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4,9-diaminoacridines and 4-aminoacridines as dual-stage antiplasmodial hits

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Supporting information for this article is given via a link at the end of the document.

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Abstract: Multi-stage drugs have been prioritized in antimalarial drug discovery, as targeting more than one process in the *Plasmodium* life cycle is likely to increase efficiency, while decreasing the chances of emergence of resistance by the parasite. Herein, we disclose two novel acridine-based families of compounds that combine the structural features of primaquine and chloroquine. Compounds prepared and studied thus far retained the *in vitro* activity displayed by the parent drugs against the erythrocytic stages of chloroquine-sensitive and -resistant *P. falciparum* strains, and against the hepatic stages of *P. berghei*, hence acting as dual-stage antiplasmodial hits.

The eradication of malaria, one of the deadliest infectious diseases in the world,[1] remains to be achieved. This is partly due to the emergence of parasite resistance to virtually all the antimalarial drugs introduced in the clinic over the last decades.[1-2] In endemic areas, where chloroquine-resistant *P. falciparum* prevails, artemisinin combination therapies (ACT) are the antimalarial treatment currently recommended by the WHO. However, reported resistance to ACT in western Cambodia and across the Greater Mekong sub-region is threatening the efficacy of these first-line therapies, for which suitable alternatives remain unavailable.[1-3] Therefore, while an effective antimalarial vaccine remains elusive,[4] new efforts bend towards the discovery of multi-target antiplasmodials, capable of targeting more than one process in the malaria parasite's life cycle.[5] In the pursuit of such efforts, one must bear in mind that only cost-effective approaches towards affordable medicines will have a real impact in the fight against malaria, which mainly affects low to middle income countries. Thus, priority should be given to the repurposing of existing drugs for malaria,[6] or to the rescuing of antimalarial pharmacophores towards the development of new multi-target compounds.[7]

Quinacrine (**QN**, Figure 1) was the first clinically tested synthetic antimalarial drug;[8] it is a potent blood schizonticide, but its serious adverse effects led to its rapid replacement by chloroquine (**CQ**, Figure 1), whose efficiency, oral bioavailability,

