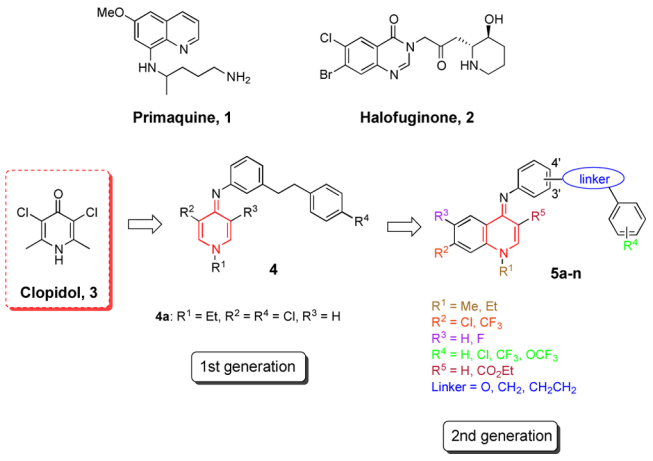
ABSTRACT: We present a novel series of quinolin-4(1*H*)-imines as dual-stage antiplasmodials, several-fold more active than primaquine in vitro against *Plasmodium berghei* liver stage. Among those, compounds **5g** and **5k** presented low nanomolar IC_{50} values. The compounds are metabolically stable and modulate several drug targets. These results emphasize the value of quinolin-4(1*H*)-imines as a new chemotype and their suitable properties for further drug development.

INTRODUCTION

Malaria remains a major public health threat worldwide, being responsible for high mortality and morbidity burdens in malaria-endemic countries.[1] Discovery of novel effective and safe antimalarials has been traditionally focused on targeting asexual parasitic stages in host erythrocytes that cause clinical symptoms.[2,3] However, full eradication of the disease requires intervention at the various developmental stages of the parasite within the host as well as in the mosquito vector.[4]

The liver stage of *Plasmodium* spp. infection is a mandatory life cycle step toward the generation of intraerythrocytic forms causing clinical symptoms.[5] Thus, targeting exoerythrocytic forms (EEFs) of the parasite offers clear advantages, as only full blocking of the clinically silent liver stage leads to true causal prophylaxis and consequently arrest of parasitic transmission. In some *Plasmodium* species, such as *Plasmodium ovale* and *Plasmodium vivax*, EEFs may persist in cryptic forms: hypnozoites, which can remain dormant for several years and, upon reactivation, lead to relapses, posing a massive challenge for malaria eradication.[6,7] Despite the benefits of targeting the liver stage of malaria,[8] only in the recent past efforts have been made to discover new drugs. Primaquine (Scheme 1, **1**) is the only clinically approved drug that eliminates liver forms of *Plasmodium*, including hypnozoites, while displaying moderate blood stage potency. However, the methemoglobinemia side effect, shared with other 8-aminoquinolines,[9,10] limits its clinical use and urges the discovery of safer and more effective liver stage antimalarial drugs. Breaking with traditional antimalarial drug discovery efforts, focused mainly on the intraerythrocytic stage of infection, we and others have initiated programs toward the discovery of antimalarials with new chemotypes and differentiated modes of action that target the underexploited liver stage of infection.[11−19] For example, halofuginone, **2**, was discovered to block early- and late-stage parasite development in the liver.[20]

Scheme 1. Structures of Primaquine, Halofuginone, Clopidol, 4a, and Quinolin-4(1*H*)-imines 5a−n



Here, we disclose a series of novel, dual-stage antiplasmodial quinolin-4(1*H*)-imine inhibitors. In particular, **5g** and **5k** kill *Plasmodium* liver stages in low nanomolar concentrations. We

