**N-Cinnamoylation of Antimalarial Classics: Quinacrine Analogues with Decreased Toxicity and Dual-Stage Activity**

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*Malaria is caused by protozoan parasites of the genus Plasmodium. During the last decade (2000–2010), mortality associated with malaria has dropped by over 25%. Still, more than half a million children continue to die from this disease every year.[1] Moreover, *P. falciparum*, the organism that causes the most lethal form of the disease, is becoming increasingly resistant to most drugs available in the antimalarial compendium.[2,3] Thus, the search for new chemotherapeutic strategies to fight malaria is urgently needed.

Quinacrine (1), formerly marketed as Atebrin and also known as mepacrine, is an acridine-based compound that was first synthesized in 1931 (Bayer, Germany) and initially used for prophylaxis and treatment of malaria.[3] It was the first synthet-ic substitute for quinine,[4] a natural product obtained from the cinchona tree, and the first effective therapy used against malaria. Quinacrine was superseded by chloroquine (2), a more effective, less toxic, and affordable antimalarial agent, which was first synthesized in 1934 but only advanced as a therapeutic agent after World War II.[5] While quinacrine is no longer commonly employed as an antimalarial, it has been used “off-label” to treat giardiasis, systemic lupus erythematosus, and rheuma-toid arthritis.[6–8]

A convenient approach to antimalarial drug discovery is the development of analogues of classical antimalarials aiming at altering their unwanted properties and/or optimizing their original effects, in order to restore their relevance in antimalarial chemotherapy. Following the covalent biotherapy concept proposed by Meunier and co-workers,[9,10] our group recently developed hybrid compounds (3–5) through cinnamic acid conjugation with heterocyclic moieties from well-known antimalarials, such as 4-amino-7-chloroquinoline from chloroquine, 8-amino-6-methoxyquinoline from primaquine or the acridine ring from quinacrine. These hybrid agents exhibited improved antimalarial activity when compared with their parent drugs.[11–14] Furthermore, hybrids bearing either the chloroquine or the acridine core displayed dual-stage activity with IC50 values of 11–892 nM against *P. falciparum* W2 strain and 1.1–6.5 μM against liver forms of *P. berghei*, respectively, which compares rather favorably with the activities displayed by the reference drugs against blood (IC50(DQ) = 138 nM) and liver (IC50(PQ) = 7.5 μM) stage parasites.[12,13]

It was previously reported that introduction of a methoxy group at position 2 and a chlorine at position 6 of the acridine core increases the inhibitory potency by three- and ninefold on chloroquine-resistant and chloroquine-sensitive strains, respectively.[15] Based on this, we herein report the synthesis and in vitro evaluation of *N*-cinnamoylated quinacrine surrogates, 9-(*N*-cinnamoylaminobutyl)-8-amino-6-chloro-2-methoxyacridines (7a–f), which resulted highly potent against both blood-stage *P. falciparum* and liver-stage *P. berghei* parasites. The choice of the benzene ring substituents and of the aminobutyl spacer between the acridine and the cinnamoyl moieties was based...
on previously established structure–activity relationships (SAR) regarding N-cinnamoylated chloroquine analogues.[14]

The preparation of compounds 7a–f was inexpensive and facile; given the areas affected by malaria, a constant concern when developing potential antimalarial agents is to ensure the synthesis is as straightforward as possible. Briefly, the synthetic route to access compounds 7a–f involved two main reaction steps: a nucleophilic aromatic substitution between butan-1,4-diamine and 6,9-dichloro-2-methoxyacridine to obtain compound 6, according to an adaptation of the method described by Anderson and co-workers,[15] followed by a nucleophilic addition–elimination (condensation) reaction between 6 and the relevant cinnamic acid (Scheme 1). The global synthesis yields were around 13–32%, generally higher than those previously obtained in the synthesis of acridine derivatives 5 (3–22%).[12]

N-Cinnamoylated quinacrine analogues 7a–f were evaluated in vitro for their 1) activity against erythrocytic forms of P. falciparum (chloroquine-sensitive 3D7, and chloroquine-resistant W2 and Dd2 strains), 2) activity against liver-stage of W2 and Dd2 strains, 7c) cytotoxicity towards human hepatocellular carcinoma (HepG2) cells. The corresponding assays carried out are described in detail in the Experimental Section. Chloroquine and quinacrine were chosen as reference drugs for blood-stage activity assays, whereas primaquine was the reference drug for liver-stage studies.