and safety were far superior to those of QN.[2] Interestingly, "disassembly" of the QN structure shows it as a "merge" of the structure of CQ with the heterocycle core of primaquine (**PQ**, Figure 1). PQ is another emblematic antiplasmodial, active against all liver forms of the parasite, and able to block parasite transmission from the human host to the mosquito vector.[2] In view of this, and based on previous promising results for similar compounds,[9] we hypothesized that 4,9-diaminoacridines (**1**) and 4-aminoacridines (**2**) might act as multi-target antiplasmodials, since compounds **1** embed the structures of both CQ and PQ, and compounds **2** combine the heterocyclic core of CQ with PQ.
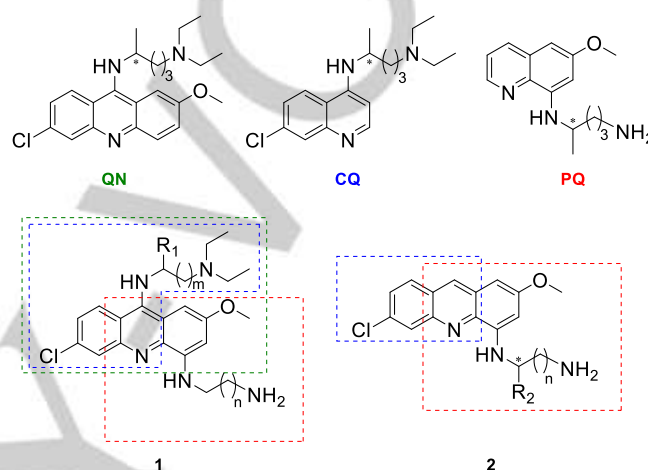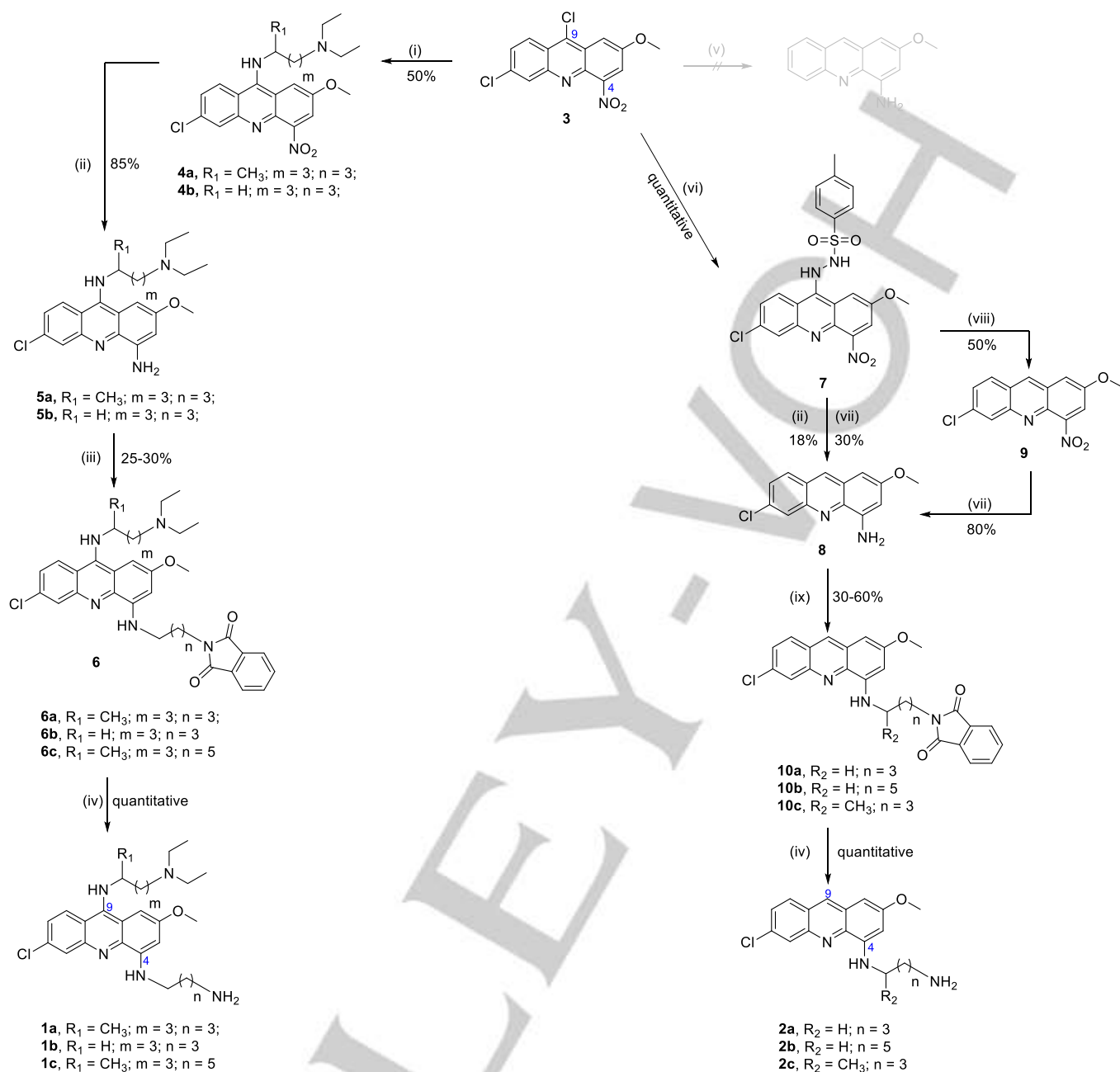
Figure 1. Structures of antimalarials quinacrine (**QN**) chloroquine (**CQ**), and primaquine (**PQ**) – top row; and of 4,9-diaminoacridines (**1**) and 4-aminoacridines (**2**) in this work – bottom row.