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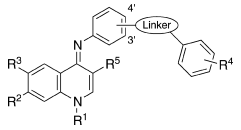
Table 1. Structure and Antiplasmodial Activity of Quinolin-4(1*H*)-imines 5a−n, 4a, Clopidol, Primaquine (PQ), Chloroquine (CQ), and Halofuginone (HF) and Atovaquone (ATV)



							antiplasmodial activity (IC_{50}, μM)				
compd	R^1	R^2	R^3	R^4	R^5	linker	blood stage[a]	liver stage[b]	log (K_a, μM)[c]	hemozoin inhibition (IC_{50}, μM)	CC_{50} (μM)[d] (Sel. Index[e])
5a	Et	Cl	H	4-OCF_3	H	3′-$(CH_2)_2$	3.11	0.227	5.2	ND[f]	3.94 (17.4)
5b	Et	CF_3	H	4-OCF_3	H	3′-$(CH_2)_2$	1.67	0.235	4.9	ND	6.09 (25.9)
5c	Et	Cl	H	H	H		0.54	0.200	5.6	18.9	4.61 (23.6)
5d	Et	CF_3	H	H	H		0.59	0.575	5.7	17.0	7.04 (12.2)
5e	Et	Cl	H	H	H	4′-CH_2	1.69	0.589	5.0	ND	2.89 (4.91)
5f	Et	Cl	H	H	H	4′-O	0.89	0.214	5.4	ND	5.33 (24.9)
5g	Et	Cl	H	4-Cl	H	4′-O	1.09	0.087	5.1	16.2	2.69 (30.9)
5h	Et	Cl	H	4-OCF_3	H	4′-O	0.96	0.145	4.9	13.5	3.53 (24.3)
5i	Et	Cl	H	4-CF_3	H	4′-O	1.26	0.234	4.7	ND	3.20 (13.7)
5j	Me	Cl	H	4-CF_3	H	4′-O	1.08	0.229	4.9	ND	3.08 (13.4)
5k	Et	Cl	H	3-OCF_3	H	4′-O	1.18	0.081	5.1	15.4	5.16 (63.7)
5l	Et	CF_3	H	3-OCF_3	H	4′-O	1.56	0.537	3.9	ND	12.89 (24.0)
5m	Me	H	F	H	CO_2Et		5.88	>10	ND	ND	ND
5n	Et	H	F	H	CO_2Et		5.32	>10	ND	ND	ND
4a							3.46[21]	0.407	ND	ND	ND
clopidol							9.73[21]	1.46	4.7	ND	ND
PQ							3.3[32]	7.5	ND	ND	ND
CQ							0.052	ND	4.8	6.6	ND
HF							ND	0.017	ND	ND	ND
ATV							0.0012[21]	3.76×10^{-4}	ND	ND	ND

[a]Choroquine-resistant *P. falciparum* W2 strain. [b]*P. berghei*. [c]Binding to hematin. [d]HepG2 cells. [e]Selectivity index = CC_{50}/IC_{50}. [f]ND: not determined.

also present our efforts to unveil their mechanisms of action and potential polypharmacological profile.

RESULTS AND DISCUSSION

Having noticed that our previously reported pyridon-4(1*H*)-imines **4**, designed to target the mitochondrial cytochrome bc_1 complex from the parasite, using clopidol as starting point for the optimization process, were only moderately active against the multiresistant *Plasmodium falciparum* W2 strain[21] but significantly potent against *Plasmodium berghei*-infected Huh-7 hepatoma cells (Supporting Information (SI)), we reasoned that **4a** could serve as a lead structure for further improvement of anti liver stage activity. Importantly, **4a** showed higher potency than primaquine (IC_{50} = 0.4 vs 7.5 μM, Table 1 and Figure 1) in a standard in vitro assay using the Huh-7 hepatoma cell line. To optimize activity of **4a** against EEFs, we conducted docking studies and envisaged that an additional aromatic ring at the core scaffold would favor hydrophobic interactions at the Q_o binding pocket of cytochrome bc_1, a prominent drug target in liver stage malaria (SI).

5a−n were synthesized following procedures similar to those used to prepare their first generation counterparts[21] (Scheme 2A). 4-Phenoxyanilines were prepared via aromatic nucleophilic substitution followed by reduction of the nitro group to the aniline intermediates **8a−e** (Scheme 2B). Access to **5m** and **5n** required synthesis of the corresponding quinolone precursor, **9**, which was subsequently converted into the corresponding 4-chloroquinoline by reaction with $POCl_3$ (Scheme 2C). X-ray analysis of **5c**, and NOESY spectra in other cases, confirmed the

