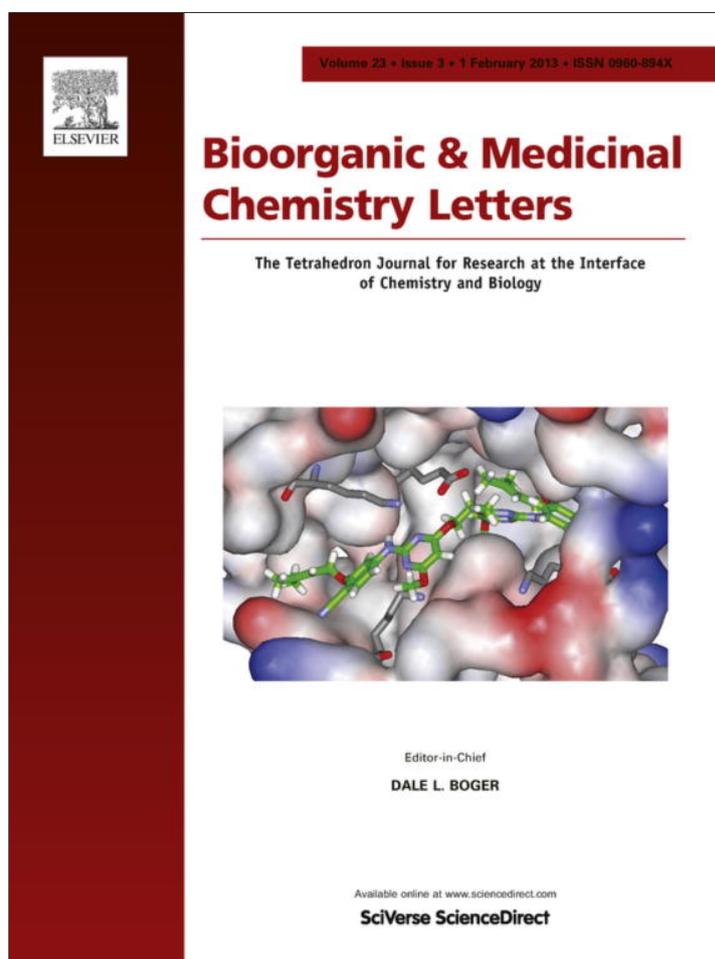


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## In vitro efficiency of 9-(*N*-cinnamoylbutyl)aminoacridines against blood- and liver-stage malaria parasites

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## ABSTRACT

Novel 9-aminoacridine derivatives were synthesized by linking the heteroaromatic core to different cinnamic acids through an aminobutyl chain. The test compounds demonstrated mid-nanomolar in vitro activity against erythrocytic stages of the chloroquine-resistant W2 strain of the human malaria parasite *Plasmodium falciparum*. Two of the most active derivatives also showed in vitro activity against liver-stage *Plasmodium berghei*, with activity greater than that of the reference liver-stage antimalarial primaquine. The compounds were not toxic to human hepatoma cells at concentrations up to 5  $\mu$ M. Hence, 9-(*N*-cinnamoylbutyl)aminoacridines are a new class of leads for prevention and treatment of malaria.

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Malaria, one of the world's deadliest diseases, primarily affects developing countries. According to the last World Malaria Report, there were around 3.3 billion people at risk of infection in 2010.<sup>1</sup> Although the malaria mortality rate has probably decreased since 2000, the disease remains widespread in most endemic areas, and the continued spread of resistance mandates efforts to identify new antimalarial drugs.<sup>1</sup> In particular, the declining efficacy of artemisinin, the most active available antimalarials, highlights the need for the discovery of novel antimalarials (Dondorp, NEJM, 2010).

