N-Cinnamoylation of Antimalarial Classics: Effects of Using Acyl Groups Other than Cinnamoyl toward Dual-Stage Antimalarials

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In a follow-up study to our reports of N-cinnamoylated chloroquine and quinacrine analogues as promising dual-stage antimalarial leads with high in vitro potency against both blood-stage *Plasmodium falciparum* and liver-stage *Plasmodium berghei*, we decided to investigate the effect of replacing the cinnamoyl moiety with other acyl groups. Thus, a series of N-acylated analogues were synthesized, and their activities against blood- and liver-stage *Plasmodium* spp. were assessed along with their in vitro cytotoxicities. Although the new N-acylated analogues were found to be somewhat less active and more cytotoxic than their N-cinnamoylated counterparts, they equally displayed nanomolar activities in vitro against blood-stage drug-sensitive and drug-resistant *P. falciparum*, and significant in vitro liver-stage activity against *P. berghei*. Therefore, it is demonstrated that simple N-acylated surrogates of classical antimalarial drugs are promising dual-stage antimalarial leads.

A child dies nearly every minute from malaria. Despite the fact that malaria-associated deaths in Africa have decreased by about 54% since 2000, eradication is far from being attained in the near future.[1] There are well-identified obstacles to the eradication of malaria, including the complexity of the malaria parasite’s life cycle, widespread resistance to less expensive and most popular antimalarials such as chloroquine (CQ, 1), the toxicity of some classical antimalarial drugs, e.g., quinacrine (QC, 2), the lack of effective vaccines, and the scarcity of multi-stage antimalarials that are able to efficiently deplete liver- and blood-stage forms of *Plasmodium* parasites from the human body. Another drawback in malaria containment is the high cost of first-line treatments, such as artemisinin combination therapies (ACT), which instigates the trafficking of counterfeit antimalarial drugs.[2,1] One strategy to accelerate the development of new drugs is to start from the chemical frameworks of known antimalarials, i.e., to recycle classical drug scaffolds.[6–10] In this regard, our group has been working extensively on the chemical recycling of classical antimalarials with rather promising results, including the recent discovery of N-cinnamoylated chloroquine (3) and N-cinnamoylated quinacrine (4) analogues as dual-stage antimalarial leads.[6–10] These conjugates display high in vitro potency against both blood-stage *P. falciparum* (CQ-sensitive and -resistant strains), and liver-stage *P. berghei*, representing promising dual-stage antimalarial leads.[6–10] These were unprecedented findings, as dual-stage activity for CQ- or QC-based molecules had not been reported previously. Furthermore, some of the compounds developed in these studies also displayed in vivo antimalarial activity.[8]

Interestingly, compounds 3 and 4 lack a basic aliphatic amine, classically considered a prerequisite for the potent blood-stage antimalarial activity of aminoquinolines and related structures.[11] Moreover, the potent blood-stage activity of CQ analogues 3 against CQ-resistant *P. falciparum* suggests that insertion of the N-cinnamoyl group produces a resistance-reversal effect, possibly due to either chemosensitization,[12] or a mechanism of action (MOA) different from that classically attributed to CQ, i.e., inhibition of β-hematin.[13]

In fact, we found that the antiplasmodial activity of compounds 3 could not be fully explained by β-hematin inhibition. We have also ruled out that these compounds could owe their potent blood-stage activity to falcipain inhibition, through irreversible Michael-type addition of the enzyme’s catalytic Cys
thiol to the compounds’ α,β-vinylcarbonyl moiety in the cinnamoyl group.\(^6\),\(^{14}\)

In the absence of a confirmed MOA for compounds \(3\) and \(4\), we decided to investigate the importance of the cinnamoyl group for antimalarial activity. To this end, a series of N-acylated CQ (compounds \(9\)) and QC (compounds \(10\)) analogues were synthesized (Scheme 1), and their antimalarial activities against blood- and liver-stage \(P. falciparum\) spp., as well as in vitro toxicity to human hepatocellular carcinoma (HepG2) cells, were assessed (Table 1). The results are reported and discussed herein.