Scheme 1. Synthetic routes towards 4,9-diaminoacridines **1** and 4-aminoacridines **2**. *Reagents and conditions:* (i) phenol (15 equiv.), Cs$_2$CO$_3$ (1 equiv.), anhydrous DMSO, 4 Å molecular sieves, 100 °C, 2 h, then diamine (4 equiv.), 100 °C, 4 h; (ii) SnCl$_2$ (5 equiv.), 37% aq. HCl, 0→40 °C, 30 min; (iii) *N*-(n-bromoalkyl)phthalimide (3 equiv.), CH$_3$COONa (3 equiv.), CH$_3$CH$_2$OH, microwave (MW) heating (100 W, 120 °C) in a pressurized vial (100 psi), 2.5 h; (iv) hydrazine monohydrate (40 equiv.), tetrahydrofuran (THF), 60 °C, 72 h; (v) H$_2$ (50 psi), Pd/C, CH$_3$OH, rt, 5 h; (vi) *p*-TSH (1 equiv.), CHCl$_3$, rt, 24 h; (vii) hydrazine monohydrate (10 equiv), Pd/C (10% wt), CH$_3$OH, 80 °C, 1 h – 3 h; (viii) ethylene glycol/H$_2$O (3:1), Na$_2$CO$_3$ (0.0625 M), 95 ºC, 1.5 h; (ix) *N*-(n-bromoalkyl)phthalimide (3 equiv.), Et$_3$N (3 equiv.), CH$_3$CH$_2$OH, MW heating (100 W, 120 °C) in a pressurized vial (100 psi), 3 h.

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In view of the above, we have synthesized 4,9-diaminoacridines **1a-c** and 4-aminoacridines **2a-c**, as depicted in Scheme 1. The synthetic route towards 4,9-diaminoacridines **1** had been previously reported by us,[10] whereas the synthesis of 4-aminoacridines **2** starting from **3**, the common precursor to both compounds **1** and **2**, was established in the present work, as follows. The first step was the reduction of the nitro group, along with the removal of chlorine in position 9 of the acridine ring in 6,9-dichloro-2-methoxy-4-nitroacridine **3**; classical catalytic hydrogenation using $H_2(g)$ and Pd/C (step v in Scheme 1) did not afford the desired compound, as non-selective removal of the two chlorine atoms occurred (data not shown). Alternatively, we applied a procedure early reported by Scovill *et al.*,[9] whereby **3** was converted into the *p*-toluene sulfonyl hydrazide (*p*-TSH) **7** (step vi). This intermediate was subsequently reduced to aniline **8**, for which either $SnCl_2$/HCl (step ii) or hydrazine hydrate and Pd/C (step vii) were used. In both cases, the desired product was obtained, at 18 and 30% yields, respectively. An alternative two-step (steps viii and vii) procedure, via cleavage of the *p*-TSH moiety in **7** using Na_2CO_3 in aqueous ethylene glycol to give **9** and subsequent reduction of the latter with hydrazine hydrate and Pd/C to afford **8**, increased the overall yield to 40%. Aniline **8** was next alkylated with the appropriate *N*-(*N*-bromoalkyl)phthalimide, as previously reported by us.[10] This afforded compounds **10a-c** in moderate yields (30-60%), which were quantitatively converted into the respective final 4-aminoacridines **2a-c** by hydrazinolysis (step iv) using excess hydrazine monohydrate in refluxing tetrahydrofuran (THF). Detailed procedures and relevant spectroscopic data on intermediate and final compounds are given in the Supplementary Information.