As shown in Table 1, all compounds were exceptionally active across all three strains of erythrocytic-stage parasites, with IC₅₀ values ranging from 17 to 39 nM for chloroquine-sensitive strain 3D7, and 27.1–131 nM and 3.2–41.2 nM for chloroquine-resistant strains Dd2 and W2, respectively. Hence, in general, quinacrine analogues displayed decreasing antimalarial activity against those three strains in the following order: W2 > 3D7 > Dd2. Moreover, while all compounds except 7c were one- to approximately twofold less active than chloroquine against the chloroquine-sensitive strain, the opposite was observed for both chloroquine-resistant strains: all quinacrine analogues were considerably more active, by two- to fourfold and five- to 70-fold, than chloroquine against P. falciparum Dd2 and W2, respectively, with the exception of 7d against P. falciparum Dd2.

Compared with the parent antimalarial quinacrine, compounds 7a–f exhibited more potent activities across all three strains. Also, the activities of compounds 7a–f against chloroquine resistant strains were on average five- to 27-fold higher than those obtained for chloroquine-sensitive strains. Furthermore, the compounds exhibited activity against the chloroquine-resistant Dd2 and W2, respectively, with the exception of 7c which was one- to approximately twofold less active than chloroquine against the chloroquine-sensitive strain, the opposite was observed for both chloroquine-resistant strains: all quinacrine analogues were considerably more active, by two- to fourfold and five- to 70-fold, than chloroquine against P. falciparum Dd2 and W2, respectively, with the exception of 7d against P. falciparum Dd2.

Table 1. In vitro cytotoxicity and antimalarial activities of test compounds 7a–f and reference drugs against blood- and liver-stage parasites.

| Compd | R   | logP[a] | IC₅₀ [nM][b] | CC₅₀ [μM][c] | SI[f] | IC₅₀ [μM][f] |
|-------|-----|---------|--------------|-------------|------|-------------|
|       | PF 3D7 | PF Dd2 | PF W2 | HepG2 | | liver stage |
| 7a    | p-Me | 5.90 | 33.3 | 42.2 | 3.2 | 8.6 | 204 | – |
| 7b    | p-Pr | 6.63 | 39.0 | 27.1 | 10.5 | 4.1 | 105 | – |
| 7c    | p-OMe | 5.23 | 17.0 | 47.9 | 41.2 | 20.5 | 428 | 4.9 |
| 7d    | p-F  | 5.53 | 29.8 | 130.0 | 17.8 | 164.7 | 1257 | 1.6 |
| 7e    | p-Cl | 5.99 | 27.6 | 28.9 | 8.9 | 4.3 | 149 | 1.8 |
| 7f    | p-Br | 6.15 | 37.0 | 50.6 | 13.1 | 3.4 | 67 | – |
| Chloroquine | – | 21.0 | 107.5 | 225.8 | – | > 15 | – |
| Quinacrine | 100th | 200th | 159th | 7.4 | 37 | 13 | – |
| Primaquine | – | – | – | – | – | 7.5th | – |

[a] LogP values were calculated using Marvin 6.1.0 (calculator plugin) from ChemAxon (http://www.chemaxon.com); (b) Blood-stage antimalarial activity was determined against chloroquine-resistant strains W2 and Dd2 and chloroquine-sensitive strain 3D7 of P. falciparum (PF); (c) Cytotoxicity was determined against a human hepatocellular carcinoma (HepG2) cell line; data are expressed as the cytotoxic concentration 50 (CC₅₀), which is the concentration required to reduce the cell viability by 50%; (d) Liver-stage antimalarial activity was determined against P. berghei; (e) Values taken from Ref. [17]; (f) Selectivity index (SI): cytotoxicity/antiplasmodial ratio, calculated using the least potent IC₅₀ value determined against the R. falciparum strains. (g) Value taken from Ref. [11].
quinite-resistant strain W2 were significantly better (IC\textsubscript{50} = 3.2–41.2 nm) than those of the respective 9-(N-cinnamoylbutyl)aminooxadiazines \(5\) (IC\textsubscript{50} = 126–225 nm),\(^{11,12}\) as expected due to the presence of the 2-methoxy and 6-chloro substituents in the acridine core of \(7\).\(^{13,14}\) For instance, \(7a\) \((R = p\text{-Me})\) displayed an IC\textsubscript{50} value of 3.2 nm against \(P.\) \textit{falciparum} W2, almost 70 times more potent than that displayed by its counterpart in compounds \(5\) (IC\textsubscript{50} = 225 nm).