To evaluate the effect of replacing the \(N\)-cinnamoyl moiety with other \(N\)-acyl groups, compounds \(9\) and \(10\) were designed in which the cinnamoyl group of \(3\) and \(4\), respectively, was substituted with either alkanoyl groups of various lengths, including linear (\(9a-d, 10a-d\)), branched (\(9e-f, 10e-f\)), and fluorinated (\(9g-j, 10g-j\)) groups, or by nonaromatic α,β-vinylcarbonyls (\(9h-j, 10h-j\)), as shown in Scheme 1. Accordingly, the CQ and QC derivatives \(9\) and \(10\) reported herein were obtained by starting from 4,7-dichloroquinoline (5) and 6,9-dichloro-2-methoxyacridine (6), respectively, through a couple of simple steps: a nucleophilic aromatic substitution followed by a nucleophilic addition–elimination (condensation) reaction (Scheme 1). Detailed procedures are described under the Experimental Section below. The small number of synthesis steps, inexpensive starting materials, and straightforward workup procedures (cf. Experimental Section) clearly compensate for some of the lower yields obtained in the synthesis of the target compounds. These were obtained in high purity, as determined by high-performance liquid chromatography with diode array detection (HPLC-DAD), and their structures were confirmed by \(^1\)H and \(^{13}\)C NMR spectroscopy, as well as by mass spectrometric analysis with electrospray ionization and ion-trap quadrupole detection (ESI-IT MS); relevant data are presented in the Experimental Section.

Blood-stage activity of compounds \(9\) and \(10\) was determined by previously reported methods\(^{[10]}\) against three strains of \(P. falciparum\): CQ-sensitive 3D7 and CQ-resistant Dd2 and W2. Notably, compounds \(9\) and \(10\) were unable to inhibit β-hematin formation (i.e., heme polymerization) in vitro (data not shown). Close inspection of the data listed in Table 1 enables the following general observations:

1) Remarkably, all compounds display nanomolar activities against the three \(P. falciparum\) strains, with a noteworthy IC\(_{50}\) value of 4.60 nM for CQ analogue \(9b\) against CQ-resistant strain W2, and with \(9f\) being the best performer against the three strains tested (IC\(_{50}\): 5.30, 27.0, and 12.3 nM against 3D7, Dd2, and W2, respectively).

2) CQ analogues \(9\) (5.30 ≤ IC\(_{50}\) ≤ 254 nM) were generally more active than QC analogues \(10\) (11.0 ≤ IC\(_{50}\) ≤ 493 nM), with \(10h\) being the best performer amongst the latter series (IC\(_{50}\): 38.5, 11.0, and 32.4 nM against 3D7, Dd2, and W2, respectively).

3) With only a few exceptions, all compounds are globally more active than the respective parent drugs, CQ or QC\(^{[15]}\) against the blood-stage \(P. falciparum\) strains used in these assays.

4) A clear trend regarding the compounds’ differential activities against the three \(P. falciparum\) strains could not be identified; for instance, for N-alkanoylated CQ analogues \(9b-f\), compounds \(9b-d\) were increasingly active in the order Dd2 < 3D7 < W2, \(9c\) in the order Dd2 < W2 < 3D7, \(9e\) in the order Dd2 < 3D7 < W2, and \(9f\) in the order 3D7 < W2 < Dd2.
5) N-Acetylated analogues 9a and 10a were substantially less active than all other compounds tested, with activity against the 3D7 strain being enhanced by increasing the length of the alkyl chain from one to three carbons (9a to 9c, and 10a to 10c); this enhancement is also observed in terms of the activity against both CQ-resistant strains, when going from one to two carbons (9a versus 9b, 10a versus 10b), but no other consistent correlations between activity and alkyl chain length could be identified.

6) Upon comparing equally sized alkyl groups R, as in 9d,e,f or 10d,e,f, replacing their linear chain (9d, 10d) by their β-branched (9e, 10e) or α-branched (9f, 10f) isomers leads to a decrease or an increase, respectively, in activity against all strains.

7) Replacement of a methyl with a trifluoromethyl group in CQ analogues, that is, 9e versus 9g, enhances activity against 3D7 and Dd2, but not against W2; in turn, the same replacement in QC analogues, i.e., 10e versus 10g, leads to a decrease in activity against the three strains.

8) Interestingly, introducing a double bond has opposite effects in CQ and QC analogues: the activity decreases when going from 9g to its vinylic counterpart 9h, whereas activity is increased in 10h relative to 10g.

9) α,β-Vinylcarbonyl moieties introduced in 9i,j or 10i,j did not contribute to any significant enhancement in activity, as compared with the remaining test compounds.

