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Targeting the Erythrocytic and Liver Stages of Malaria Parasites with *s*-Triazine-Based Hybrids

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A diversity-oriented library of *s*-triazine-based hybrids was screened for activity against the chloroquine-resistant *Plasmodium falciparum* W2 strain. The most striking result was sub-micromolar activity against cultured erythrocytic-stage parasites of hybrid molecules containing one or two 8-aminoquinoline moieties. These compounds were not clearly toxic to human cells. The most effective blood-schizontocidal *s*-triazine derivatives were then screened for activity against the liver stage of malaria parasites. The *s*-triazine hybrid containing two 8-aminoquinoline moieties and one chlorine atom emerged as the most potent against *P. berghei* liver-stage infection, active in the low nanomolar region, combined with good metabolic stability in rat liver microsomes. These results indicate that *s*-triazine-8-aminoquinoline-based hybrids are excellent starting points for lead optimization as dual-stage antimalarials.

Introduction

Malaria is a potentially life-threatening disease caused by infection with parasites of the genus *Plasmodium* and transmitted to humans through the bite of *Anopheles* mosquitoes.[1,2] There are five known *Plasmodium* species that infect humans; the most common are *P. vivax*, which is predominant in South America and Asia, and *P. falciparum*, which is responsible for most of the mortality worldwide. A large and increasing problem is resistance of malaria parasites, in particular *P. falciparum*, to almost all available drugs.[3] Thus, the discovery and development of new effective and affordable antimalarial drugs remains essential to control and eliminate the disease.[3–5]

The development of hybrid antimalarial agents has been pursued as a strategy to improve potency and to overcome the emergence of drug resistance.[6,7] A key feature of hybrid drugs is the presence of different mechanisms of action against either a single target or different targets.[8] In addition, hybrid compounds have the potential to act against different life-cycle stages of malaria parasites, a required feature to facilitate the ultimate goal of eradicating malaria.[9] The asymptomatic, obligatory developmental phase of the parasite in the liver,[10] which precedes the formation of red blood cell infective forms, is of particular relevance in the context of multistage antimalarial agents. Targeting the liver stage of infection can provide a causal prophylactic strategy, and this could eliminate the parasites before they cause symptomatic bloodstream infections.[11] Action against liver-stage parasites is also critical to the control of *P. vivax* and *P. ovale* infections, as the life cycles of these parasites include cryptic hypnozoites that persist in the liver for long periods of time and that, upon reactivation, are responsible for relapses of malaria.[12] The 8-aminoquinoline primaquine (**1**, Figure 1) is the only registered drug for radical cure (elimination of dormant hypnozoites) of *P. vivax* and *P. ovale* malaria, and it is also active against the transient liver forms of all *Plasmodium* species.[3,13] Primaquine is also a potent gametocytocidal agent active against the blood-circulating sexual forms of the parasite that are transmitted to the mosquito upon a blood meal, and thus it is capable of interrupting the transmission of infection from the human host to mosquito vectors.[4,13] Primaquine is also active against the blood stage of malaria parasites, although its potency is low;[4,14] this limitation has been addressed by developing multistage hybrid compounds.[15,16]

s-Triazine (1,3,5-triazine) is a versatile scaffold that has found a wide range of applications in materials and health sciences.[17] *s*-Triazine derivatives have been reported to display different pharmacological properties,[18] including antimicrobial,[19] anticancer,[19,20] antiviral,[21,22] and antimalarial activities.[23–26] Dihydrofolate reductase (DHFR), which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, is considered the target of *s*-triazine-based antimalarials,[27] including cycloguanil (**2**, Figure 1), the active metabolite of proguanil.[28]

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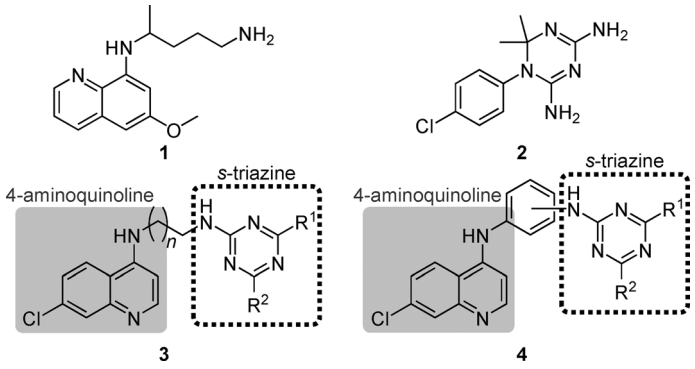


Figure 1. Structures of primaquine (**1**), cycloguanil (**2**), and antimalarial 4-aminoquinoline-*s*-triazine hybrids **3** and **4**. R^1 and R^2 are chlorine and/or substituted amines (aryl; alkyl; and tertiary, secondary, and primary amines).[31–40]

Resistance of the parasite to cycloguanil and other DHFR inhibitors is common and is mainly caused by mutations around the active site of the enzyme, which leads to dramatic decreases in the activities of the DHFR inhibitors.[29,30] For this reason, *s*-triazine-based hybrids have been developed with the aim to improve antimalarial activity and to prevent the emergence of resistant *P. falciparum* strains. Recent studies reported that 4-aminoquinoline *s*-triazine conjugates (e.g., compounds **3** and **4**, Figure 1) have promising activity against blood-stage *P. falciparum*.[31–40] Triazines have also been conjugated with endoperoxides,[41] a class of antimalarial agents that are highly active against the asexual erythrocytic stage of infection. Endoperoxides appear to require activation by iron(II), which accumulates inside the parasite food vacuole after digestion of large quantities of host hemoglobin, to interact with their target.[42–46] Overall, the activity of reported *s*-triazine hybrids against *P. falciparum* is improved relative to that of chloroquine, but their potential as dual-stage agents, capable of acting against the blood and liver stages of malaria parasites, remains to be explored.