Quinacrine (QA, **1** in Fig. 1), based on the 9-aminoacridine heteroaromatic core, was initially approved in the 1930s as an antimalarial drug and was one of the first synthetic surrogates for quinine, whose supply from Indonesia was blocked during World War II. During that war, quinacrine was the standard suppressive and therapeutic antimalarial of the Allied forces.<sup>2</sup> From 1946, and for over 50 years, chloroquine (CQ, **2** in Fig. 1), an analogue of QA with the 6-chloro-2-methoxyacridine core replaced by a 4-amino-7-chloroquinoline moiety, replaced quinacrine as a first-line

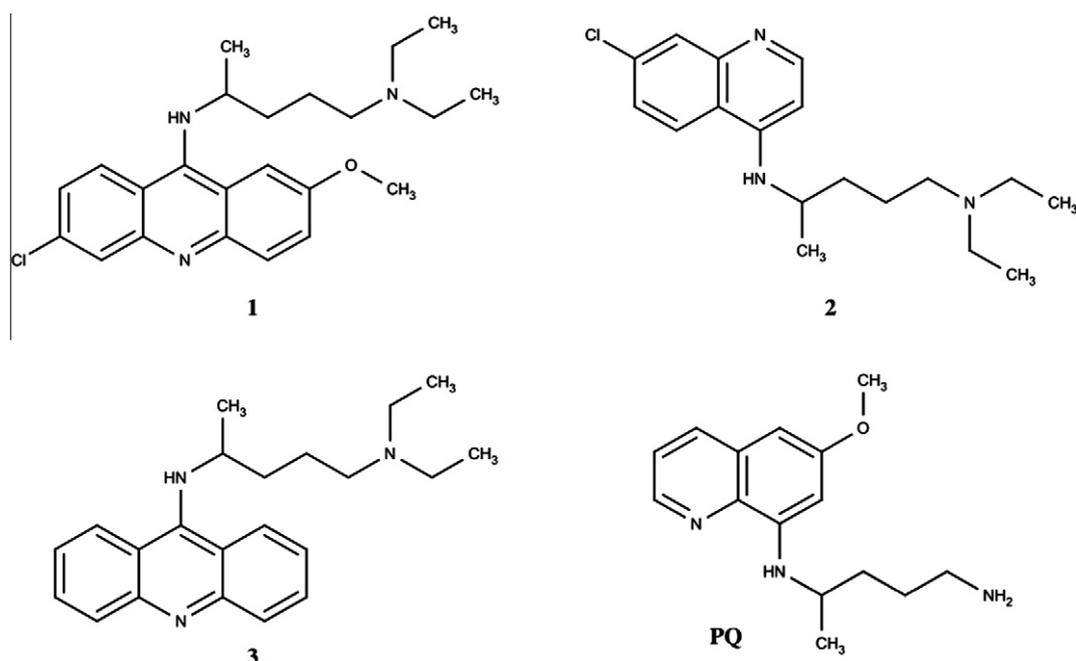
antimalarial, given its greater potency and safety, and lower cost.<sup>2</sup> However, the use of CQ to treat *falciparum* malaria has been gradually abandoned worldwide, due to evolution and spread of CQ-resistant parasites, especially *Plasmodium falciparum*.<sup>1</sup> However, cross-resistance is not uniformly seen among related compounds, and new acridine derivatives may arise again as CQ substitutes. If possible, such derivatives should act as multi-stage antimalarials, capable of killing liver, blood, and mosquito-stage parasites.<sup>3</sup>

Recently, our group showed that *N*-alkylcinnamoylation of the aminoquinoline core of known antimalarials, such as primaquine (PQ, Fig. 1) or CQ, led to substantially enhanced antimalarial activity as compared to those classical drugs.<sup>4–6</sup> In view of this, we have investigated whether a similar effect would be obtained by coupling a butylcinnamoyl moiety to the amino group of 9-aminoacridine, the unsubstituted heterocyclic scaffold of quinacrine. In this context, we report the synthesis and in vitro evaluation of novel 9-(*N*-butylcinnamoyl)aminoacridines, **6**, which were found to have greater activity than the acridine analogue of CQ, **3** (Fig. 1), and PQ, against blood-stage *P. falciparum* and liver-stage *Plasmodium berghei* malaria parasites.