10) Comparing blood-stage activities reported herein for compounds 9 and 10 with those formerly determined for their N-cinnamoylated counterparts (compounds 3 and 4) reveals that despite the latter having an overall slightly better performance than the former, differences are not significant—IC₅₀ values of compounds 3 against Dd2 and W2 strains were in the ranges of 15.6–78.1 and 11.0–58.8 nM,[8] whereas IC₅₀ values for compounds 4 on 3D7, Dd2, and W2 strains were respectively 17.0–39.0, 27.1–131, and 3.20–41.2 nM.[10]

In view of the above, although compounds 3 and 4 display slightly better activities against intra-erythrocytic parasites[8,10]...

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Table 1. In vitro cytotoxicity (HepG2 cells) and activity against blood-stage *P. falciparum* strains and liver-stage *P. berghei* displayed by compounds 9 and 10.[8]

| R       | Compd | Blood-stage IC₅₀ [nM] | HepG2 CC₅₀ [μM] | S[II] | Liver-stage IC₅₀ [μM] |
|---------|-------|-----------------------|-----------------|-------|-----------------------|
|         |       | PF 3D7                | PF Dd2          | PF W2 |                        |
| Me      | 9a    | 134                   | 172             | 13.7  | 53.9                  |
|         | 10a   | 202                   | 493             | 0.76  | 1.50                  |
| Et      | 9b    | 23.6                  | 4.60            | 12.5  | 245                   |
|         | 10b   | 87.7                  | 185             | 2.90  | 15.7                  |
| Pr      | 9c    | 6.60                  | 52.7            | 22.3  | 418                   |
|         | 10c   | 145                   | 213             | 1.39  | 6.50                  |
| Bu      | 9d    | 30.4                  | 10.9            | 13.2  | 232                   |
|         | 10d   | 48.2                  | 70.8            | 3.65  | 38.9                  |
| tBu     | 9e    | 86.1                  | 56.0            | 22.8  | 261                   |
|         | 10e   | 48.5                  | 88.3            | 3.29  | 31.5                  |
| secBu   | 9f    | 5.30                  | 12.3            | 125   | 4618                  |
|         | 10f   | 107                   | 122             | 3.63  | 29.8                  |
| CF₃     | 9g    | 16.5                  | 89.3            | 37.6  | 421                   |
|         | 10g   | 82.8                  | 166.1           | 8.11  | 48.8                  |
| CF₃     | 9h    | 94.7                  | 125             | 9.20  | 45.5                  |
|         | 10h   | 38.5                  | 32.4            | 2.07  | 53.8                  |
| CF₃     | 9i    | 97.3                  | 153             | 4.35  | 28.4                  |
|         | 10i   | 61.0                  | 61.0            | 2.86  | 46.9                  |
| HCF₂    | 9j    | 48.8                  | 64.0            | 16.9  | 99.4                  |
|         | 10j   | 34.0                  | 77.9            | 3.34  | 42.9                  |
| –       | CQ    | 21.0                  | 226             | 97.2[11] | 22.9 > 15   |
| –       | QC    | 100[15]              | 159[12]         | 7.40  | 37.0                  |
| –       | PO    | 180[14]              | 180[14]         | 7.5[5] |

[a] Chloroquine (CQ), quinacrine (QC), and primaquine (PQ) were used as reference classical antimalarial drugs. [b] Selectivity index: ratio of (CC₅₀ HepG2)/ (highest IC₅₀ against Pf strains).
than their analogues 9 and 10 reported herein, overall antiplasmodial performance of the latter clearly shows that simple N-acylation of classical antimalarials like CQ and QC leads to remarkable nanomolar activities in vitro against CQ-sensitive and CQ-resistant \textit{P. falciparum} strains.