Our previous experience with the synthesis of functionalized *s*-triazines[47–49] and our ongoing research on antimalarial compounds[16,50,51] led us to enlarge and screen our existing library of *s*-triazines against *Plasmodium* liver and erythrocyte stages. This library was enriched with the 8-aminoquinoline structural motif to target the liver stage. Herein, we report preliminary structure–activity data from this screen, which led to the identification of highly potent and metabolically stable dual-stage *s*-triazine conjugates.

Results and Discussion

Chemistry

The synthesis of substituted *s*-triazines from cyanuric chloride is a widely used method owing to the diversity of possible derivatizations.[48] The facile displacement of chlorine by various nucleophiles in the presence of a hydrochloride acceptor is a temperature-controlled process that allows the stepwise addition of nucleophiles.[52] The *s*-triazine library was generated by stepwise nucleophilic substitution of cyanuric chloride (**5**) with amines, alcohols, and one thiol (Figure 2) on the basis of our previously published procedures (Scheme 1).[48] Compounds **8c**, **8e**, and **8f** containing only one 8-aminoquinoline moiety were synthesized from intermediates **7a**, **7g**, and **7d**, respectively, by using a method based on reported procedures.[48] Compound **7b** containing two 8-aminoquinoline moieties was prepared directly from cyanuric chloride and was then converted into conjugate **8d** by heating at reflux in

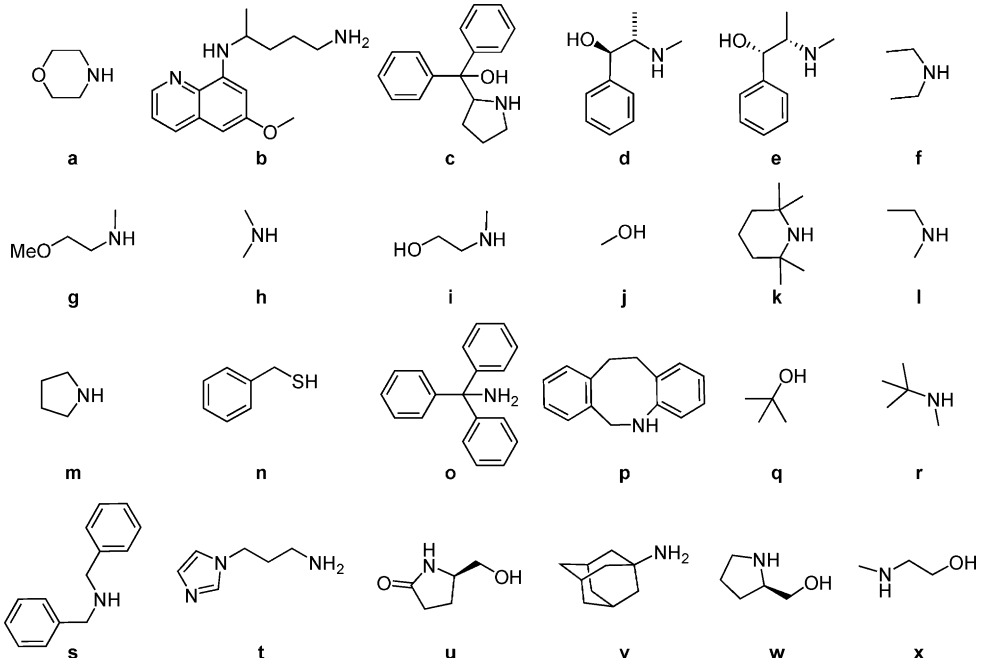
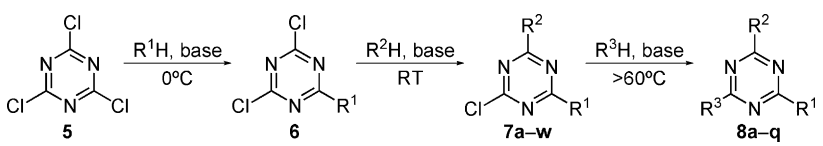


Figure 2. Diversity of *s*-triazine substituents used in this study.



Scheme 1. Temperature-controlled nucleophilic substitution of cyanuric chloride.