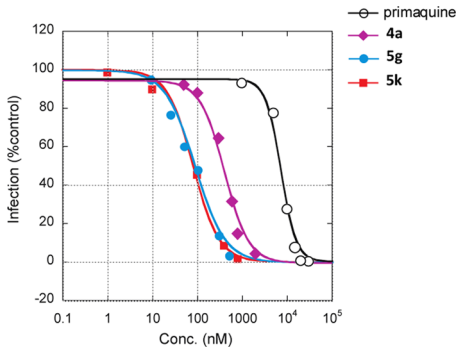


Figure 1. Concentration−response sigmoidal curves of primaquine, **4a**, **5g**, and **5k**. Huh-7 hepatoma cells infected with *P. berghei* were incubated with increasing concentrations of inhibitor.

stereochemistry of the C═N bond (SI). Only the *E* isomer was obtained, in accordance with previous reports.[22,23]

Having a series of novel quinolin-4(1*H*)-imines in hand, we tested the ability of these compounds to inhibit the liver stage of *P. berghei* in vitro (SI). In general, compounds **5** revealed moderate to high potency against EEFs, with IC_{50} values in the nanomolar range (Table 1). Derivatives **5g** and **5k** were ca. 94-fold more active than the reference compound primaquine in vitro (Figure 1) and present IC_{50} values in the same order of magnitude as the recently disclosed imidazopiperazines.[24] Also, **5g** and **5k** displayed an IC_{50} value in the same order of magnitude of halofuginone (IC_{50} = 17 nM) but were less potent than atovaquone (IC_{50} = 0.376 nM). For this series of compounds, an

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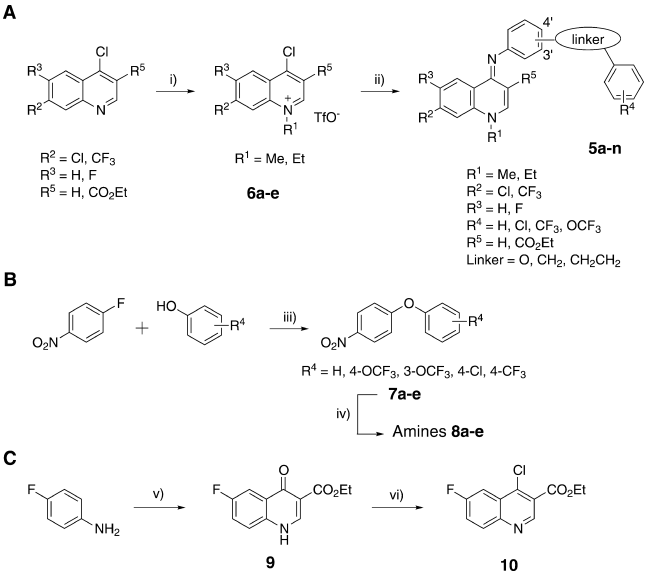
Scheme 2. Synthetic Pathway for 5a−n[a]



[a]Reagents and conditions: (i) toluene, methyl or ethyltriflate, rt; (ii) EtOH, TEA, amine, reflux; (iii) DMF, Na_2CO_3, reflux; (iv) MeOH, DCM, TES, Pd−C 10% or Sn, HCl, reflux; (v) diethyl dimethylaminomethylene malonate, reflux, (vi) $POCl_3$, reflux.

oxygen linker appears to be important for activity against the liver stage of infection (**5g**, **5k**). Furthermore, the anti liver stage activity does not correlate with the substituent electronic[25] and lipophilic[26] constants (**5f** vs **5g** vs **5h** vs **5j**). In contrast, **5m−n** exhibited only poor activity at the highest concentration tested of 10 μM. **5k** presented a selectivity index of ca. 64 and did not show detectable aggregation up to 10 μM, which could have led to measuring artifacts and false positive results (SI).

Next, we evaluated the antiplasmodial activity of the quinolin-4(1*H*)-imines **5a−n** against the *P. falciparum* W2 strain to determine whether these compounds would also be active against the blood stage of the infection. Contrasting with their potent anti liver stage activity, **5a−n** were only moderately active against the blood stage of infection (Table 1). Biaryl compounds **5c** and **5d** were the most potent within the series, presenting IC_{50} values of ca. 0.55 μM and suggesting that introduction of spacers between the two aromatic systems is detrimental for intra-erythrocytic activity. Regarding the 4-phenoxy derivatives, the substituent effect on the activity is apparently opposed to what had been reported for 4(1*H*)-pyridones[27] and pyridon-4(1*H*)-imines.[21] Overall, while the differences of activity could be explained on the grounds of dissimilar assay conditions and different parasite species, it is also possible that different mechanisms of action are at play in either life cycle stage.

