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Cinnamic Acid/Chloroquinoline Conjugates as Potent Agents against Chloroquine-Resistant *Plasmodium falciparum*

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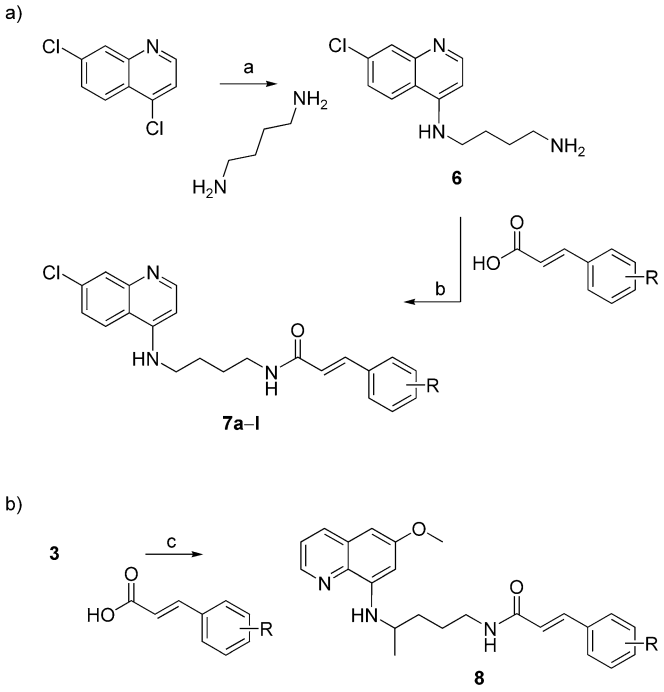
Malaria, particularly the cases of the disease caused by *Plasmodium falciparum*, is the most widespread and deadly parasitic disease, causing approximately one million deaths each year. Strains of *P. falciparum* have emerged that are commonly resistant to many available drugs, including chloroquine (**1**) and antifolates, which have been classical antimalarial agents for over half a century;[1] however, drug therapy remains a mainstay of malaria control. Most clinically available antimalarial agents target the blood-stage parasite, but specific mechanisms of action are, in many cases, unknown.[2] Still, the major mechanism of action of **1** is quite well understood—it is believed to block the biocrystallization of heme, the main process through which *Plasmodia* eliminate toxic heme after it is released from parasite digestion of host erythrocyte hemoglobin.[3]

A recent rational approach of antimalarial drug design characterized as "covalent bitherapy" involves linking two molecules with individual intrinsic activity to create a single agent, thus packaging dual activity into a single hybrid molecule.[4,5] In this regard, we have synthesized twelve novel compounds (**7**) with the heteroaromatic core of **1**, 4-amino-7-chloroquinoline, linked to differently substituted cinnamoyl groups (CIN) through a flexible aminobutyl spacer (Scheme 1 a). Compound design rationale was based on 1) relevance of **6** for inhibition of heme biocrystallization, and consequently, of the development of erythrocytic malaria parasites;[3] and 2) previous reports on cinnamic acid derivatives with promising antimalarial properties.[6–8] Moreover, we have recently demonstrated that 1) a spacer between the chloroquinoline and cinnamoyl moieties is required for antiplasmodial activity, as conjugates **5**, bearing a dipeptide spacer between those moieties were active, whereas their counterparts **4** lacking such spacer were not; and 2) the higher the lipophilicity of conjugates **5**, the higher the antiplasmodial activity.[7] Additionally, it is known that a basic nitrogen together with a flexible polymethylene chain linked to the aminoquinoline core favor drug accumulation in the acidic parasite digestive vacuole and, consequently, have a positive effect of the antiplasmodial activity of the compound.[9] Therefore, we designed second-generation conjugates **7**, where both the chloroquinoline and cinnamoyl cores were preserved, but where the rigid and hydrophilic dipeptide spacer of first-generation conjugates **5** has been replaced by a flexible and more hydrophobic butyl chain, which also increases the overall basicity of the compounds.



Scheme 1. Synthesis of a) 4-amino-7-chloroquinoline/cinnamic acid conjugates **7** and b) 8-amino-6-methoxyquinoline analogue **8** (for details of substituent R, see Table 1). *Reagents and conditions*: a) 100 °C, 2 h, 64%; b) TBTU/DIEA, RT, 24 h, 8–67%; c) TBTU/DIEA, RT, 24 h, 78% (R = *p*-*i*Pr).