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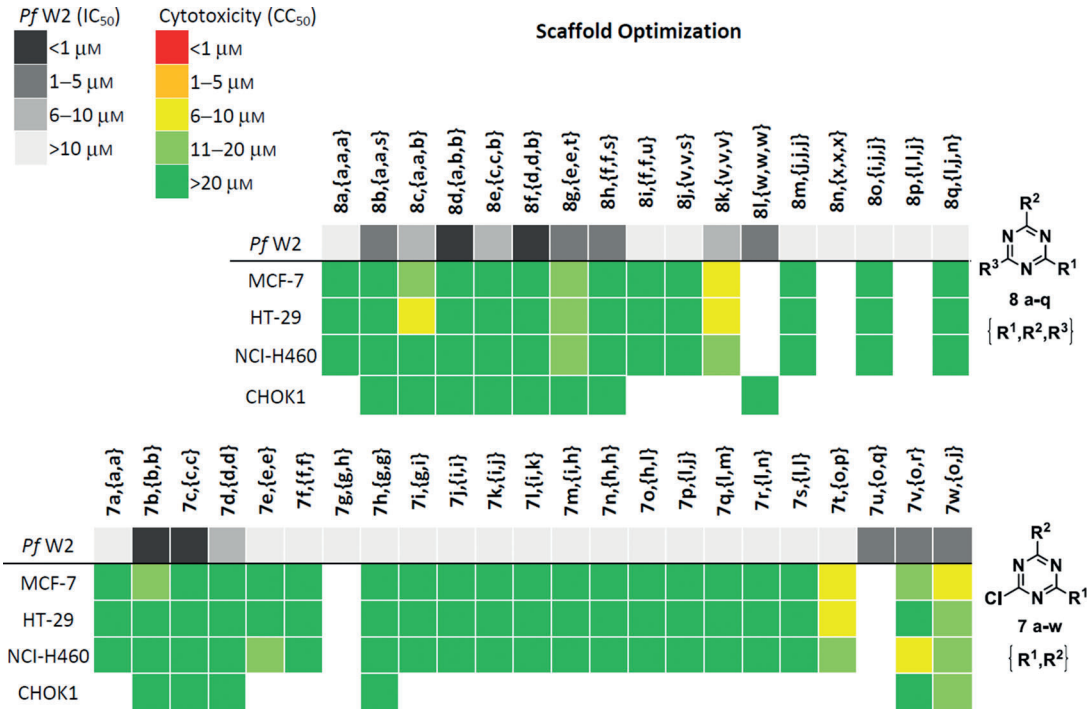

Figure 3.
Activity of all tested compounds against *P. falciparum* (*Pf*W2) and human cancer cell lines from breast (MCF-7), colon (HT-29), and lung (MCI-H460).

dioxane. Novel compounds not previously reported in the literature were characterized by both NMR spectroscopy and LC–MS.

Blood schizontocidal activity and cytotoxicity

The *s*-triazine-based library was evaluated in vitro for schizontocidal activity against the *P. falciparum* W2 strain (Figure 3, Table 1). The in vitro cytotoxicity of compounds **7** and **8** was evaluated by using mammalian CHOK1 cells. The selectivity index (SI) as expressed by the ratio $CC_{50}^{CHOK1}/IC_{50}^{W2}$ (CC_{50} = half-maximal cytotoxicity concentration, IC_{50} = half-maximal inhibitory concentration) calculated for these three cell lines ranges from 1 to >90, and compounds **7b**, **7c**, **8d**, and **8f** display the highest SI values (>30 to >90; Table 1). In addition, toxicity was also assessed against three cancer cells lines: MCF-7 (breast), HT-29 (colon), and NCI-H460 (lung). Most of the *s*-triazines were poorly active against these cancer cells lines.

Compounds **7b** containing two 8-aminoquinoline moieties and **7c** with two 2-(hydroxydiphenylmethyl)pyrrolidine groups present the highest potency among compounds **7**, both with an IC_{50} value of 0.6 μM (Table 1). Interestingly, compounds **7** containing two 2-amino-1-phenylethanol (ephedrine) moieties (i.e., compounds **7d** and **7e**) reveal a strong dependence of activity on the stereochemistry at C1 and C2. Whereas derivative **7d** with two (1*R*,2*S*)-ephedrine groups displays moderate antiplasmodial activity (IC_{50} = 7.7 μM), its counterpart with two (1*S*,2*S*)-ephedrine moi-

Table 1. In vitro antimalarial activity and cytotoxicity of *s*-triazine hybrids.

Compd	Log *P*[a]	IC_{50} [μM][c]	CC_{50} [μM][c]				SI[d]
		*Pf*W2[b]	MCF-7	HT-29	NCI-H460	CHOK1	
7b,{b,b}	6.40	0.60±0.01	20.6±8.4	>25	>25	>20	>33
7c,{c,c}	6.21	0.60±0.01	>25	>25	>25	>20	>33
7d,{d,d}	4.37	7.7±0.2	>25	>25	>25	>20	>3
7v,{o,r}	6.98	1.1±0.3	10.8±3.4	>25	8.95±1.7	>20	>18
7w,{o,j}	5.66	2.7±0.1	<10	20.5±1.2	16.4±1.2	11.5±1.0	>4
8b,{a,a,s}	3.81	3.5±0.1	>25	>25	>25	>20	>6
8c,{a,a,b}	3.47	5.6±0.3	11.6±1.3	<10.0	>25	>20	>4
8d,{a,b,b}	5.97	0.48±0.38	>25	>25	>25	>20	>42
8e,{c,c,b}	8.00	8.3±5.2	>25	>25	>25	>20	>2
8f,{d,d,b}	6.11	0.22±0.05	>25	>25	>25	>20	>91
8g,{e,e,t}	4.23	1.6±0.2	13.8±1.0	12.4±1.4	13.6±3.7	>20	>12
8h,{f,f,s}	6.01	3.4±0.2	>25	>25	>25	>20	>3
8k,{v,v,v}	6.30	6.4±0.3	10.3±1.1	9.47±1.62	12.8±2.0	ND	ND
8l,{w,w,w}	0.59	2.2±0.4	ND	ND	ND	>20	>9
ART	2.52	0.02±0.003	>20	>20	>20	>20	>1000
CQ	5.28	0.03±0.009	>20	10.4±3.3	>20	8.5±2.7	>283
PQ	2.76	3.3[53]	>25	>25	>25	>20	>6

[a] Calculated log *P* values (source: www.vcclab.org/lab/alogps/start.html). [b] *Plasmodium falciparum* W2 (*Pf*W2). [c] Data represent the mean±SD of $n=2$ independent experiments performed in quadruplicate. [d] Selectivity index (SI): CC_{50} (CHOK1)/IC_{50}. Abbreviations: artemisinin (ART), chloroquine (CQ), primaquine (PQ), not determined (ND).