The toxicity of compounds 9 and 10 against HepG2 cells was determined (Table 1) as previously described.\textsuperscript{10} All compounds except 9f (CC\textsubscript{50} = 125 \textmu M) were more cytotoxic than CQ (CC\textsubscript{50} = 97.2 \textmu M),\textsuperscript{10} displaying CC\textsubscript{50} values below 40 \textmu M. CQ analogues 9 (9.20 \textmu M ≤ CC\textsubscript{50} ≤ 125 \textmu M) were less cytotoxic than QC analogues 10, the latter being more cytotoxic (0.76 \textmu M ≤ CC\textsubscript{50} ≤ 3.65 \textmu M) than the parent drug QC (CC\textsubscript{50} = 7.40 \textmu M). Hence, compounds 9 and especially 10 presented poor selectivity indices (SI, taken as the ratio between CC\textsubscript{50} and the highest IC\textsubscript{50} observed amongst the \textit{P. falciparum} strains used; Table 1), except in the case of 9f, the SI for which was 4618. Comparison of these findings with previous data for N-cinnamoylated analogues of QC, 4, against the same cell line (3.4 \mu M ≤ CC\textsubscript{50} ≤ 165 \mu M) shows that replacement of the cinnamoyl group by other acyl moieties as in 10 is somewhat disadvantageous regarding regard cytotoxicity.

The activity of compounds 9 and 10 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 (data not shown).\textsuperscript{10} Cell confinement measurements confirmed cytotoxicity of most of the compounds to hepatic cells (data not shown), preventing us from obtaining reliable quantitative data on their liver-stage activity. For this reason, we determined IC\textsubscript{50} values only for compounds with SI values > 250 (9c-e-g), with the following exceptions:

- 9d (SI = 232), given its interest as a structural isomer of compounds 9ef;
- 9h (SI = 45.5), to assess the effect of introducing a double bond, as compared with 9g;
- 9j (SI = 99.4), to assess the effect of replacing a linear saturated by a linear unsaturated R group;
- 10g (SI = 48.8), to observe the impact on liver-stage activity upon replacing the 4-aminoquinoline core in 9g with an acridine moiety.

The results obtained (Table 1) show that, like their N-cinnamoylated counterparts, these compounds display dual-stage activity. Hence, the compounds tested are remarkably active in vitro against liver forms of \textit{P. berghei}, with IC\textsubscript{50} values ranging from 0.52 to 7.7 \textmu M, all of which are lower than those of their parent drugs, and lower than or similar to that of the reference drug for liver-stage malaria, primaquine (PQ): 7.5 \textmu M.\textsuperscript{7} The range of IC\textsubscript{50} values is too narrow to build any meaningful structure–activity relationships, although it can be observed that increasing the length of the alkyl chain R from three to four carbons (9c versus 9d) improves activity by a factor of ~3, whereas going from linear (9d) to \beta-branched (9e) or \alpha-branched (9f) isomers only slightly increases IC\textsubscript{50} values. Replacing a methyl in 9e by a trifluoromethyl in 9g also slightly improves liver-stage activity, whereas replacing a linear alkane group (9d) by a linear alkyldiene (9j) has no apparent effect. Introduction of a double bond, as in 9g versus 9h, only slightly increases activity, whereas replacing the 4-aminoquinoline moiety in 9g by an acridine, in 10g, clearly increases activity, but at the expense of safety. Nevertheless, although 10g is more cytotoxic, its IC\textsubscript{50} against liver-stage parasites (0.52 \textmu M) is about 16-fold lower than its CC\textsubscript{50} against liver HepG2 cells (8.11 \textmu M). Therefore, in vitro liver-stage activity of compounds 9 and 10 is not markedly lower than those previously reported for compounds 3 (1.06 \textmu M ≤ IC\textsubscript{50} ≤ 1.86 \textmu M, average IC\textsubscript{50}: 1.79 \textmu M)\textsuperscript{9} or some of compounds 4, like 4a (R = p-F, IC\textsubscript{50} = 1.60 \textmu M), or 4b (R = p-Cl, IC\textsubscript{50} = 1.70 \textmu M),\textsuperscript{10} although none of them was able to display lower cytotoxicity toward HepG2 cells than the reference drug, PQ (CC\textsubscript{50} = 180 \textmu M).\textsuperscript{17} Altogether, these results show that replacement of the N-cinnamoyl moiety with other N-acyl groups is not deleterious concerning in vitro liver-stage antimalarial activity.