No obvious influence regarding the stereoelectronic properties of the cinnamoyl moieties, was observed on the antiplasmodial activities of compounds \(7a\)–\(7f\). Nevertheless, a similar trend is observed for the halogenated derivatives against the chloroquine-resistant strains Dd2 and W2, as in both cases their activities followed the order \(7e > 7f > 7d > 7a\) (p-Cl). Interestingly, para-chloro substitution of the cinnamoyl moiety has also been previously found to lead to most potent activities of \(N\)-cinnamoylated 4- and 8-aminooxadiazines (3 and 4), and acridine (5) analogues against \(P.\) \textit{falciparum} W2.\(^{11–13}\)

In order to assess the selectivity of compounds \(7a\)–\(7f\) for parasites over human cells, their cytotoxicity was evaluated in human hepatocellular carcinoma (HepG2) cells. The selectivity index (SI) is defined as the ratio of cytotoxicity over antiplasmodial activity—the CC\textsubscript{50} value determined against HepG2 cells over the least potent IC\textsubscript{50} value determined against \(P.\) \textit{falciparum} strains 3D7, W2 or Dd2. Although some compounds presented moderate cytotoxicity (CC\textsubscript{50} = 4.1 (7b), 4.3 (7e), and 3.4 (7f) \(\mu\)M), all compounds except \(7f\) (SI = 67) displayed high selectivity indices (SI > 100). Biological efficacy is generally considered to not be due to in vitro cytotoxicity when an SI value is \(> 10.\)\(^{11}\) Consequently, these results suggest that the activity exhibited by compounds \(7a\)–\(7f\) is unlikely due to general cellular toxicity but rather to specific antiplasmodial activity. Note-worthily, compounds with the para-methoxy and para-fluoro R groups (7c and 7d, respectively) exhibited considerably higher SI values (428 and 1257, respectively) than the other quinacrine derivatives, whose SI values ranged from 67 to 204. This could be related to two key facts: 1) the methoxy- and fluoro-substituted analogues are the least lipophilic in the series (Table 1), since high lipophilicity is known to give rise to increased tendency for toxicity,\(^{20}\) and 2) aromatic compounds containing a fluorine atom typically exhibit good stability, solubility, and bioavailability.\(^{21}\) Thus, compounds 7c and 7d show a promising profile as potential leads for safe antimalarial therapy.

The recent unprecedented finding of liver-stage antimalarial activity of \(N\)-cinnamoylated chloroquine analogues\(^{22}\) prompted us to assess the activity of compounds \(7a\)–\(7f\) against liver forms of the rodent malaria parasite \(P.\) \textit{berghlei} (Table 1). The activity of compounds \(7a\)–\(7f\) was evaluated at 1, 5 and 10 \(\mu\)M using a previously described bioluminescence-based method to quantify overall parasite infection of Huh-7 cells, a human hepatoma cell line.\(^{19}\) The toxicity of the compounds was also assessed against this human cell line through the fluorescence measurement of cell confluency (Figure 1). Compounds \(7a\)–\(7f\) were all active against liver-stage \(P.\) \textit{berghlei} parasites, and IC\textsubscript{50} values were determined for the three best compounds, according to both their activity and cytotoxicity (Table 1 and Figure 1). Remarkably, as for the blood-stage activities, compounds \(7a\)–\(7f\) resulted more active against liver-stage \(P.\) \textit{berghlei} (IC\textsubscript{50} = 1.6–4.9 \(\mu\)M) than the reference drug for this stage, primaquine (IC\textsubscript{50} = 7.5 \(\mu\)M), or the parent drug, quinacrine (IC\textsubscript{50} = 13 \(\mu\)M). The compounds exhibiting the best activity/cytotoxicity ratio, \(7d\) \((R = p\text{-F}; \text{IC}_{50} = 1.6 \mu\text{M})\) and \(7e\) \((R = p\text{-Cl}; \text{IC}_{50} = 1.8 \mu\text{M})\) resulted about four to five times more active than primaquine and over ten times more active than quinacrine. Finally, electron-withdrawing R groups seem to be more effective than electron-donating groups in terms of the enhancement of liver-stage activity, as suggested by the fact that \(7c\) \((R = p\text{-OMe})\) was at least twofold less active (IC\textsubscript{50} = 4.9 \(\mu\)M) than \(7d\) or \(7e\) \((R = p\text{-F} \text{ or } p\text{-Cl}, \text{respectively})\).