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eties (i.e., compound **7e**) is inactive. Nevertheless, replacement of the chlorine moiety in **7e** with an *N*-(3-*a*minopropyl)imidazole group leads to a significant increase in potency (**8g**: $IC_{50}=1.62$ μM). The triphenylmethylamine subseries (i.e., compounds **7t**–**w**) also includes members with good activity, in particular compound **7v** with an IC_{50} value of 1.1 μM.

The most effective trisubstituted *s*-triazines **8b**–**h**, **8k**, and **8l** present IC_{50} values ranging from 0.22 to 8.31 μM. The most striking result is the high antiplasmodial activity of hybrid members containing two (i.e., compound **8d**) or one (i.e., compound **8f**) primaquine moiety, with IC_{50} values of 0.48 and 0.22 μM, respectively. Interestingly, other trisubstituted *s*-triazines containing only one 8-aminoquinoline substituent are significantly less potent than **8f** (i.e., **8c**: $IC_{50}=5.63$ μM, **8e**: $IC_{50}=8.3$ μM), which suggests that the (1*R*,2*S*)-ephedrine groups in **8f** might be beneficial for activity. This result, together with that obtained for (1*R*,2*S*)-ephedrine derivative **7d**, is in line with the recent finding that 2-amino-1-phenylethanol derivatives are useful scaffolds to develop potent antimalarial agents.[54] Other trisubstituted *s*-triazines with good antiplasmodial activity are those containing dibenzylamine groups, that is, compounds **8b** and **8h**, or a *N*-(3-aminopropyl)imidazole group, that is, compound **8g**. The remaining compounds resulting from combinations of the *s*-triazine core with other amines, alcohols, and benzylmercaptan were found to be inactive.

Liver schizontocidal activity

s-Triazine derivatives active against the erythrocytic stage of infection (Table 1) were further evaluated for their ability to inhibit the development of malaria parasites in liver cells. A total of 14 compounds were assayed at two different concentrations by using an in vitro infection model that employs a human hepatoma cell line (Huh7) infected with *P. berghei* expressing firefly luciferase. Results were compared with those obtained with primaquine (Figure 4). Most compounds were poorly active at the lowest concentration tested (1 or 2 μM). Exceptions were 8-aminoquinoline-containing derivatives **7b**, **8d**, and **8f**, which, at this dose, led to >50% inhibition of the infection relative to the controls, whereas they almost suppressed infection at 10 μM without significantly affecting Huh7 cell proliferation. These results are in sharp contrast to those for 8-aminoquinoline-containing derivatives **8c** and **8e**, which did not demonstrate liver-stage activity; this indicates that the presence of the 8-aminoquinoline moiety does not necessarily convey potency against the liver stage. Compounds **7c**, **7v**, and **8g**, lacking an 8-aminoquinoline substituent, also displayed excellent activity at 10 μM concentrations without affecting Huh7 cell confluence.

With these promising results in hand, we then determined the IC_{50} values for **7b** and **8f** against the liver stage of *P. berghei* infection of Huh7 cells. These compounds were 345 and 8 times more potent than primaquine, respectively (Table 2). Of note, primaquine is only moderately active in this assay, as the drug requires metabolic activation to display potent activity.[55] Also, hepatoma cells are metabolically less competent than hepatocytes in activating primaquine.[56] Compounds **7b** and **8f** were less potent than atovaquone, an inhibitor of parasite cytochrome bc_1 that has potent liver-stage activity and that does not require metabolic activation.[57] Overall, these results suggest that the 8-aminoquinoline pharmacophore does not

Table 2. IC_{50} values for the inhibition of infection of human hepatoma cells (Huh7) by *P. berghei*, and half-lives for the degradation of **7b** and **8f** in rat liver microsomes.

Compd	IC_{50} [nM][a]	$t_{1/2}$ [h]	k_{obs} [h^{-1}]
7b	27.5±1.5	2.4	0.32
8f	1210±53.8	5.9	0.12
PQ	9500±2300	ND	ND
ATV	1.10±0.03	ND	ND

[a] Data represent the mean±SD of $n=3$ independent experiments performed in triplicate; ND: not determined.