The synthesis of the target molecules was straightforward (Scheme 1) making the chemistry underlying their preparation

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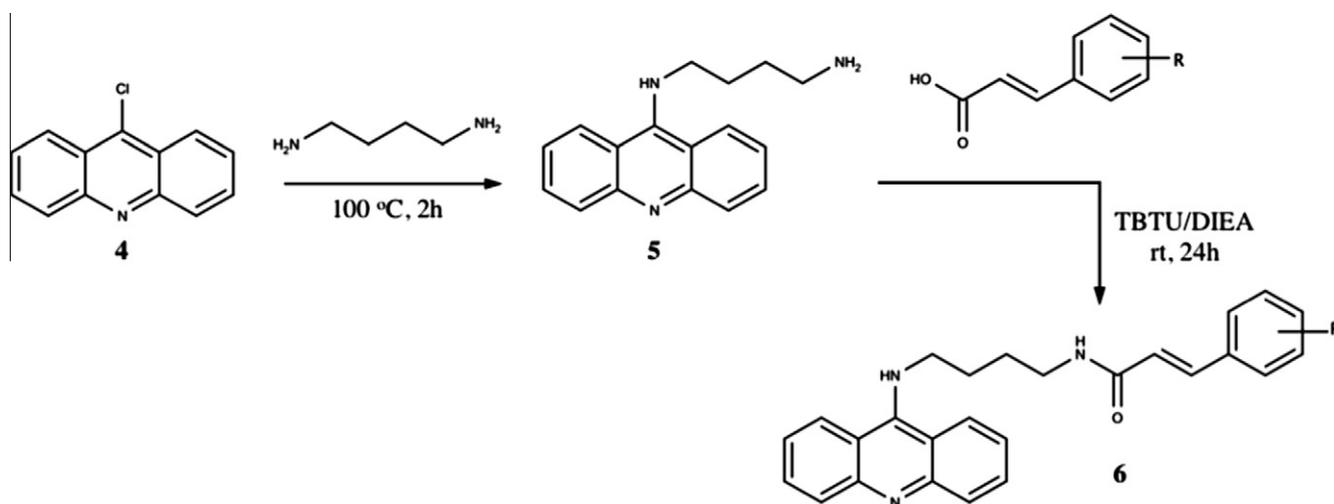


**Figure 1.** Molecular structures of quinacrine (1), chloroquine (2), acridine analogue of chloroquine (3) and primaquine (PQ).

simple and cheap, which must be a constant concern when dealing with development of potential antimalarials, which should be very inexpensive. The 9-(*N*-cinnamoylbutyl)aminoacridines **6** were synthesized by first reacting 9-chloroacridine (**4**) with a large excess of butane-1,4-diamine to produce 9-(*N*-aminobutyl)aminoacridine, **5**. This compound was then condensed to the respective cinnamic acid by a standard peptide coupling method, namely, activation of the carboxylic acid with *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) in the presence of *N*-ethyl-*N,N*-diisopropylamine (DIEA), in *N,N*-dimethylformamide (DMF) as solvent. Crude products thus obtained were further purified by liquid chromatography on silica columns, to give the target compounds **6** in high purity, as confirmed by high-performance liquid chromatography (HPLC). Structural analysis of compounds **6** by electrospray ionization-ion trap mass spectrometry (ESI-IT MS), as well as by proton ( $^1\text{H}$ ) and carbon-13 ( $^{13}\text{C}$ ) nuclear magnetic resonance (NMR) provided confirmation of the expected