Compounds **7** were evaluated in vitro as inhibitors of 1) the development of intraerythrocytic parasites of the chloroquine-resistant *P. falciparum* W2 strain, 2) heme biocrystallization, and 3) falcipain-2 (FP2) activity. As shown in Table 1, all compounds **7** were exceptionally active against erythrocytic parasites, with IC_{50} values ranging from 11 to 59 nM. All compounds were significantly more active than **1**, and two of them (**7c** and **7h**) were about as active as artemisinin (**2**), the prototype for the most important new class of antimalarial agents.

Compounds **7** did not display any enzymatic activity up to 50 μM and thus, do not exert their potent antiplasmodial activity through FP2 inhibition. This was expected, as first-generation compounds **5** were not FP2 inhibitors either.[7] Computational studies run in our group support this finding by predict-

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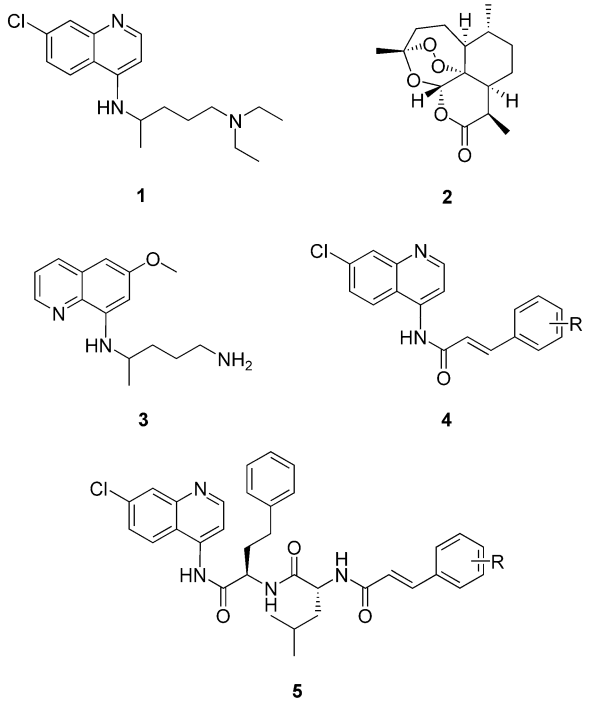


1 2 3 4 5

ing that compounds **7** are inappropriate ligands for FP2. Although docking calculations showed that the vinyl bond in **7** is placed at a distance that allows the formation of a covalent bond between the ligand and the catalytic Cys residue of the enzyme (Figure S1 in the Supporting Information), molecular dynamics determined that, after a few picoseconds, the distance between the catalytic Cys and the putative site of nucleophilic attack of compounds **7** increases from ~3.5 to ~5–5.5 Å (Figure S2 in the Supporting Information). As such, the interaction between the enzyme and compounds **7** is predicted to be insufficiently strong enough to form a stable complex, eventually leading to irreversible alkylation of the enzyme, as the ligand rapidly moves away from the catalytic Cys. Hence, it was not surprising that compounds **7** do not inhibit FP2 (Table 1). Still, replacing the dipeptide spacer in conjugates **5** by the more flexible and hydrophobic butyl chain, producing conjugates **7**, led to the anticipated increase in antiplasmodial potency.

Table 1. In vitro data for compounds **7** against *P. falciparum* (W2 strain). Chloroquine (**1**) and artemisinin (**2**) were used as reference drugs; compound **8**, the primaquine analogue of **7c**, is also included for comparison.