The mechanism of action (MoA) of quinacrine against malaria parasites remains unclear, but it has been demonstrated that the drug can interact with \(\beta\)-hematin, consequently inhibiting hemozoin formation and the development of intraerythrocytic parasites.\(^{20}\) In view of this, we further investigated whether compounds \(7a\)–\(7f\) could owe their activity against blood-stage parasites to inhibition of \(\beta\)-hematin polymerization. To this end, compounds \(7a\)–\(7f\) were evaluated in vitro as inhibitors of hemozoin formation by previously described methods.\(^{13–21}\) With the exception of compound \(7a\), which was a moderate inhibitor of hemozoin formation, as compared to chloroquine,
none of the compounds was able to inhibit hemozoin formation in vitro (data not shown). This result is in agreement with our previous observations for acridine analogues. Consequently, inhibition of hemozoin formation does not appear to be the main MoA of compounds 7a-f against blood-stage Plasmodium spp. Moreover, even if compounds 7a-f were able to inhibit this intraerythrocytic process, that would not explain their ability to also inhibit infection by liver-stage parasites.

Other mechanisms that might be responsible for the antimalarial activity of compounds 7a-f include the activation of p53 and the inhibition of NF-κB pathways. It is well known that the p53 and NF-κB pathways play important roles in diverse cellular functions, including cell growth, apoptosis, and tumorigenesis. Indeed, mutations that inactivate the p53 gene and trigger NF-κB pathway activation are common occurrences in human cancers. However, these pathways have also been reported as critical for parasite survival; as the activation of NF-κB in response to pathogens stimuli is normally associated with the initiation of protective immunity, it is believed that several parasites have developed strategies to interfere with NF-κB activation in order to decrease the host immune response to allow parasite survival. In fact, Tato and co-workers reported the ability of R. falciparum schizonts to activate NF-κB in host vascular endothelium and, thus, contribute to parasite survival. Moreover, Kaushansky and co-workers recently disclosed their finding that perturbation of the hepatocyte p53 pathway critically impacts parasite survival, as mice that expressed increased levels of p53 showed decreased liver-stage parasite burden, whereas p53-knockout mice displayed increased liver-stage parasite burden. In this context, and based on literature accounts that report quinacrine as a compound that simultaneously activates p53 and inhibits NF-κB pathways, these two processes arise as putative MoA for compounds 7a-f, as they would explain both the liver- and blood-stage activities. Nevertheless, other previously proposed mechanisms for quinacrine derivatives with antimalarial activity, such as binding to DNA, either by intercalation or groove binding, inhibition of mitochondrial bc1 complex or DNA topoisomerase II, cannot be ruled out. Studies aiming at the establishment of the MoA of these compounds are currently underway.

In summary, we have presented a novel family of N-cinnamoylated quinacrine analogues as dual-stage antimalarial compounds. The compound with the best profile of the series was 7d (R= P-F), which exhibits 1) activities comparable or higher than those of reference chloroquine against blood-stage 3D7, Dd2 and W2 P. falciparum parasites, 2) the most potent activity against liver-stage P. berghei, and 3) the weakest cytotoxicity against human cells. The latter adds to the fact that fluorinated compounds are known to have higher bioavailability, solubility, and metabolic stability compared with their nonfluorinated analogues. The MoA of the compounds remains to be elucidated, but results suggest that inhibition of hemozoin formation can be ruled out. Ongoing studies will hopefully elucidate the MoA, bringing new insights into the development of new antimalarial agents and enabling resurgence for quinacrine-related compounds as dual-stage antimalarial leads. Finally, it must be stressed that, altogether, these and previous findings from our group strongly suggest that the N-cinnamoyl moiety is a relevant pharmacophore to boost antimalarial activity of classic antimalarial agents, based on either the [4-/8]-aminoquinoline or acridine cores.