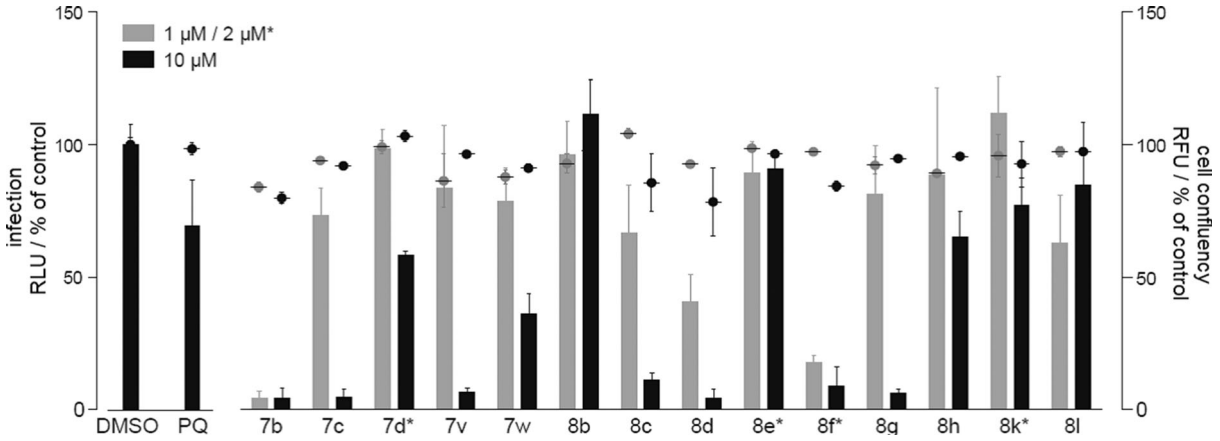

Figure 4. Luminescence-based measurement of dose-dependent effects of selected *s*-triazine derivatives on *P. berghei*-infected Huh7 cells. Bars represent infection loads and dots represent cell confluency. Results are expressed as mean±SD (triplicate wells). PQ: primaquine, used as positive control. DMSO: solvent-treated control.

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require metabolic activation if associated with the *s*-triazine core.

Metabolic stability

The microsomal stability of *s*-triazine hybrids **7b** and **8f** was determined by using rat liver microsomes (Table 2). Both **7b** and **8f** displayed good metabolic stability, with half-lives of 2.4 and 5.9 h, respectively. Neither primaquine nor its oxidative deamination product, carboxyprimaquine,[13] were detected in the incubation mixtures, which suggests that other metabolic pathways might be operating for **7b** and **8f**. In addition, these hybrids did not degrade upon incubation in 80% human plasma for 3 days.

Conclusions

We reported that *s*-triazines **7** and **8** decorated with 8-aminoquinoline side chains are a new family of efficient dual-stage antiplasmodial agents endowed with good metabolic stability. *s*-Triazine derivatives inhibited the development of intra-erythrocytic forms of *P. falciparum,* with IC_{50} values ranging from the low micromolar to the nanomolar range, while displaying low cytotoxicity against mammalian cells. The most effective compounds against the erythrocytic stage of *P. falciparum* were then screened for activity against the liver stage. Analysis of the structure–activity relationships revealed that the presence of (1*R*,2*S*)-ephedrine and 8-aminoquinoline moieties was beneficial for dual antiplasmodial activity. However, the presence of an 8-aminoquinoline moiety was not an absolute requirement to deliver dual-acting *s*-triazines, as some compounds without this moiety (e.g., **7c**, **7v**, and **8g**) were also active. Compounds **7b** and **8f** are the more promising from this library; they presented potent activity against both blood (IC_{50} = 0.60 and 0.22 μM, respectively) and liver (IC_{50} = 0.028 and 1.21 μM, respectively) stages combined with high selectivity indices. These compounds are significantly more potent than primaquine. Additionally, none of the compounds significantly affected Huh7 cell proliferation, which indicates that triazine–primaquine hybrids are selective and nontoxic antimalarial agents.

Experimental Section

Chemistry

General: All reagents used were purchased from Aldrich or Merck and were used without further purification. Evolution of the reactions was followed by TLC by using silica Merck Kieselgel 60 F_{254} plates with inspection under ultraviolet light at λ = 254 and 325 nm or stain solution of phosphomolybdenum acid in ethanol. Purifications were performed by preparative TLC plates by using Merck Kieselgel 60 F254 silica for thin-layer chromatography. The compounds were characterized by NMR spectroscopy with a Bruker Avance II 400 and 300 Ultrashield Plus, by elemental analysis performed at the Faculty of Pharmacy of University of Lisbon services, by infrared spectroscopy with a Shimadzu IRAffinity-1 FTIR spectrometer by using KBr platelets, and by mass spectrometry with a Micromass Quattro Micro triple quadrupole (Waters, Ireland) with an electrospray (ESI) ion source.

***N*^{1},*N*$^{1'}$-(6-Chloro-1,3,5-triazine-2,4-diyl)bis[*N*^{4}-(6-methoxyquinolin-8-yl)pentane-1,4-diamine] (7b)**: Primaquine biphosphate was first treated with aqueous KOH (6 equiv) to remove phosphoric acid. The solution was then extracted with dichloromethane, dried with Na_2CO_3, filtered, and concentrated under vacuum. Dioxane (2 mL) was added at 0 °C to a mixture of cyanuric chloride (15.7 mg, 0.085 mmol), primaquine (70 mg, 0.268 mmol, 3.15 equiv), and *N*,*N*-diisopropylethylamine (DIPEA; 51 μL, 0.293 mmol, 3.4 equiv). After 45 min of stirring, the solution was warmed up to room temperature, stirred for another 1.25 h, and then heated at reflux for 20 h. After cooling, the solvent was removed under vacuum, and the resulting crude material was purified by preparative TLC (EtOAc/hexane 1:1); the fraction at R_f = 0.50 gave the desired compound as a yellow foam. Yield: 27.3 mg (53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.51 (s, 1H), 7.89 (d, J = 6.7 Hz, 1H), 7.38–7.16 (m, 1H), 6.28 (d, J = 17.2 Hz, 2H), 6.10–5.88 (m, 2H), 3.86 (d, J = 6.0 Hz, 3H), 3.61 (s, 1H), 3.37 (s, 2H), 1.68 (s, 4H), 1.26 ppm (d, J = 4.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.2, 165.9, 159.4, 144.4, 144.9, 135.3, 134.7, 129.9, 121.8, 96.7, 91.6, 55.1, 47.7, 40.8, 33.8, 25.9, 20.5 ppm. IR (KBr): $\tilde{\nu}$ = 3385, 3263, 2958, 2931, 2856, 1610, 1577, 1544, 1388 cm^{-1}. MS (ESI+): *m*/*z*: 652.42 $[M+Na]^+$, 630.40 $[M+H]^+$. HRMS (IEA): *m*/*z*: calcd for $C_{33}H_{40}ClN_9O_2$: 629.2993; found: 629.3001.