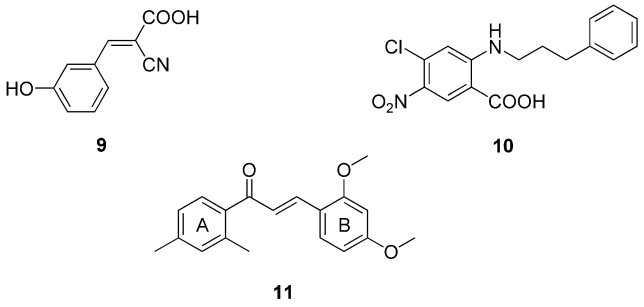
Compd	R	β-Heme inhibition[a]	IC_{50}[b] [nM]	clog *P*[c]	Yield [%]
7a	H	NA	46.6 ± 5.5	4.51	52
7b	*p*-Me	NA	16.9 ± 1.2	4.90	32
7c	*p*-*i*Pr	+	11.0 ± 6.2	5.72	28
7d	*p*-OMe	+	20.0 ± 2.6	4.45	30
7e	*p*-NH_2	NA	58.8 ± 1.5	3.77	52
7f	*m*-F	+	34.1 ± 4.2	4.68	52
7g	*p*-F	+	19.7 ± 0.4	4.69	34
7h	*p*-Cl	+	11.6 ± 0.4	5.12	16
7i	*p*-Br	++	18.2 ± 2.8	5.26	16
7j	*o*-NO_2	+	38.3 ± 4.2	4.37	67
7k	*m*-NO_2	NA	26.2 ± 5.0	4.41	36
7l	*p*-NO_2	++	23.5 ± 1.3	4.43	8
8	*p*-*i*Pr	NA	4840	5.56	78
Artemisinin		ND	9.5	2.86 (2.90)	–
Chloroquine		++	138[e]	4.49 (4.63)	–

[a] β-Heme inhibition was determined as the ability of a compound to inhibit hemozoin formation in vitro. Results were calculated as a percentage of the inhibitory effect displayed by chloroquine in the same experiment. Test compounds were ranked as follows: 50–75% (+); ≥75% (++); not active (NA); [b] IC_{50} values were determined in *P. falciparum* (W2 strain). Data represent the mean ± SD of at least three independent assays; [c] clog *P* was calculated using ALOGPS 2.1 (http://www.vcclab.org/lab/alogps/). Experimental values for artemisinin and chloroquine are given in parentheses; [d] Value taken from Ref. [12].

The remarkable activities of compounds **7** against erythrocytic parasites, along with their general ability to inhibit heme biocrystallization, suggest that these compounds share their antimalarial mechanism of action with chloroquine. To test this, we prepared and evaluated compound **8** (Scheme 1b), an analogue of **7c** (R = *p*-*i*Pr), where the 4-amino-7-chloroquinoline moiety is replaced by 8-amino-6-methoxyquinoline, the heteroaromatic ring of the antimalarial primaquine (**3**), which is not an efficient inhibitor of heme biocrystallization.[2] Such replacement led to loss of heme biocrystallization inhibitory activity, providing evidence that this activity is mainly due to the chloroquine part of the molecule and not to the cinnamoyl moiety. Still, the ability of compounds **7** to inhibit heme biocrystallization did not fully correlate with their antimalarial activity; that is to say, the most active antiplasmodial agents were not necessarily the best inhibitors of biocrystallization in vitro. For instance, compound **7b**, which did not inhibit heme biocrystallization, was determined to have antimalarial activity (16.9 nM) comparable to that of compound **7i** (18.2 nM), which is one of the best inhibitors of heme biocrystallization.

Based on literature accounts, the antimalarial activities might be ascribed to the presence of the 4-amino-7-chloroquinoline as a heme-targeted pharmacophore, with the remaining structure acting as a potential pharmacophore targeting the new permeability pathways (NPP), created on red blood cells (RBCs) by erythrocytic *P. falciparum* parasites.[10,11] Induced in the host RBC plasma membrane several hours after parasite invasion, NPP are a single or set of routes that have the functional characteristics of chloride channels, being also able to transport a range of other structurally unrelated molecules.[10] They have been proposed to play roles in nutrient uptake (e.g., sugars, amino acids and nucleotides), waste removal (e.g., lactic acid), volume regulation, and ion balance.[11] Because the properties of the NPP are significantly different to channels found in normal human RBCs, they are considered to be an important antimalarial drug target.

Kanaani et al. found that cinnamic acid derivatives (e.g., **9**) inhibit the growth of intraerythrocytic *P. falciparum* by inhibiting the NPP induced in the host cell membrane by the parasite.[14] Interestingly, they observed that NPP inhibitory activity was correlated with the hydrophobic character of the molecules. Additionally, several compounds that have previously been shown to inhibit anion transport pathways, such as 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; **10**), also inhibit NPP.[10] Generally, these compounds have a heterocyclic moiety linked to another aromatic ring through a flexible poly-



methylene chain. Kirk and Horner reported NPPB derivatives with an aromatic moiety bearing an anionic head group and an uncharged tail of varying size and polarity as NPP inhibitors.[15] According to the same authors, the ability of these NPPB derivatives to inhibit NPP improves with the length and lipophilicity of the hydrophobic tail. Chalcones like **11** are structurally related to the cinnamoyl moiety and were also reported as NPP inhibitors by Go et al.[16] and Sisodia et al.[17]