**Experimental Section**

**Chemistry**

**General:** Starting materials 6,9-dichloro-2-methoxy-acridine, butane-1,4-diamine, and phenol were acquired from Sigma–Alrich; O-(Benzotriazol-1-yl)-N,N,N’,N’-tetra-methyluronium tetrafluoroborate (TBTU) was bought from Bachem, cinnamic acids from Acros Organics, and solvents from VWR International. NMR analyses were carried out on a Brucker Avance III 400 MHz spectrometer, and samples were prepared in CDC13 with tetramethylsilane (TMS) as an internal reference. Mass spectrometry (MS) spectra were obtained on a Thermo Finnigan LCQ Deca XP Max LC/MSn instrument with electrospray ionization and ion-trap mass analysis (ESI-IT MS). Purity of the compounds was confirmed to be of at least 98% by HPLC using the following conditions: 10–70% of B in A (A: 0.05% aq trifluoroacetic acid; B: CH3CN) over 25 min at a flow rate of 1 mL/min. On a Purospher STAR, RP-C18 column (150×4.0 mm; particle size, 5 μm), using a Merck-Hitachi Lachrom Elite instrument equipped with a diode-array detector (DAD) and a thermostat (Peltier effect) autosampler.

**General procedure for the synthesis of 9-(N-cinnamoylanilino)-6-chloro-2-methoxyacridines (7a-f):** 6,9-Dichloro-2-methoxy-acridine (1 equiv), phenol (5 equiv), Cs2CO3 (1 equiv) and anhyd DMSO (2 mL) were stirred at 100 °C for 2 h with 3 A molecular sieves. Then, butane-1,4-diamine (10 equiv) was added directly to the mixture, and the reaction was stirred at 100 °C for an additional 3 h. The mixture was cooled to RT, diluted in CH3Cl2 (25 mL), and washed with 5% aq Na2CO3 (3×25 mL), after which the organic layer was dried over anhyd Na2SO4, filtered and concentrated in vacuo to yield 9-(N-(aminobutyl)amino)-6-chloro-2-methoxyacridine (6) as an orange solid, which was used in the next step without further purification.

Next, a mixture of the appropriate cinnamic acid (1.1 equiv), TBTU (1.1 equiv) and N,N-diisopropylethylamine (2 equiv) in DMF (2 mL) was stirred at 0 °C for 10 min. Then, a solution of 6 (1 equiv) in DMF (2 mL) at RT was added, and the reaction was stirred for 24 h at RT. The reaction mixture was diluted with CH3Cl2 (25 mL), washed with 5% aq Na2CO3 (3×25 mL), dried over anhyd Na2SO4, filtered and concentrated in vacuo to yield 9-(N-(aminobutyl)amino)-6-chloro-2-methoxyacridine (6) as an orange solid (13–32%). Complete spectroscopic and analytical data are provided in the Supporting Information.

**Biology**

In vitro inhibition of β-hematin polymerization: Stock solutions of compounds 7a-f were prepared at 0.1–1 mM in DMSO. The assays were run in triplicate, and DMSO and water were used as negative controls, and chloroquine was used as a positive control. Each well of a 96-well plate contained varying concentrations of test compound, hemin chloride in DMSO (5.2 mg mL-1; 50 μL) and acetate buffer (0.2 M, pH 4.4, 100 μL), and the plate was incubated at 37 °C for 48 h. Samples were centrifuged at 3000 rpm for 15 min. After
discarding the supernatant, the pellet was washed with DMSO (4 x
200 μL) and then dissolved in 0.2 mL aq NaOH (200 μL). The solubi-
ligated aggregates were further diluted with 0.1 mL aq NaOH (1:6),
and absorbance was recorded at 405 nm.

**Drugs and antibodies:** The two commercial monoclonal antibodies
(Immunology Consultants Laboratory Inc., Newberg, OR, USA) di-
rected against *P. falciparum*-specific HR2P were from MPFM-45A
(MPFM-55A), an immunoglobulin M (IgM) antibody used as the capture
antibody, and MPFG-45P (MPFG-55P), a horseradish peroxidase-conju-
gated IgG antibody used as the indicator antibody. Chloroquine
(C6628), bovine serum albumin (BSA; A2153), Tween 20 (P1379),
3,3',5,5'-tetramethylbenzidine (T0440), thiazolyl blue tetrazolium
bromide (M2128) were purchased from Sigma–Aldrich and
RPMI 1640 medium (51800019), AlbuMAX II (11021037) from Life
Technologies.