***N*^{1}-(4,6-Dimorpholino-1,3,5-triazin-2-yl)-*N*^{4}-(6-methoxyquinolin-8-yl)pentane-1,4-diamine (8c)**: Dioxane (1 mL) was added at 0 °C to a mixture of cyanuric chloride (22.2 mg, 0.119 mmol), primaquine (30.2 mg, 0.114 mmol, 1 equiv), and DIPEA (75 μL, 0.433 mmol, 3.6 equiv). After stirring for 15 min, the solution was warmed to room temperature and morpholine (52 μL, 0.602 mmol, 5 equiv) was added. The mixture was stirred at room temperature for 50 min and then heated at reflux for 19 h. After cooling, the solvent was removed under vacuum, and the resulting crude material was purified by preparative TLC (EtOAc/hexane 1:1); the fraction at R_f = 0.40 gave the desired compound as a yellow foam. Yield: 38.6 mg (57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.54 (dd, J = 4.2, 1.6 Hz, 1H), 7.94 (dd, J = 8.3, 1.6 Hz, 1H), 7.33 (dd, J = 8.2, 4.2 Hz, 1H), 6.35 (d, J = 2.5 Hz, 1H), 6.29 (d, J = 2.5 Hz, 1H), 6.03 (d, J = 8.3 Hz, 1H), 3.91 (s, 3H), 3.73 (s, 8H), 3.71 (s, 8H), 3.66 (m, 1H), 3.51–3.29 (m, 2H), 1.91–1.62 (m, 4H), 1.32 ppm (d, J = 6.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 159.4, 145.0, 144.3, 135.3, 134.8, 129.9, 121.8, 96.6, 91.5, 66.8, 55.2, 47.8, 43.6, 40.6, 34.2, 26.6, 20.6 ppm. IR (KBr): $\tilde{\nu}$ = 3368, 2959, 2918, 2895, 2851, 1499, 1431, 1389 cm^{-1}. MS (ESI+): *m*/*z*: 531.37 $[M+Na]^+$, 509.56 $[M+H]^+$. HRMS (IEA): *m*/*z*: calcd for $C_{26}H_{36}N_8O_3$: 508.2910; found: 508.2924.

***N*^{1},*N*$^{1'}$-(6-Morpholino-1,3,5-triazine-2,4-diyl)bis[*N*^{4}-(6-methoxyquinolin-8-yl)pentane-1,4-diamine] (8d)**: Dioxane (2 mL) was added at 0 °C to a mixture of cyanuric chloride (27.1 mg, 0.147 mmol), primaquine (81.9 mg, 0.315 mmol, 2.1 equiv), and DIPEA (99 μL, 0.566 mmol, 3.8 equiv). After stirring for 20 min, the solution was warmed to room temperature. The mixture was stirred at room temperature for 50 min. Morpholine (23 μL, 0.275 mmol, 5 equiv) was added, and the mixture was heated at reflux for 18 h. After cooling, the solvent was removed under vacuum, and the resulting crude material was purified by preparative TLC (EtOAc/hexane 1:1); the fraction at R_f = 0.20 gave the desired compound as a yellow foam. Yield: 71.1 mg (63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.52 (dd, J = 4.2, 1.6 Hz, 2H), 7.91 (dd, J = 8.2, 1.5 Hz, 2H), 7.29 (dd, J = 8.2, 4.2 Hz, 2H), 6.32 (d, J = 2.5 Hz, 2H), 6.27 (d, J = 2.0 Hz, 2H), 6.01 (d, J = 8.1 Hz, 2H), 3.88 (s, 6H), 3.76–3.54 (m, 10H), 3.36 (d, J = 5.7 Hz, 4H), 1.70 (qd, J = 14.5, 8.2 Hz, 8H, 4CH_2), 1.28 ppm (d, J = 6.3 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 159.4, 145.0, 144.3, 135.4, 134.8, 129.91, 121.8, 96.7, 91.6, 66.8, 55.2, 47.8, 43.6, 40.6, 34.1, 26.6,

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20.6 ppm. IR (KBr): $\tilde{\nu}$ = 3377, 3329, 3314, 3267, 3246, 2959, 2926, 2853, 1545, 1501, 1443, 1422, 1387 cm^{-1}. MS (ESI+): *m/z*: 681.63 $[M+H]^+$. HRMS (IEA): *m/z*: calcd for $C_{37}H_{48}N_{10}O_3$: 680.3911; found: 680.3911.