Our recent discovery of the exceptional in vitro dual-stage activity of compounds 3 and 4 unveiled a key role for N-cinnamoylation of CQ and QC scaffolds as a means to both reverse resistance of blood-stage parasites to classical antimalarial scaffolds and enhance their activity against liver-stage parasites.\textsuperscript{8,10} Exactly how and why N-cinnamoylation leads to such remarkable improvement of in vitro antimalarial properties has yet to be determined. Thus far, it has been possible to rule out inhibition of either falcipains or \beta-hematin formation (i.e., heme polymerization) as major MOA of those compounds,\textsuperscript{6,8,10} additionally, such MOA could only explain blood-stage activity, not that observed against the parasite’s liver stages. Preliminary data (not confirmed) have suggested that inhibition of new, permeability pathways (NPPs) formed in \textit{P. falciparum}-infected red blood cells might be one possible MOA underlying blood-stage activity of compounds 3.\textsuperscript{6} Although these NPPs have been exclusively associated with blood-stage infection, recent evidence that similar NPP activity may be elicited upon invasion of hepatocytes by \textit{P. berghei} parasites might provide a unifying explanation for the dual-stage activity displayed.\textsuperscript{13,14} This hypothesis agrees with earlier evidence that cinnamic acid derivatives are NPP inhibitors.\textsuperscript{20} One other possible and perhaps more likely MOA underlying antimalarial performance of compounds 3 and 4 is associated with the redox properties of cinnamonic moieties\textsuperscript{21} and other structurally related compounds, e.g., curcumins,\textsuperscript{22} hence, N-cinnamoylated compounds might owe their dual-stage antimalarial action to the generation of reactive oxygen species in a manner similar to that described for ferroquine.\textsuperscript{23} Still, we now further demonstrate that using acyl groups other than cinnamoyl, as in compounds 9 and 10, globally preserves in vitro nanomolar activities against blood-stage CQ-sensitive and -resistant \textit{P. falciparum} strains as well as remarkable activity against liver forms of \textit{P. berghei}. Taken together, our findings show that simple N-acylated surrogates of antimalarial classics like chloroquine and quinacrine are relevant leads worthy of further exploration toward the development of dual-stage antimalarial candidates.
Experimental Section

Chemicals and instrumentation: All solvents and common reagents were obtained from Sigma–Aldrich (Spain), whereas the coupling agent TBTU was from Bachem (Switzerland). NMR spectra were acquired on a Bruker Avance III 400 spectrometer from solutions in either deuterated chloroform (CDCl3), deuterated methanol (CD3OD) or deuterated dimethyl sulfoxide (D2)DMSO), containing tetramethylsilane as internal reference. Multiplicity of proton NMR signals is given as: s, singlet; d, doublet; t, triplet; q, quartet; qi, quintuplet; ss, sextet; bs, broad singlet; bt, broad triplet; dd, doublet doublet; m, unresolved multiplet.

Mass spectrometry (MS) analyses were run on a Thermo Finnigan LCQ Deca XP LC MS2 instrument operating with electrospray ionization and ion-trap (ESI-IT) quadrupole detection. The target compounds were confirmed to have at least 95% purity, based on peak areas obtained through HPLC analyses that were run using the following gradient elution: 10→70% B in A (A = H2O with 0.05% trifluoroacetic acid; B = acetonitrile) over 25 min, at a flow rate of 1 mL min⁻¹, on a Merck–Hitachi Lachrom Elite instrument equipped with a diode-array detector (DAD) and thermostatted (Peltier effect) autosampler, using a Purospher STAR RP-18e column (150x4.0 mm; particle size: 5 μm).

Synthesis of 4-(N-aminobutyl)amino-7-chloroquinoline (7): Compound 7 was prepared as previously described, and its structural analyses were in agreement with previously reported data.[6]

Synthesis of 9-(N-aminobutyl)amino-6-chloro-2-methoxycacidine (8): Compound 8 was prepared as previously described, and used without further purification.[10]

Synthesis of compounds 9a and 10a: Synthesis of N-acetylated surrogates of CQ (9a) and QC (10a) was carried out by addition of acetic anhydride (20 equiv) to 7 and 8, respectively (1 equiv), allowing the reaction to run for 1 h at room temperature (RT). The reaction mixture was then taken to dryness on a rotary evaporator, and the solid residue was re-solubilized in CH2Cl2 (20 mL). The resulting organic layer was washed three times with 5% aqueous Na2CO3, dried with anhydrous Na2SO4, filtered, and evaporated to dryness on a rotary evaporator. The impure solids obtained were purified by liquid chromatography on a silica gel column using CH2Cl2/MeOH 4:1 (v/v) as mobile phase to obtain the final products in high purity.