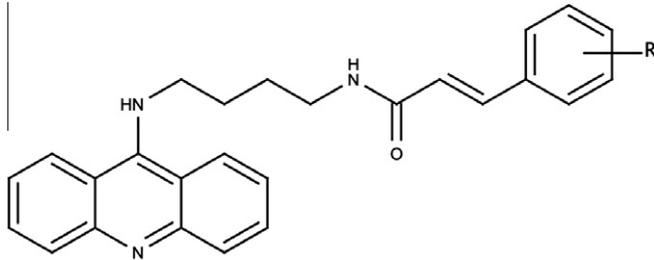
structures. The synthetic route depicted in Scheme 1 led to low global synthesis yields (2–22%), but allowed fast production of compounds **6** for immediate screening as potential dual-stage antimalarials. Attempts to increase the yields by optimization of both  $\text{S}_{\text{N}}\text{Ar}$  and condensation steps were carried out: (a) pre-activation of 9-chloroacridine **4** with phenol, followed by addition of an amine in anhydrous conditions, to avoid extensive formation of the respective acridones, according to Anderson and co-workers;<sup>7</sup> (b) use of condensation reagents of usually higher efficiency than TBTU, such as (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP). These procedures led to reaction mixtures apparently cleaner by thin layer chromatography (TLC), especially in the  $\text{S}_{\text{N}}\text{Ar}$  step, but reaction yields were not significantly improved.

The compounds were evaluated for their antiplasmodial activity against (i) erythrocytic stages of the human parasite *P. falciparum* (CQ-resistant W2 strain), Table 1, and (ii) liver stages of the rodent



**Scheme 1.** Synthesis route towards 9-(*N*-cinnamoylbutyl)aminoacridines **6**. Reagents and conditions: (i) 10 M equiv (equiv) of butane-1,4-diamine, 100 °C, 2 h; (ii) TBTU (1.1 equiv), respective cinnamic acid (1.1 equiv), DIEA (2 equiv), DMF, 24 h. Substituents R are given on Table 1.

**Table 1**  
In vitro data on compounds **6** for their blood- and liver-stage antimalarial activity



Compound	R	Pf W2 IC <sub>50</sub> <sup>a</sup> (nM)	Liver stage IC <sub>50</sub> <sup>b</sup> (μM)
<b>6a</b>	H	892 ± 152	–
<b>6b</b>	<i>p</i> -Me	225 ± 3	–
<b>6c</b>	<i>p</i> -iPr	126 ± 3	–
<b>6d</b>	<i>p</i> -OMe	138 ± 2	3.2
<b>6e</b>	<i>m</i> -F	345 ± 32	–
<b>6f</b>	<i>p</i> -F	145 ± 6	–
<b>6g</b>	<i>m</i> -NO <sub>2</sub>	142 ± 2	–
<b>3</b>		721 <sup>c</sup>	–
CQ		138 <sup>d</sup>	>15
PQ		3300 <sup>e</sup>	8

<sup>a</sup> Blood-stage antiparasmodial activity was determined against the CQ-resistant *P. falciparum* strain W2 as previously described.<sup>8</sup>

<sup>b</sup> Inhibition of liver stage infection by compounds **6** was determined by measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *PbGFP-Luc<sub>con</sub>*, as previously described.<sup>9</sup>

<sup>c</sup> EC<sub>50</sub> determined through inhibition of *Plasmodium falciparum* 3D7 LDH activity, using an LDH reporter assay; test compounds present at 2 M. Molecule ChEMBL ID: CHEMBL532286.

<sup>d</sup> Value taken from Ref. 10.