In compounds **7**, the heterocyclic core is linked to a butyl-cinnamoyl group that reasonably matches relevant structural factors described above, reinforcing the structural suitability of the compounds as NPP inhibitors. Heme seems to be a main target of compounds **7**, which agrees with their structural similarity to chloroquine. Hence, the activity of **7** could be attributed to a dual mode of action (heme biocrystallization + NPP inhibition), unveiling the butyl-cinnamoyl building block as an NPP inhibitor chemotype. Furthermore, results with known NPP inhibitors are consistent with the NPP having hydrophobic components and with the observation that the pathway shows a higher permeability to hydrophobic solutes than to hydrophilic solutes of similar sizes.[10]

Thus, it is reasonable to assume that bulkiness and lipophilicity of the putative NPP inhibition motif in **7** would favor antiplasmodial activity. In fact, there is a clear correlation between IC_{50} values found for **7** and compound lipophilicity, as estimated by calculated log P (Table 1 and Figure 1). A similar correlation is also found between the IC_{50} value and the Charton's steric parameter (ν)[12] of *p*-alkyl substituents on the aryl ring in **7**, as shown for a subset of these compounds, derivatives **7a**–**c**, with Charton's steric parameter values of 0.0, 0.52 and 0.76, for H (**7a**), *p*-Me (**7b**) and *p*-*i*Pr (**7c**) substituents, respectively.[12] Ongoing studies on the NPP inhibitory activity of compounds **7** will provide grounds to confirm the above theories, but at this stage we cannot exclude the possibility that these compounds have alternative mechanism(s) of action within the infected cell, such as, for example, bypassing the chloroquine resistance transporter (PfCRT).

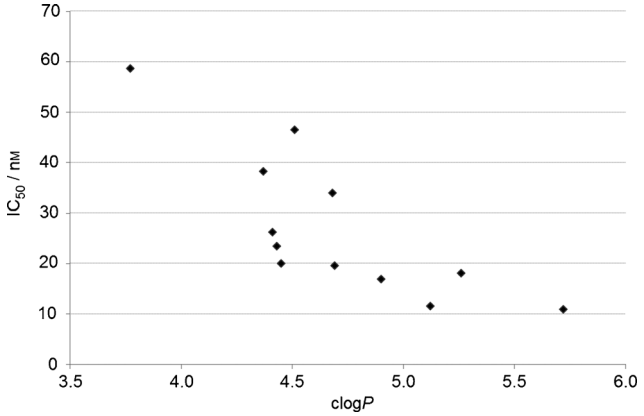


Figure 1. Plot of IC_{50} versus clog *P* values for compounds **7**.

It has been shown that resistance to chloroquine is not due to loss of activity against the chloroquine therapeutic target, heme. Rather, it is due to mutations in a putative transmembrane chloroquine transporter, PfCRT, which appears to allow the drug to rapidly efflux from the parasite digestive vacuole, preventing sufficient inhibition of heme polymerization to block parasite development. Chloroquine chemosensitizers, also known as chloroquine-resistance-reversing agents, are thought to interact with PfCRT in such a way that drug efflux is impeded.[18,19] Compounds **7** described here match relevant structural factors of chloroquine-chemosensiziting agents recently reported by Kelly[9] and Lavrado,[20] such as a hydrogen-bond acceptor (nitrogen) or aminobutyl chains between the heterocyclic core of the compound and its N-substituted terminal amine. Therefore, we cannot rule out that conjugates **7** act also through this mechanism.

In summary, novel chloroquine analogues in which the chloroquinoline core is linked to a cinnamoyl motif through a flexible aminobutyl chain have been found to be highly active against erythrocytic *P. falciparum* parasites. The in vivo activity of the two most potent compounds is presently under investigation, and preliminary data indicate that these chloroquine derivatives are nontoxic in vitro to human Huh7 hepatoma cells (data not shown). Compounds **7**, like **1**, inhibit heme biocrystallization; however, such inhibitory activity does not fully account for the potent antiplasmodial activities recorded against the chloroquine-resistant *P. falciparum* strain used. Our results strongly suggest that 1) the mode of action of **7** is similar to that of chloroquine, and 2) the butyl-cinnamoyl moiety possibly acts as a NPP inhibitor and/or a chemosensitizer motif. Confirmation of these hypotheses is being pursued in ongoing studies, and preliminary data (not shown here) are promising regarding the NPP-inhibitory ability of these compounds. This will, hopefully, establish a novel family of dual action *P. falciparum* inhibitors, bringing new insights in to the development of new antimalarial agents and offering new potential for chloroquine-related compounds in malaria therapy.

