Journal of Medicinal Chemistry

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/jm301654b • Publication Date (Web): 30 Dec 2012

N-cinnamoylated chloroquine analogues as dual-stage antimalarial leads

Bianca C. Pérez,[1] Cátia Teixeira,[1,2] Inês S. Albuquerque,[3] Jiri Gut,[4] Philip J. Rosenthal,[4] José R. B. Gomes,[2] Miguel Prudêncio,[3] Paula Gomes[1,*]

[1] Centro de Investigação em Química da Universidade do Porto, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, R. do Campo Alegre 687, P-4169-007 Porto, Portugal

[2] CICECO, Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[3] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal

[4] Department of Medicine, San Francisco General Hospital, University of California, CA 94143-0811, USA

Abbreviations: ^{13}C-NMR, carbon-13 nuclear magnetic resonance; ^{1}H-NMR, proton nuclear magnetic resonance; ACT, artemisinin-based combination therapies; CQ, chloroquine; DCM, dichloromethane; DIEA, *N*-ethyl-*N,N*-diisopropylamine; DMF, *N,N*-dimethylformamide; DMSO-d_6, deuterated dimethylsulfoxide; ESI-IT, electrospray ionization and ion-trap quadrupole detection; HEFLECINs, heterocyclic-cinnamic conjugates; HPLC-DAD, high performance liquid chromatography with diode array detection; MS, mass spectrometry; NPP, new permeation pathways; PQ, primaquine; PRIMACINs, primaquine-cinnamic acid conjugates; RBC, red blood cell; SAR, structure-activity relationship; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate.

* To whom correspondence should be addressed: P. Gomes, Centro de Investigação em Química da Universidade do Porto, Rua do Campo Alegre, 687, P-4169-007, Porto, Portugal; Phone and Fax: +351220402563; E-mail: pgomes@fc.up.pt.

ABSTRACT

The control of malaria is challenged by drug resistance, and new antimalarial drugs are needed. New drug discovery efforts include consideration of hybrid compounds as potential multi-target antimalarials. Previous work from our group has demonstrated that hybrid structures resulting from cinnamic acid conjugation with heterocyclic moieties from well known antimalarials present improved antimalarial activity. Now, we report the synthesis and SAR analysis of an expanded series of cinnamic acid derivatives displaying remarkably high activities against both blood- and liver-stage malaria parasites. Two compounds judged most promising, based on their *in vitro* activity and drug-likeness according to the Lipinski rules and Veber filter, were active *in vivo* against blood stage rodent malaria parasites. Therefore, the compounds reported represent a new entry as promising dual-stage antimalarial leads.

Keywords: antimalarials, blood-schizontocidal, chloroquine, cinnamic acid derivatives, dual-action, liver stage, *Plasmodium falciparum*, *Plasmodium berghei*.

Introduction

Several concerted efforts to control malaria and achieve its eradication have been made through history.[1] Despite initial success, with regional elimination in Southern Europe and some countries in North Africa and the Middle East, malaria has not been controlled in many other countries due to a number of factors.[2] Two main reasons behind malaria remaining such a burden to humanity are the widespread resistance of the malaria parasite to available drugs and its complex life cycle.[2] Human malaria is triggered by infection by an intracellular *Plasmodium* parasite, which is transmitted to human hosts via an infected female *Anopheles* mosquito bite. Once in the host circulation, *Plasmodium* sporozoites initiate the asexual phase of the life cycle by quickly moving to the liver. During this clinically silent liver-stage infection, thousands of merozoites are formed and released into the bloodstream, where they invade red blood cells, thus starting the pathogenic asexual erythrocytic cycle, resulting in the various clinical manifestations of malaria.[3]

Resistance of malaria parasites to previous generations of medicines, such as chloroquine (**1**), became widespread in the 1970s and 1980s, undermining malaria control efforts. During the last decade, artemisinin-based combination therapies (ACTs) have been adopted globally as the first line of treatment. ACTs include a rapidly acting artemisinin component and a partner drug to improve efficacy and hinder emergence of resistance to artemisinins.[4] However, there are now worrisome signs that malarial parasites are developing resistance to artemisinins (**2**),[5] and so the dependence of all new drug combinations on artemisinin derivatives is a matter of concern. While most available antimalarial agents target the blood-stage parasites, relatively few drugs are known to inhibit liver stage parasites. Primaquine (**3**) remains the only drug in clinical use worldwide that acts against liver stages of all *Plasmodium* species,[6] including *P. vivax* and *P. ovale* hypnozoites, which are latent forms unique to these species that cause relapsing malaria long after the original infection, complicating control efforts.[7, 8] Taking into account concerns of drug resistance and difficult-to-treat chronic liver stages, optimal therapy should be effective against multidrug-resistant parasite strains and be active against both liver and erythrocytic parasite stages.