Quinolin-4(1*H*)-imines **5** are structurally related to 4(1*H*)-quinolones, and several compounds of the latter class have shown antiplasmodial, blood and liver stage activities, via cytochrome bc_1 inhibition.[11,14−16,28−30] Hence, we investigated whether the activity of the present series of compounds was a consequence of bc_1 complex inhibition (SI). The mitochondrial electron transport-chain is used for maintaining the electrochemical gradient across the mitochondrial membrane and as ubiquinone regenerator for dihydroorotate dehydrogenase (DHODH) enzymatic activity. DHODH is a key enzyme for de novo pyrimidine biosynthesis, which *Plasmodium* spp. cannot salvage.[28] We selected one of the most (**5k**) and one of the least active (**5d**) compounds for these biochemical assays. As presented in Table 2, both compounds can inhibit cytochrome bc_1 but only in the micromolar range. Given the order of magnitude of these IC_{50} values, it is unlikely that inhibition of cytochrome bc_1 is their primary mode of antiplasmodial action. To confirm this hypothesis, we then carried out a series of experiments on a *Saccharomyces cerevisiae* cytoplasmatic DHODH-expressing *P. falciparum* Dd2 strain. In short, this transgenic strain can bypass the *P. falciparum* DHODH counterpart by using ScDHODH. Interestingly, ScDHODH uses cytoplasmatic fumarate instead of mitochondrial ubiquinone as final electron acceptor, thereby making the parasite resistant to both PfDHODH and bc_1 complex inhibitors.[31] Addition of proguanil restores sensitivity of the transgenic Dd2-yDHODH strain to bc_1 but does not show any effect with specific PfDHODH inhibitors. Overall, the results suggest that the mitochondrial electron transport chain is not the primary target of **5d** and **5k** (SI), as both compounds did not show loss of activity in the transgenic Dd2-yDHODH, with or without proguanil, versus the control Dd2 line.

Table 2. Sensitivity of Ubiquinol:Cytochrome bc_1 Activity in Isolated Mitochondria to 5d and 5k

IC_{50} ± SD (μM)		
5d	**5k**	atovaquone
28 ± 4.2	1.2 ± 0.21	0.0003 ± 0.0001

Alongside the mitochondrial electron transport chain, Tarun et al. had previously reported the redox metabolism, and the fatty acid synthesis II pathway as highly active in liver stage.[33] Thioredoxin reductase is a putative enzyme expressed in liver stage with ideal characteristics for drug targeting.[34] Furthermore, enoyl-acyl carrier protein reductase catalyzes the rate-limiting step of de novo fatty acid biosynthesis and its blockage is associated with reduced infectivity of the human host.[35,36] Screening of **5d** and **5k** against both enzymes did not show noticeable activity up to 50 μM (data not shown), ruling out these pathways as drug targets.

Because these compounds display a quinoline-based scaffold, it was also investigated whether they would bind to hematin, in resemblance to chloroquine. UV−visible spectroscopy was used to determine accurately the binding equilibrium constant, K_a (Table 1).[37] All experimental data fitted best to a 1:1 binding stoichiometry model. Under these experimental conditions, chloroquine presented a log K_a of 4.8, which is in accordance with literature values.[38] **5a−l** also bound to hematin with similar K_a as chloroquine, suggesting the inhibition of heme crystallization to hemozoin as a possible mechanism of action in blood stages of infection but not in liver stage, where this metabolic process does not occur. Building on these preliminary results, **5c**, **5d**, **5g**, **5h**, and **5k** were confirmed to inhibit heme crystallization (Table 1). Altogether, and opposing the current paradigm of compound selectivity, our data show that compounds **5** may exert dual stage inhibition through modulation of several distinct targets.