Experimental Section

4-(*N*-Aminobutyl)amino-7-chloroquinoline: 1,4-Diaminobutane (10 equiv) and 4,7-dichloroquinoline (1 equiv) were stirred at 100 °C for 3 h. After cooling to RT, the mixture was diluted with CH_2Cl_2 (25 mL), and the solution was washed with 5% aq Na_2CO_3 (×3). The organic layer was separated, dried over anhyd Na_2SO_4, filtered and concentrated to yield the title compound. Further purification was not required.

General procedure for the synthesis of 7a–l: A solution of substituted cinnamic acid (1.1 equiv), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU; 1.1 equiv) and *N*-ethyl-

N,N-diisopropylamine (DIPEA; 2.2 equiv) in *N,N*-dimethylformamide (DMF; 5 mL) was stirred at 0 °C for 10 min. Then, a solution of 4-(*N*-aminobutyl)amino-7-chloroquinoline (1.1 equiv) in DMF was added, and the reaction proceeded at RT for an additional 24 h. The mixture was diluted with CH_2Cl_2 (25 mL), and the solution was washed with 5% aq Na_2CO_3 (×3). The organic layer was separated, dried over anhyd Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography (EtOAc/MeOH, 4:1 *v/v*) to give the desired compound **7**. Compound **8**, the primaquine analogue of **7c**, was prepared by a similar method. Full spectrocopic and analytical data on the synthesized compounds are given in the Supporting Information.

In vitro heme biocrystallization inhibition assay: The assay was performed as previously described.[21,22] Briefly, test compound in DMSO at various concentrations (0.1–1 mM) was added to a solution of hemin chloride in DMSO (50 μL, 5.2 mg mL^{-1}). Assays were performed in triplicate. Controls contained equal volumes of water (or DMSO). Biocrystallization was initiated by the addition of acetate buffer (100 μL, 0.2 M, pH 4.4), and the plates were incubated at 37 °C for 48 h. Samples were centrifuged (SIGMA 3–30 K) at 3000 rpm for 15 min. After discarding the supernatant, the pellet was washed with DMSO (200 μL×4) and then dissolved in 0.2 M aq NaOH (200 μL). The solubilized aggregates were further diluted with 0.1 M aq NaOH (1:6), and absorbance was recorded at 405 nm (Biotek Powerwave XS with software Gen5 1.07).

In vitro falcipain-2 inhibition assay: The assay was performed as previously described.[23] Briefly, recombinant falcipain-2 (1 nM) was incubated with various concentrations of test compound (taken from 10 mM DMSO stock solutions) in sodium acetate (100 mM; pH 5.5) and dithiothreitol (10 mM) for 30 min at room temperature before addition of the substrate, benzoxycarbonyl-Leu-Arg-7-amino-4-methyl-coumarin (final concentration 25 μM). Fluorescence was continuously monitored for 30 min at room temperature in a Labsystems Fluoroskan II spectrofluorometer, and IC_{50} values were determined from plots of activity versus enzyme concentration created using GraphPad Prism software.

In vitro blood-schizontocidal activity determination: The activity was determined using a method previous reported by us.[24] Synchronized ring-stage W2 strain *P. falciparum* was cultured with test compound at various concentrations (taken from 1000× DMSO stock solutions) in RPMI 1640 medium containing 10% human serum or 0.5% Albumax serum substitute. After incubation for 48 h, when control cultures contained new rings, parasites were fixed with 1% formaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 48 h at room temperature and then labeled with YOYO-1 (1 nM; Molecular Probes) in 0.1% Triton X-100 in PBS. Parasitemia was determined from dot plots (forward scatter versus fluorescence) acquired on a FACSort flow cytometer using CELLQUEST software (Becton Dickinson). Growth inhibition (IC_{50}) was determined from plots of parasitemia (%) against inhibitor concentration generated using GraphPad Prism software. In each case, the curve fit presented an R^2 value of >0.95.

Molecular docking and molecular dynamics simulations: Computational methods were used to predict the structures and stabilities of **7**–FP-2 complexes. Additional details about these calculations are supplied in the Supporting Information.

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