{1,1′-[6-({4-[(6-Methoxyquinolin-8-yl)amino]pentyl}amino)-1,3,5-triazine-2,4-diyl]bis (pyrrolidine-2,1-diyl)}bis(diphenylmethanol) (8e): A solution of compound **7c** (25.3 mg, 0.041 mmol), primaquine (11.5 mg, 0.045 mmol, 1.1 equiv), and DIPEA (10 μL, 0.057 mmol, 1.4 equiv) in dioxane (1 mL) was heated at reflux for 20 h. After cooling, the solvent was removed under vacuum, and the resulting crude material was purified by preparative TLC (EtOAc/hexane 2:8); the fraction at R_f = 0.14 yielded the desired compound as a yellow foam. Yield: 26 mg (76%). ^{1}H NMR (400 MHz, DMSO, 80 °C): δ = 8.52 (dd, *J* = 9.5, 3.4 Hz, 1H), 8.05 (d, *J* = 8.3 Hz, 1H), 7.46–7.11 (m, 20H), 6.70 (s, 1H), 6.47 (d, *J* = 2.3 Hz, 1H), 6.28 (d, *J* = 1.9 Hz, 1H), 6.09 (d, *J* = 7.2 Hz, 1H), 3.84 (s, 3H), 3.69 (d, *J* = 7.8 Hz, 2H), 3.61 (d, *J* = 5.4 Hz, 1H), 3.20 (s, 2H), 2.97 (s, 2H), 2.00 (s, 2H), 1.85 (s, 2H), 1.71–1.34 (m, 8H), 1.21 (d, *J* = 7.1 Hz, 3H), 1.01 ppm (s, 2H).^{13}C NMR (101 MHz, [D$_6$]DMSO, 80 °C): δ = 164.7, 159.7, 147.5, 145.4, 145.4, 144.6, 135.3, 135.1, 130.1, 128.3, 128.1, 127.6, 127.5, 127, 126.9, 122.4, 96.7, 92.6, 82.1, 55.5, 47.8, 40.6, 34.3, 29.3, 26.5, 22.8, 20.8 ppm. IR (KBr): $\tilde{\nu}$ = 3275, 3169, 2961, 2930, 2874, 1535, 1448, 1425, 1389 cm^{-1}. MS (ESI+): *m/z*: 841.39 $[M+H]^+$. HRMS (ESI-FIA-TOF): *m/z*: calcd for $[C_{52}H_{56}N_8O_3+H]^+$: 841.4548; found: 841.4550.

(1*R*,1′*R*,2*S*,2′*S*)-2,2′-{[6-({4-[(6-Methoxyquinolin-8-yl)amino]pentyl}amino)-1,3,5-triazine-2,4-diyl]bis(methylazanediyl)}bis(1-phenylpropan-1-ol) (8f): A solution of compound **7d** (101.2 mg, 0.23 mmol), primaquine (66 mg, 0.25 mmol, 1.1 equiv), and DIPEA (47 μL, 0.27 mmol, 1.2 equiv) in dioxane (2 mL) was heated at reflux for 24 h. After 24 h, unreacted **7d** remained; more primaquine (33.2 mg, 0.125 mmol, 0.55 equiv) and dioxane (2 mL) were added, and the mixture was heated at reflux for an additional 24 h. After cooling, the solvent was removed under vacuum, and the resulting crude material was purified by preparative TLC (EtOAc/hexane 1:1); the fraction at R_f = 0.53 yielded the desired compound as a yellow foam. Yield: 51 mg (33%).^{1}H NMR (400 MHz, [D$_6$]DMSO, 80 °C): δ = 8.53 (dd, *J* = 4.1, 0.8 Hz, 1H), 8.05 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.40 (dd, *J* = 8.3, 4.2 Hz, 1H), 7.36 (d, *J* = 7.4 Hz, 4H), 7.22 (t, *J* = 7.3 Hz, 4H), 7.15 (t, *J* = 7.2 Hz, 2H), 6.47 (d, *J* = 2.4 Hz, 1H), 6.29 (d, *J* = 2.3 Hz, 1H), 6.12 (d, *J* = 8.4 Hz, 1H), 5.21 (s, 1H), 4.93 (s, 2H), 4.75 (d, *J* = 5.3 Hz, 2H), 3.83 (s, 3H), 3.65 (s, 1H), 3.29 (d, *J* = 4.4 Hz, 2H), 2.91 (s, 6H), 1.77–1.54 (m, 4H), 1.25 (d, *J* = 6.3 Hz, 3H), 1.14 (d, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, [D$_6$]DMSO, 80 °C): δ = 165.9, 165.4, 159.7, 145.3, 144.7, 144.6, 135.2, 135.1, 130.1, 128.0, 127.0, 126.7, 122.4, 96.8, 92.7, 75.7, 55.5, 55.3, 47.9, 40.8, 34.4, 30.1, 26.7, 20.7, 12.62 ppm. IR (KBr): $\tilde{\nu}$ = 3385, 3329, 3292, 3265, 3246, 3215, 3171, 3084, 3058, 2961, 2934, 2868, 2552, 2502, 1560, 1518, 1481, 1450, 1391, 1356, 1219, 1163, 1051, 1030, 822, 750, 702 cm^{-1}. MS (ESI+): *m/z*: 665.36 $[M+H]^+$. HRMS (IEA): *m/z*: calcd for $C_{38}H_{48}N_8O_3$: 664.3849; found: 664.3857,

Biology

Activity against erythrocyte-stage P. falciparum: Human erythrocytes infected with 1% ring-stage W2-strain *P. falciparum* strains synchronized with 5% sorbitol were incubated with test compounds in 96-well plates at 37 °C for 48 h in RPMI-1640 medium, supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 10% heat inactivated human serum (or 0.5% Albumax, 2% human serum), and 100 μM hypoxanthine under an atmosphere of 3% O_2, 5% CO_2, and 91% N_2. After 48 h, the cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS), transferred into PBS with 100 mM NH_4Cl, 0.1% Triton X-100, 1 nM YOYO-1, and infected erythrocytes were counted in a flow cytometer (FACSort, Beckton Dickinson; EX 488 nm, EM 520 nm). Values of IC_{50} based on comparisons with untreated control cultures were calculated by using GraphPad PRISM software. Two independent experiments were performed, each with four replicates for each of the experimental conditions.