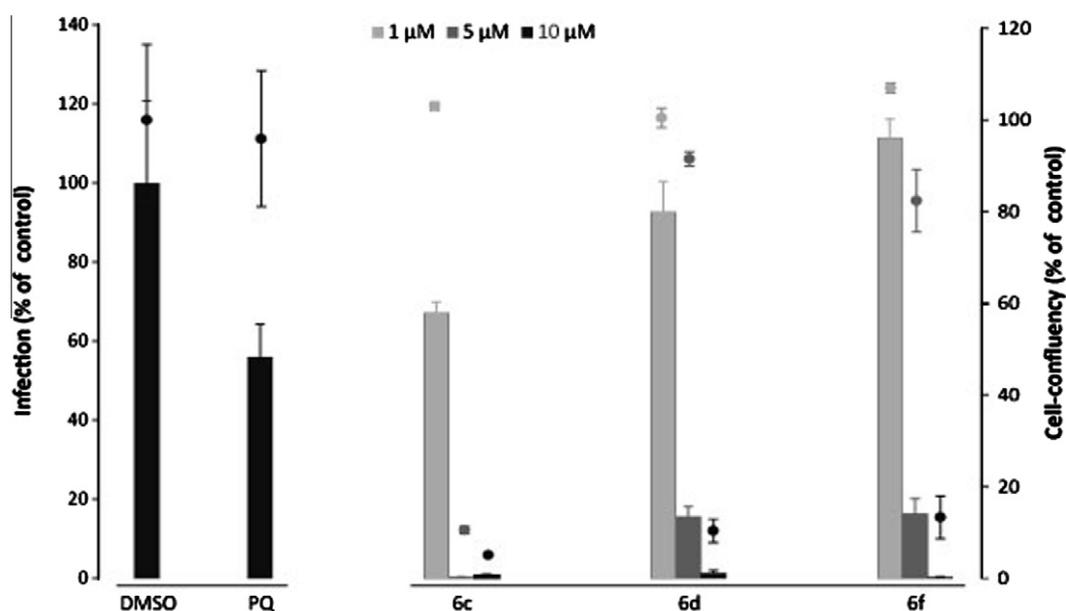
<sup>e</sup> Value taken from Ref. 8.

parasite *P. berghei* (Fig. 2, Table 1), as well as for their cytotoxicity to Huh7 human hepatoma cells (Fig. 2). Methods employed were as previously reported by us.<sup>4,5,8</sup> CQ and its acridine analogue **3** were chosen as reference drugs for blood-stage assays, whereas PQ was the reference compound for liver-stage studies.

As shown in Table 1, all the compounds except **6a** presented mid-nanomolar inhibitory activity against erythrocytic parasites,

with IC<sub>50</sub>s ranging between 126 and 345 nM. These IC<sub>50</sub>s, determined against CQ-resistant *P. falciparum* W2, were significantly lower than those reported for reference compound **3** against CQ-sensitive *P. falciparum* 3D7 (720 nM). Furthermore, compounds **6c**, **6d**, **6f** and **6g** displayed activities in the same order of magnitude as CQ, while the remaining hybrids were slightly less active. The antiplasmodial activity was significantly improved by introducing a substituent on the cinnamoyl moiety: the unsubstituted derivative, **6a**, showed an IC<sub>50</sub> of 892 nM while the substituted compound, **6e**, is approximately 3-fold more active (IC<sub>50</sub> 345 nM). The most active compound of the series, **6c**, bears an isopropyl group in the *para*-position, similar to what was previously observed by our group for 4- or 8-(*N*-cinnamoylalkyl)aminoquinolines,<sup>5,6</sup> suggesting that a bulky lipophilic cinnamic substituent is preferred to enhance antiparasitic activity. However, in our previous work we observed the best homogeneity in activity amongst cinnamic derivatives belonging to the same series, meaning that substituent R had only a slight effect on activity.<sup>4,5</sup> In contrast, the influence of cinnamoyl substituent R on the antiplasmodial activity of compounds **6** is apparent, but the SAR is unclear: the best three compounds, **6c**, **6d** and **6g** (IC<sub>50</sub> values of 126, 138 and 142 nM, respectively), exhibit quite different substituents regarding their lipophilicity and electron-donating/-withdrawing properties. Yet, comparison of compounds **6e** (R = *m*-F; IC<sub>50</sub> = 345 nM) and **6f** (R = *p*-F; IC<sub>50</sub> = 145 nM) demonstrates a clear preference for substituents in the *para*- over the *meta*-position.