Target compounds **1a-c** and **2a-c**, as well as **4a**, **5a**, and **6a**, all of which are synthetic precursors of **1a**, were evaluated *in vitro* for their (i) activity against erythrocytic forms of CQ-sensitive 3D7, and CQ-resistant W2 *P. falciparum* strains, (ii) haemolytic effects, (iii) activity against the hepatic stages of *P. berghei*, and (iv) toxicity to human hepatic cells (Huh7 cell line). All the compounds exhibited antimalarial activity against blood forms of CQ-sensitive (3D7) and CQ-resistant (W2) *P. falciparum* parasites (Table 1), although those of 4-aminoacridines **2** were quite modest. Interestingly, the 4,9-diaminoacridines **1** showed better activity than compounds **2**, emphasizing the importance of the aminoalkyl chain in position 9 of the acridine ring, in close similarity to the structures of **QN** and **CQ**. Also, our data suggest that the length of the side chains at both positions 9 and 4 of the acridine ring influences the activity of 4,9-diaminoacridines **1**. Specifically, increasing the length of the side chain at position 4 from four (**1a**) to six (**1c**) carbons improved the activity against 3D7 and W2 strains of *P.*

falciparum two-fold and twelve-fold, respectively. In turn, changes in the side chain at position 9 did not significantly affect activity against the 3D7 strain, but the IC_{50} value against the W2 strain decreased by nearly ten-fold when changing from four (**1a**) to three (**1b**) carbon atoms. Yet, since these two compounds also differ in R_1 (methyl in **1a** and hydrogen in **1b**), we cannot establish, at this stage, which of these two structural features, or both, lead to the observed difference, and why. Intermediates **4a**, **5a** and **6a** also showed interesting results, particularly against the CQ-sensitive 3D7 strain. Remarkably, neither any of these synthetic precursors of **1a**, nor any of the target compounds **1a-c** or **2a-c** were haemolytic up to 10 μM (Figure S1, Supp. Information).

Table 1. *In vitro* activity of **1a-c**, **2a-c**, and **9-11a**, against blood-stage *P. falciparum* (CQ-sensitive and -resistant strains 3D7 and W2, respectively) and liver-stage *P. berghei* parasites. Data for reference drugs are also included, for comparison.

Compound	IC_{50} (μM) blood-stage[c]		IC_{50} (μM) liver-stage[c]
	Pf 3D7	*Pf* W2	
4a	0.28 ± 0.20	0.91 ± 0.23	ND[a]
5a	0.45 ± 0.22	0.49 ± 0.24	ND
6a	0.16 ± 0.22	0.98 ± 0.24	ND
1a	0.68 ± 0.24	6.17 ± 0.23	11.02 ± 0.44
1b	0.44 ± 0.21	0.66 ± 0.25	ND
1c	0.26 ± 0.35	0.49 ± 0.23	ND
2a	5.64 ± 0.22	5.14 ± 0.23	2.22 ± 0.51
2b	>10	4.17 ± 0.23	1.64 ± 0.30
2c	>10	2.39 ± 0.24	2.04 ± 0.38
CQ	0.02[b]	0.23[b]	-
QN	0.10[b]	0.16[b]	-
PQ	-	-	7.5[b]