Antiproliferative assays: Human cancer cell lines from breast (MCF-7), colon (HT-29), and lung (NCI-H460) were purchased from ATCC and cultivated in RPMI-1640 with L-glutamine and 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were plated in 96-well plates with a density of 5×10^4 (NCI-H460), 1×10^5 (HT-29), and 1.5×10^5 cells per well (MCF-7) and cultured for approximately 24 h. Stock solutions of the compounds to be tested were prepared in ethanol and then diluted with the cell culture medium with 0.5% FBS. Cells were incubated with the compounds at 0–20 μM concentration for 48 h. Cells were then washed with PBS and incubated with 0.5% FBS cell culture medium containing 50 μg mL^{-1} neutral red. Three hours later, cells were washed again with PBS, and the amount of neutral red retained by the cells was extracted with an organic solution (20 mL distilled water, 20 mL ethanol, and 400 μL glacial acetic acid). Absorbance of the samples was measured at λ = 540 nm in a plate reader after gentle shaking. Viability was determined by the ratio of absorbance of treated and control cells. Two independent experiments were performed, each with four replicates for each of the experimental conditions. IC_{50} values were determined by using GraphPad Prism 5.

Antiplasmodium liver-stage activity: Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% *v/v* fetal calf serum (FCS), 1% *v/v* nonessential amino acids, 1% *v/v* penicillin/streptomycin, 1% *v/v* glutamine, and 10 mM HEPES, pH 7, and maintained at 37 °C with 5% CO_2. Inhibition of *P. berghei* liver-stage infection was determined by measuring the luminescence of Huh-7 cell lysates 48 h after infection with a firefly luciferase-expressing *P. berghei* line, PbGFP-Luccon, as previously described[11,58] Briefly, cells (12×10^3 cells per well) were seeded in 96-well plates the day before drug treatment and infection. Tested compounds were prepared in the following way: 10 mM stock solutions were obtained by dissolving accurately weighed compounds in MeOH and dilutions were subsequently made with medium to the desired concentration. Medium was replaced by fresh medium containing the appropriate concentration of each compound 1 h prior to infection. Sporozoites (10000 spz per well), freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes, were added to the wells 1 h after compound addition. Sporozoite addition was followed by centrifugation at 1700 g for 5 min. At 24 h post-infection, medium was again replaced by fresh medium containing the appropriate concentration of each compound. Parasite load was determined 48 h after infection by luminescence measurement by using Biotium's Firefly Luciferase Assay Kit. The effect of the compounds on the viability of Huh-7 cells was assessed by the Alamar Blue assay (Invitrogen, UK) by using the manufacturer's protocol. Nonlinear regression analysis was employed to fit the normalized results of the dose-response curves, and IC_{50} values were determined by using SigmaPlot software. IC_{50} values presented are the mean IC_{50} value averaged from three independent experiments, each performed in triplicate wells.

Metabolism studies: The time courses of unchanged compounds **7b** and **8f** in microsomes were determined. Each compound was

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incubated with a reaction mixture (800 μL) consisting of rat liver microsomal protein (20 μL of 20 $mg_{protein}\,mL^{-1}$) suspended in 50 mM PBS (160 μL), water (570 μL), NADPH regenerating system solution A (40 μL, containing 31 mM $NADP^+$, 66 mM glucose 6-phosphatase, and 0.67 mM $MgCl_2$), and NADPH regenerating system solution B (8 μL, containing 40 U mL^{-1} glucose-6-phosphate dehydrogenase in 5 mM sodium citrate). After pre-incubation at 37 °C for 5 min, enzyme reactions were initiated by adding the parent solution of the drug (2 μL, 10^{-2} M) in DMSO. Samples were aliquoted (80 μL) at various time points and stopped by protein precipitation through addition of an equal volume of ice-cold acetonitrile. The mixtures were centrifuged at 10000 g for 10 min at room temperature. The supernatants were immediately analyzed, and the remaining parent drug versus time was quantified by HPLC.

The HPLC system consisted of a LichroCART 125-4 RP-18 (5 μm) analytical column on a LabChrom L7400 Merck Hitachi instrument, with a mixture of methanol/water (85:15) as eluent at a flow rate of 1 mL min^{-1}. HPLC detection was at the maximum of absorbance of compounds **7b** and **8f** ($\lambda = 266$ nm). The corresponding half-life values were determined in triplicate.

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Keywords: drug design · dual-stage antimalarials · hybrid compounds · molecular diversity · nitrogen heterocycles

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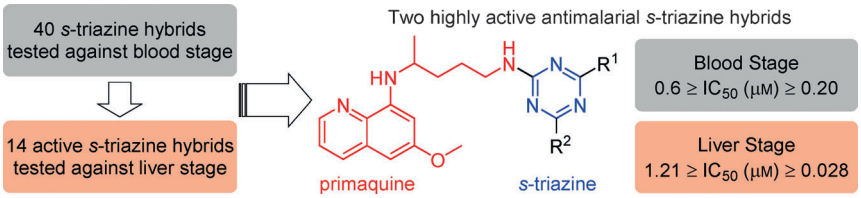

Double-edged sword to kill malaria parasites: *s*-Triazine-based hybrid compounds containing a primaquine moiety were found to be dual-stage antiplasmodial agents. The two hybrids are highly potent against both the liver and blood stages of the parasite's life cycle and show excellent metabolic stability.

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■■ – ■■

Targeting the Erythrocytic and Liver Stages of Malaria Parasites with *s*-Triazine-Based Hybrids

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