QA (**1**), like CQ (**2**), is thought to act on *P. falciparum* by disrupting the storage of toxic heme as polymeric hemozoin.<sup>11</sup> Given that both QA and compounds **6** share the acridine moiety as a central scaffold, it was conceivable that they operated via a similar mechanism. Thus, compounds **6** were evaluated in vitro as inhibitors of hemozoin biocrystallization, by methods previously reported by us,<sup>4–6</sup> but none of them was found to be capable of inhibiting this process (data not shown). Still, compounds **6** are active against *Plasmodium*, and this may be due to one of the other mechanisms earlier proposed for acridines with antimalarial activity, such as inhibition of mitochondrial *bc1* complex or DNA Topoisomerase II, and interaction with DNA.<sup>12</sup> Therefore, it would be interesting to explore, in further studies, if the acridine derivatives **6** act through any of



**Figure 2.** Activity of compounds **6** against *P. berghei* liver stages. Anti-infective activity (infection scale, bars) and toxicity to hepatoma cells (cell confluency scale, circles) are shown. Primaquine (PQ) at 10 μM was included for comparison. Infection loads of Huh7 cells, a human hepatoma cell line, were determined by bioluminescence measurements of cell lysates 48 h after infection with luciferase-expressing *P. berghei* parasites.

these mechanisms. Furthermore, work is being carried out on the synthesis and characterization of analogues of compounds **6**, where the 9-aminoacridine moiety has been replaced by the 9-amino-6-chloro-2-methoxyacridine core of QA (**1**).

It is now commonly accepted that effective and safe antimalarial drugs active against both liver and erythrocytic stage parasites will be valuable components of malaria eradication strategies.<sup>3</sup> Additionally, our recent findings on the improved liver-stage antimalarial activity of *N*-cinnamoyl derivatives of primaquine<sup>4</sup> motivated us to evaluate the activity of compounds **6c**, **6d** and **6f** against liver-stage *P. berghei* parasites (Fig. 2); the compounds were chosen according to: (i) their antiplasmodial activity against the erythrocytic stage, **6c** being the most active; (ii) their prospective propensity to present low cytotoxicity, **6d** being the most active compound complying with lead-likeness properties,<sup>13</sup> Lipinski's Rule of Five,<sup>14</sup> and Veber filter,<sup>15</sup> and (iii) their likelihood of good metabolic stability, solubility and bioavailability, the cinnamic ring of **6f** being substituted with a fluorine atom, which is known for its ability to increase the aforementioned properties when incorporated into aromatic organic compounds.<sup>16</sup> Remarkably, the three test compounds were more potent than primaquine (PQ), the reference drug for the parasite liver stage, and compounds **6d** and **6f** were non-toxic to Huh7 human hepatoma cells in vitro at up to 5  $\mu$ M, as shown by cell confluency analysis (Fig. 2). The compound presenting the best activity/cytotoxicity ratio, **6d**, had an IC<sub>50</sub> value of 3  $\mu$ M (Table 1), approximately three-fold more potent than PQ, establishing these novel acridine derivatives as promising dual-stage antimalarials. To the best of our knowledge, this is the first time that compounds devoid of an aminoquinoline moiety are reported as having in vitro activity against liver-stage *Plasmodia* greater than that of PQ; furthermore, disclosure of acridine derivatives as dual-stage antimalarial leads is unprecedented.

In summary, novel 9-aminoacridine derivatives, in which the acridine core is linked to a cinnamoyl motif through a flexible aminobutyl chain, were found to be active against two stages of malarial infection in the mammalian host, the erythrocytic- and the liver-stage, while non-toxic to human hepatoma cells at up to 5  $\mu$ M. Compounds **6** were shown to not exert their antiparasitic action by inhibition of heme biocrystallization. The results obtained in this preliminary study highlight that the absence of a substituent on the cinnamic moiety is detrimental for antiparasitic activity. Extended SAR studies are envisaged, namely the introduction of a methoxy group and a chlorine atom at positions 2 and 6, respectively, of the acridine core, as it was reported that they increase the inhibitory potency by three- and ninefold on CQ-resistant and CQ-susceptible strains, respectively.<sup>17</sup> This will, hopefully, establish a novel family of dual-stage antimalarial leads, offering new potential for acridine-related compounds in malaria chemotherapy.

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## Supplementary data

Supplementary data (details regarding synthetic procedures and analytical/spectral data for compounds **5** and **6**, procedures for in vitro *Plasmodium* blood stage infection assays and procedures for in vitro *Plasmodium* liver stage infection assays) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.12.032>.

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