[a] ND: Not determined; [b] Value taken from our previous work.[11]; [c] IC_{50} determination was carried out in two independent biological replicates. In each determination, all compound concentrations were tested in three technical replicates.
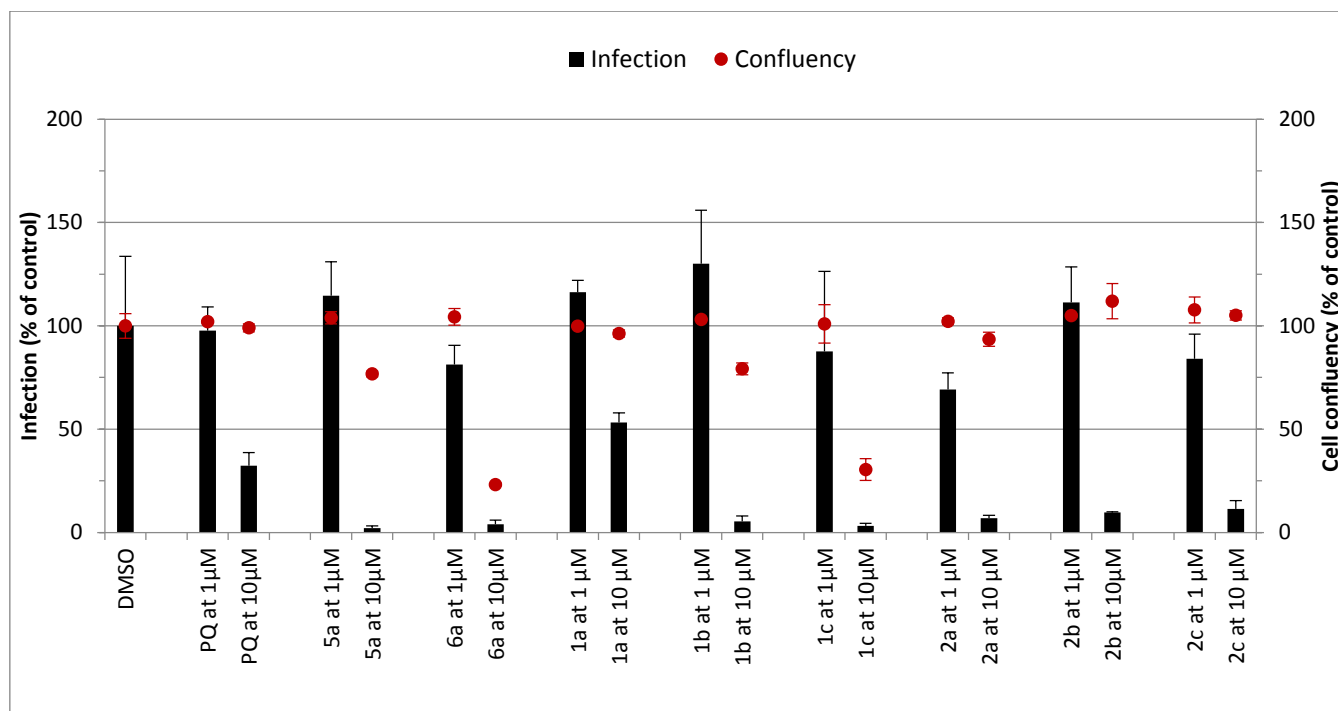
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Figure 2. Activity of **1a-c**, **2a-c**, **5a**, and **6a** against liver forms of *P. berghei*, and toxicity towards human hepatocytes (Huh7 cell line). PQ used as reference drug.

The effects of the compounds on hepatic *P. berghei* parasites and their host cells (Huh7) were further evaluated at 1 and 10 µM. As shown in Figure 2, 4-aminoacridines **2** appears to be slightly more active at 1 µM, except for **2b** that is inactive, than compounds **1** against liver stage *P. berghei*. On the other hand, at 10 µM both compounds **1** and **2** present similar activities, except for **1a** that is less active, but compounds **2** were not cytotoxic at that concentration unlike compounds **1**. The activities of compounds selected as most promising based on activity and cytotoxicity were quantitatively compared (Table 1). Relevantly, IC$_{50}$ values of compounds **2** were roughly 3 to 5 times lower than that of the reference liver stage antiplasmodial drug, **PQ**. This indicates the relevance of having the side chain at position 4 of the acridine ring, in close similarity with the structure of PQ itself, to retain selective action against liver-stage forms of *Plasmodia*, and which should be further confirmed with a more complete structure-activity relationships (SAR) study. Moreover, these results suggest that fusion of the chlorobenzene ring (affording the 4-aminoacridine moiety) to the original 8-aminoquinoline ring of parent **PQ** is advantageous for liver stage activity, with no apparent increase in compound cytotoxicity (**PQ** vs. **2a-c**, Figure 2). Regarding compounds **1** and their respective precursors, all but **1a** were significantly active at 10 µM, although some 4,9-diaminoacridines (**1c**, **5a** and **6a**) were highly cytotoxic at this concentration. Although the data set is not large enough to establish solid SAR, it seems that a decrease in length of the side chain at position 9 from **1a** to **1b** increases activity at the expense of a small increase in cytotoxicity. Interestingly, such small structural changes in the side chain at position 9, important to blood-stage activity, leads also to an increase in liver-stage activity (Figure 2). These results emphasize the importance of conducting an extensive SAR study to establish how liver stage activity can be modulated through structural changes at position 9. We also observed that

increasing length of the side chain at position 4 (**1a** vs. **1c**) seems to improve liver stage activity and cause a significant increase in cytotoxicity.