Synthesis of compounds 9b-i and 10b-i: The relevant carboxylic acids (1.1 equiv) were activated with O-(benzotriazol-1-yloxy)-N,N,N',N'-tetramethylenyluronium tetrafluoroborate (TBTU; 1.1 equiv) in the presence of N-ethyl-N,N-diisopropylamine (DIEA; 2.0 equiv) in N,N-dimethylformamide (DMF; 2 mL) for 20 min at 0°C. Then, a solution of 7 (for compounds 9b-i or 8 for compounds 10b-i) (1 equiv) in DMF (2 mL) was added, and the reaction was allowed to proceed at RT for 24 h. CH2Cl2 (25 mL) was added to the reaction mixture, and the resulting solution was washed three times with 5% aqueous Na2CO3 (30 mL), dried with anhydrous Na2SO4, and filtered. The solvent was evaporated under reduced pressure (rotary evaporator), and the crude residue was purified by liquid chromatography on a silica gel column with CH2Cl2/MeOH 4:1 (v/v) as eluent.

Synthesis of compounds 9j and 10j: Either 7 (for the synthesis of 9j) or 8 (for the synthesis of 10j) (1.0 equiv) was added to a solution of sorbic chloride (1.1 equiv) and Cs2CO3 (2.5 equiv) in DMF (2 mL), and the reaction was allowed to proceed for 3.5 h at RT. CH2Cl2 (10 mL) was then added to the reaction mixture, and the resulting solution was washed three times with 5% aqueous Na2CO3 (15 mL), dried with anhydrous Na2SO4, filtered and evaporated to dryness on a rotary evaporator. The impure solid residue was purified by liquid chromatography on a silica gel column with CH2Cl2/MeOH 4:1 (v/v) as mobile phase.

Complete spectroscopic and analytical data for compounds 9a-j and 10a-j are provided in the Supporting Information.

Inhibition of β-hematin: The β-hematin inhibition assay was performed as previously described.[24] Various concentrations (0.1–1 nm) of test compounds dissolved in DMSO were added in triplicate to 50 μL hemin chloride dissolved in DMSO (5.2 mg mL⁻¹). Negative controls were water and DMSO. β-Hematin formation was initiated by the addition of acetic buffer 0.2 μL (100 μL, pH 4.4), plates were incubated at 37°C for 48 h, and were then centrifuged at 3000 rpm for 15 min (SIGMA 3-30K). After discarding the supernatant, the pellet was washed with DMSO (4×200 μL), and finally dissolved in 0.2 mL aq NaOH (200 μL). The solubilized aggregates were further diluted 1:6 with 0.1 M aq NaOH, and absorbance values were recorded at λ 405 nm (Bioket Powerwave X5 with software Gen5 1.07).

Blood-stage activity assays: Laboratory-adapted P. falciparum Dd2, Dd2 (CQ-resistant), and 3D7 (CQ-susceptible) strains were continuously cultured under standard conditions as previously described by Trager and Jensen.[25] The antimalarial activity was determined with the SYBR Green I assay as previously described,[26] with modifications. Briefly, early ring-stage parasites (1% parasitemia and 3% hemocrit) were tested in triplicate in a 96-well plate and incubated with 12 concentrations of test compounds serially diluted (1:3), ranging from 0–10 μM, for 48 h (37°C, 5% CO2). After incubation, a solution of SG (0.001% w/v in PBS) was added to each well, incubated for 60 min, supernatant discarded, and cells re-suspended in PBS. Fluorescence was detected in a multi-mode microplate reader (TRIAD Multimode Detector, DYXEN Technologies), with excitation and emission wavelengths of 485 and 535 nm, respectively, and analyzed by nonlinear regression using GraphPad Prism 5.0 version to determine the IC50 values.

In vitro liver-stage infection assays: Inhibition of liver-stage infection by test compounds was determined as previously described.[9,10] by measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing P. berghei line, PgGFP-Luc, Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% f/v fetal calf serum, 1% f/v nonessential amino acids, 1% f/v penicillin/streptomycin, 1% f/v glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7, and maintained at 37°C with 5% CO2. For infection assays (performed in triplicate for each compound), Huh-7 cells (1.2×10⁵ per well) were seeded in 96-well plates the day before drug treatment and infection. Medium in the cells was replaced by medium 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. Sporozoite addition was followed by centrifugation at 1700 g for 5 min. At 24 h post-infection, the medium was replaced by fresh medium containing the appropriate concentration of each compound. Inhibition 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 manufacturer’s protocol.
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