**Parasite cultivation:** Laboratory-adapted *P. falciparum* DD2 (chlo-
roquine-resistant, mefloquine-resistant), W2 (chloroquine-resistant
and mefloquine-sensitive) and 3D7 (chloroquine and mefloquine-
sensitive) strains were continuously cultured and synchronized as
previously described. Staging and parasitaemia were determined
by light microscopy of Giemsa-stained thin blood smears.

**HRP2 drug assay:** Synchronized 3D7, W2 and DD2 parasites with
>90% rings were diluted to 0.5% parasitaemia with 1.5% haematocrit.
 Cultures were then incubated with serial dilutions of each compound at 37°C with 5% CO2, 5% O2, and 90% N2. After
72 hours, cultures were diluted by adding 100 μL/well of distilled
water and stored at -20°C until reading.

**HRP2 double-site antigen capture ELISA:** High-binding 96-well ELISA
plates (Costar 3590; Corning Inc., NY, USA) were coated with
100 μL/well of a 1.0 μg mL⁻¹ solution of anti-HRP2 IgM antibody
solution (MPFM-45 A) in phosphate-buffered saline (PBS). Then,
the plates were sealed and incubated overnight at 4°C. The super-
natant was discarded, and plates were saturated with 2% BSA
solution in PBS. The supernatant was discarded, and plates were
washed three times with 200 μL/well of washing solution (0.05%
Tween 20 in PBS). 100 μL of each hemolyzed (by freeze-thawing
at least twice) cultured sample were added to the precoated ELISA plates. The ELISA plates were incubated for 1 h at
room temperature and washed three times with washing solution.
Then 100 μL of the antibody conjugate (0.05 μg mL⁻¹ of MPFG-45P
in 2% BSA and 1% Tween 20 in PBS) were added to each well.
After incubation for 1 h at room temperature, the plate was
washed three times with washing solution and 100 μL of 3,3',5,5'-
tetramethylbenzidine was added to each well, and the plates were
incubated in the dark for 5–10 min. The reaction was stopped with
0.2 mL H2SO4. Plates were read at 450 nm using an ELISA
plate reader (Dynex Trixd, Alfacigen). IC50 values were estimated for
each compound by nonlinear interpolation of the dose-dependent
curve using GraphPad Software (trial version).

In vitro toxicity to HepG2 mammalian cells: HepG2 A16 hepatic cell
line viability was determined based on an MTT assay as previously
described. An in vitro culture of HepG2 cells maintained in standard
culture conditions was treated with 200 μL of fresh medium
containing seven tenfold dilutions (100 μM–1 nM) of each com-
 pound, and a negative control was performed by adding 200 μL of
compound-free medium. The plate was incubated for 24 h in condi-
tions, and the supernatant was removed and substituted by
200 μL of acidic isopropanol. Results were obtained by ELISA
reading at 570 nm, to produce a log dose-dependent curve. The
CC50 value was estimated for each compound by nonlinear interpo-
lation of the dose-dependent curve using GraphPad Software (trial
version).

In vitro liver-stage activity assays: In vitro inhibition of liver-stage in-
festation by test compounds was determined by measuring the lu-
minescence intensity in Huh-7 cells infected with a firefly luciferase
expressing *P. berghei* line, PbGFP-Lucon, as previously described.
Huh-7 cells, from a human hepatoma cell line, 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-piperazineetha-
nesulfonic acid (HEPES), pH 7, and maintained at 37°C with 5% CO2.
The Huh-7 cells (1.2 x 104 per well) were seeded in 96-well plates
the day before compound treatment and infection. Medium in
the cells was replaced by medium containing the appropriate
concentration of each compound approximately 1 h prior to infec-
tion with sporozoites freshly obtained through disruption of sali-
vary glands of infected female *Anopheles stephensi* mosquitoes. Spor-
ozoite addition was followed by centrifugation at 1700 g for 5 min.
At 24 h after infection, medium was replaced by fresh medium
containing the appropriate concentration of test compound. Inhibi-
tion of parasite development was measured 48 h after infection.
The effect of the compounds on the viability of Huh-7 cells was
assessed by the AlamarBlue assay (Invitrogen, UK) using the manu-
facturer’s protocol.

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