Collectively, our findings show that the compounds reported herein, which can be regarded as a structural merge between classical antimalarial drugs **PQ** and **CQ**, and also as analogues of **QN**, define two novel classes of antimalarial hits. Logically, optimization of these structures is needed towards the development of more potent dual-stage antiplasmodial action and lower cytotoxicity. Yet, the robust synthetic routes now established for both families, and the *in vitro* data obtained thus far pave the way to the attainment of this goal. Also, the most promising compounds will be further evaluated for their gametocytocidal activity, which may hopefully establish them as triple-stage antimalarial hits.

Experimental Section

Compounds **1** and **3-6** were prepared as previously described, and their structural analyses (Supp. Information) agreed with formerly reported data.[10]

Synthesis of 6-chloro-2-methoxy-4-nitro-9-[(*p*-toluenesulfonyl)hydrazine]acridine (7): a solution of *p*-toluenesulfonyl hydrazide in CHCl$_3$ was added dropwise to a solution of compound **3** in CHCl$_3$. After stirring for 24 h, product **7** was precipitated with cold CHCl$_3$ and isolated in quantitative yield by vacuum filtration.

Synthesis of 6-chloro-2-methoxy-4-aminoacridine (8): compound **7** and Pd/C (10% wt) were suspended in CH$_3$OH and the mixture refluxed at 80 ºC. Hydrazine hydrate (10 equiv.) was

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then added and the reaction mixture kept in reflux for 3 h or 1 h (step xiii or step xv, respectively). Pd/C was next removed by filtration through Celite, and the filtrate evaporated to dryness. The residue obtained was dissolved in CH_2Cl_2 and the solution obtained was washed with saturated aq. $NaCO_3$ (3 × 50 mL) followed by saturated aq. NaCl (1 × 50 mL). The organic layer was isolated and next dried over anhydrous $NaSO_4$, filtered, and the filtrate evaporated to dryness under reduced pressure. The crude product thus obtained was further purified by column chromatography on silica gel, using CH_2Cl_2 as mobile phase, affording **8**.

Synthesis of 6-chloro-2-methoxy-4-nitroacridine (9): compound **7** and Na_2CO_3 (6.25×10^{-2} M) were dissolved in ethylene glycol/water (18/9 mL). The reaction mixture was kept under reflux at 95 ºC for 1,5 h. Next, the mixture was poured onto cold water, and the resulting black precipitate was isolated by vacuum filtration and next dissolved in CH_2Cl_2. The resulting solution was washed with saturated aq. $NaHCO_3$ (3 × 50 mL) followed by saturated aq. NaCl (1 × 50 mL). The organic layer was isolated and then dried over anhydrous Na_2SO_4, and the filtrate evaporated to dryness under reduced pressure. The crude product thus obtained was purified by column chromatography on silica gel, using CH_2Cl_2 as mobile phase, affording **9**.

Synthesis of compounds 10a-c: triethylamine (3 equiv.) and the relevant *N-*(n-bromoalkyl)phthalimide (3 equiv.) were added to a solution of **8** in CH_3CH_2OH. The reaction mixture was transferred into a reaction vial that was next sealed and subjected to microwave (MW) irradiation (100 W); the reaction proceeded under MW heating for 3 h at 120 °C and 100 psi. Aliquots of triethylamine (3 equiv.) and the relevant *N-*(n-bromoalkyl)phthalimide (3 equiv.) were added to the reaction vial every 30 min. Once the reaction was halted, the solvent was removed under reduced pressure and the residue was next dissolved in CH_2Cl_2. The resulting solution was washed with saturated aq. $NaHCO_3$ (3 × 50 mL) and then with saturated aq. NaCl (1 × 50 mL). The organic layer was collected, dried over anhydrous Na_2SO_4 and filtered. The filtrate was next evaporated to dryness under reduced pressure, to afford the crude product that was partially purified by column chromatography on silica gel using hexane/ethyl acetate (4:1 v/v) as mobile phase. Fractions containing the target product were pooled and the solvent removed under reduced pressure. The residue was dissolved in CH_2Cl_2, 85.5% aq. H_3PO_4 was added dropwise, and the solution stirred for approximately 30 min. The liquid phase was removed by decantation and the precipitate formed, and it was washed three times with CH_2Cl_2, every time discarding the solvent also by decantation. Aq. 2 M NaOH was then added to the solid residue and the resulting solution was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was collected, dried over anhydrous Na_2SO_4 and filtered. The filtrate was next evaporated to dryness under reduced pressure to afford compounds **10a-c**.