Finally, the microsomal stability of a selected set of quinolin-4(1*H*)-imines was studied by LC-MS/MS. All tested compounds displayed remarkable metabolic stability when incubated in rat liver microsomes, with half-lives ranging from 4 to 8 h (SI). The high metabolic stability may also suggest a low potential of quinolin-4(1*H*)-imines for drug−drug interactions.[39] Search for metabolites, performed both by MS scan and multiple reaction monitoring (MRM) experiments, allowed us to detect the

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corresponding 4-anilinoquinolines, formed presumably by *N*-dealkylation of parent compounds **5** (SI).

CONCLUSIONS

We have identified a new class and chemotype of dual stage inhibitors. Two compounds presented high potency against liver stage malaria. Our efforts to unveil the mechanisms of action of the synthesized quinolin-4(1*H*)-imines did not identify an unique target, e.g., inhibition of cytochrome bc_1 in the μM range is unlikely to account for the observed potent anti liver stage activity. In fact, modulation of multiple targets and potential polypharmacology cannot be excluded for this new chemotype of antiplasmodials. The global urgency in finding new therapies for malaria, especially against the underexplored liver stage, associated with (i) excellent activity of **5g** and **5k** in a phenotypic assay, (ii) chemical tractability, and (iii) suitable ligand efficiency[40] (LE = 0.31 for **5k**), warrants this novel chemotype of liver stage antiplasmodials further development.

EXPERIMENTAL SECTION

Synthesis of 5k. Synthesis of **5k**: Intermediates **6a** (1.0 molar equiv) and **8c** (1.1 molar equiv) were dissolved in ethanol absolute (3.5 mL/mmol). TEA (1 molar equiv) was added to the solution, which was refluxed for 20−24 h. The solvent was evaporated under reduced pressure, and the crude product was purified by flash chromatography, DCM:MeOH (9.5:0.5) to afford **5k** as a yellow oil; 85%. ^{1}H NMR (CD_3OD, 400.13 MHz) δ 1.56 (3H, t, J = 7.2 Hz, CH_3), 4.61 (2H, q, J = 7.2 Hz, CH_2), 6.81 (1H, d, J = 7.2 Hz, Ar-*H*3), 6.99 (1H, br s, Ar-*H*), 7.10 (2H, m, Ar-*H*), 7.25 (2H, d, J = 9.2 Hz, Ar-*H*), 7.46 (2H, d, J = 9.2 Hz, Ar-*H*), 7.51 (1H, t, J = 9.2 Hz, Ar-*H*), 7.82 (1H, dd, J = 8.8 and 1.6 Hz, Ar-*H*), 8.26 (1H, s, Ar-*H*), 8.38 (1H, d, J = 7.6 Hz, Ar-*H*2), 8.60 (1H, d, J = 8.8 Hz, Ar-*H*). ^{13}C NMR (CD_3OD, 100.61 MHz) δ 13.48, 49.72, 100.69, 111.22, 115.52, 116.89, 117.37, 120.44, 125.74, 126.89, 127.17, 127.33, 130.92, 139.11, 140.58, 146.13, 150.08, 152.20, 154.09, 155.55, 155.80, 158.32. IR (film): ν_{max} 1613, 1555, 1504, 1440, 1248, 1160, 1025 cm^{-1}. ESI-MS m/z (abund): 458.95 $[M + H]^+$ (100). Anal. Calcd ($C_{26}H_{22}ClF_3N_2O{\cdot}0.8CF_3SO_3H$): C, 51.45; H, 3.27; N, 4.84%. Found: C, 51.23; H, 3.39; N, 4.79%.

ASSOCIATED CONTENT

Supporting Information

Syntheses, methods for biochemical and cell-based assays, in vitro data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): G.S. is a scientific consultant to pharmaceutical industry and a co-founder of AlloCyte Pharmaceuticals Ltd, Basel.

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ABBREVIATIONS USED

ATV, atovaquone; CC_{50}, half-maximum cytotoxic concentration; CQ, chloroquine; DHODH, dihydroorotate dehydrogenase; EEF, exoerythrocytic form; HF, halofuginone; K_a, association constant; LE, ligand efficiency; MRM, multiple reaction monitoring; ND, not determined; PQ, primaquine; SD, standard deviation; Sel. Index, selectivity index; SI, Supporting Information; TEA, triethylamine; TES, triethylsilane

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