Synthesis of compounds 2a-c: To a solution of the relevant compound **15** in THF, $NH_2NH_2.H_2O$ (40 equiv.) was added. The reaction mixture was left under stirring at 60 °C until the reaction was completed, according to TLC analysis (approximately 48 h). The solvent was removed by evaporation under reduced pressure, the residue dissolved in CH_2Cl_2, and washed with saturated aq. $NaHCO_3$ (3 × 50 mL) and saturated aq. NaCl (1 × 50 mL). The organic layer was collected and dried over

anhydrous Na_2SO_4, filtered, and the filtrate evaporated to dryness under reduced pressure to afford the target compounds **2a-c**. The compounds used in biological assays were confirmed to have at least 95% purity, based on peak areas obtained through HPLC analyses that were run as detailed in the Supporting Information.

***In vitro* liver-stage assays:** *In vitro* inhibition of liver-stage infection by test compounds was determined by measuring the luminescence intensity in Huh7 cells infected with a firefly luciferase-expressing *P. berghei* line, PbGFP-Luccon, as previously described.[12] Huh7 cells were cultured in RPMI 1640 medium supplemented with 10% v/v fetal calf serum, 1% v/v nonessential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2. For infection assays, Huh7 cells were seeded in 96-well plates the day before drug treatment and infection. Culture medium in the wells was replaced by infection medium (culture medium supplemented with 50 µg/mL gentamicin and 0.8 µg/mL amphotericin B) containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes. For control cells, culture medium was replaced by medium containing equivalent amounts of DMSO, the compounds solvent. After 48 h of infection, inhibition of infection was measured and the effect of the compounds on the viability of Huh7 cells was assessed by the AlamarBlue assay (Invitrogen, UK) 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 using GraphPad Prism 8.0 (GraphPad software, La Jolla California USA).

Cytotoxicity was inferred from the cell confluency data. As a control, Huh7 cells were infected in the presence of a percentage of DMSO that mimics that of compound samples (0.01%), and that is known to not be cytotoxic. All compound data was then normalized to the DMSO control. Therefore, a reduction in cell confluency, as compared to the DMSO control, is indicative of cytotoxicity.

Ethics statement: The human blood used in this work was commercially obtained from the *Banc de Sang i Teixits* (www.bancsang.net). Blood was not specifically collected for this research; the purchased units had been discarded for transfusion, usually because of an excess of blood relative to anticoagulant solution. Prior to their use, blood units underwent the analytical checks specified in the current legislation. Before being delivered to us, unit data were anonymized and irreversibly dissociated, and any identification tag or label had been removed in order to guarantee the non-identification of the blood donor. No blood data were or will be supplied, in accordance with the current Spanish *Ley Orgánica de Protección de Datos* and *Ley de Investigación Biomédica*. The blood samples will not be used for studies other than those made explicit in this research. The studies reported here were performed in accordance with the current Spanish *Ley Orgánica de Protección de Datos* and *Ley de Investigación Biomédica* and under protocols reviewed and approved by the Ethical

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Graphical abstract

We disclose two novel acridine-based families of compounds that combine the structural features of primaquine and chloroquine. Compounds prepared and studied thus far retained the in vitro activity displayed by the parent drugs against the erythrocytic stages of chloroquine-sensitive and -resistant P. falciparum strains, and against the hepatic stages of P. berghei, hence acting as dual-stage antiplasmodial hits.