A recent approach in antimalarial drug design characterized as "covalent bitherapy" involves linking two active molecules, thus packaging dual activity into a single hybrid molecule with potential to enhance efficacy, improve safety and/or reduce propensity to elicit resistance relative to the parent drugs.[9, 10] As part of our efforts to develop novel hybrids, we previously reported a first-generation of heterocyclic-cinnamic acid conjugates (**4** and **5**, Figure 1), linking the heteroaromatic ring of known antimalarials to a *trans*-acid cinnamic motif, active *in vitro* against blood-stage *P. falciparum*.[11] From these studies we discovered that a spacer between the heterocycle and the cinnamoyl moiety is required for antiplasmodial activity and that the higher the lipophilicity of conjugates **4**, the higher their *in vitro* antiplasmodial activity. Based on these results, we developed a second-generation of conjugates (**7** and **8**, Figure 1) where the cinnamoyl core was linked to the heterocyclic core of known antimalarials through a flexible and more hydrophobic butylamine chain.[12, 13] Compounds **7** (IC_{50} against cultured malaria parasites: 11 – 59 nM)[13] and **8** (IC_{50}: 1.4 – 2.4 μM)[12] had higher *in vitro* potency than their parent compounds, **1** (IC_{50}: 138 nM) and **3** (IC_{50}: 7.5 μM), against blood-stage *P. falciparum* (W2 strain) and liver-stage *P. berghei*. These findings established the cinnamoyl core as a valuable pharmacophore to enhance potency of established antimalarials.

Building on these compounds, we have performed further SAR studies and herein report the synthesis and antimalarial assessment of a series of new heterocyclic-cinnamic conjugates (in brief, HEFLECINs). In terms of SAR, the presence of aromaticity in the heterocyclic core, the number of the heteroaromatic rings, the length of the aliphatic chain, the nature (amide or ester) of the linkage between the heterocycle and cinnamic core and the nature of the cinnamic substituent were all evaluated (Figure 2). Compounds were tested *in vitro* against erythrocytic stages of the human parasite *P. falciparum*; two of them, **7d** and **7h**, were further confirmed to be active *in vivo* against the clinically relevant blood-stage infection in a *P. berghei* rodent malaria model. Additionally, our recent findings on the improved liver-stage antimalarial activity of *N*-cinnamoyl derivatives of primaquine,[12] prompted us to evaluate the activity of heterocyclic-cinnamic conjugates against liver-stage *P. berghei* parasites. Most of the HEFLECINs were structurally

related to chloroquine, presented outstanding activity against both erythrocytic and liver stages of the parasite life-cycle, and were non-cytotoxic against Huh7 human hepatoma cells. To the best of our knowledge, chloroquine-related compounds active against both liver- and blood-stage parasites have not previously been reported. These results bring new insights into the development of dual action antimalarial agents.

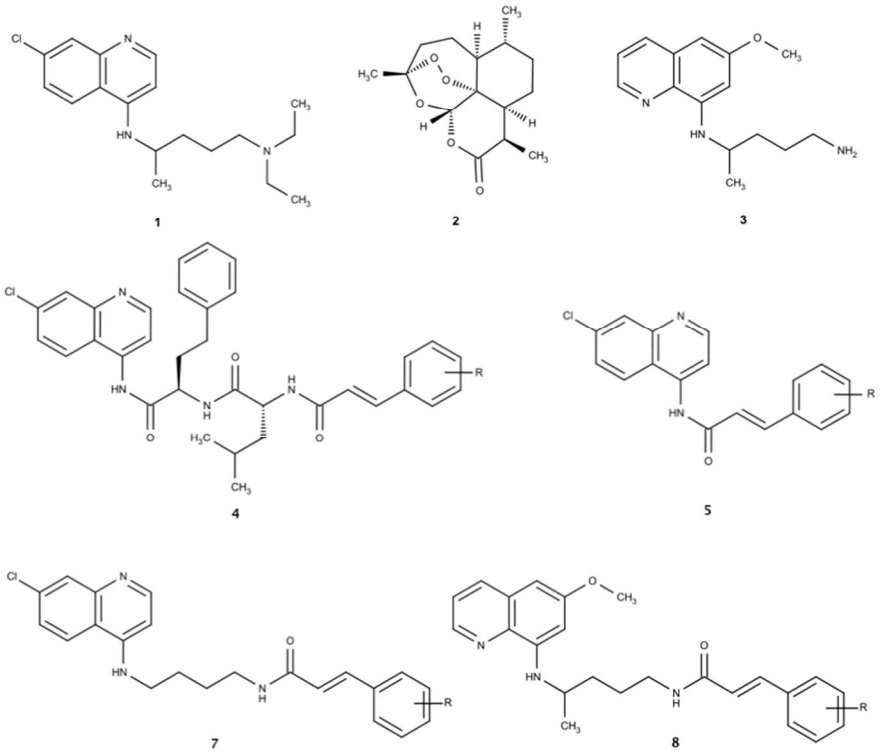
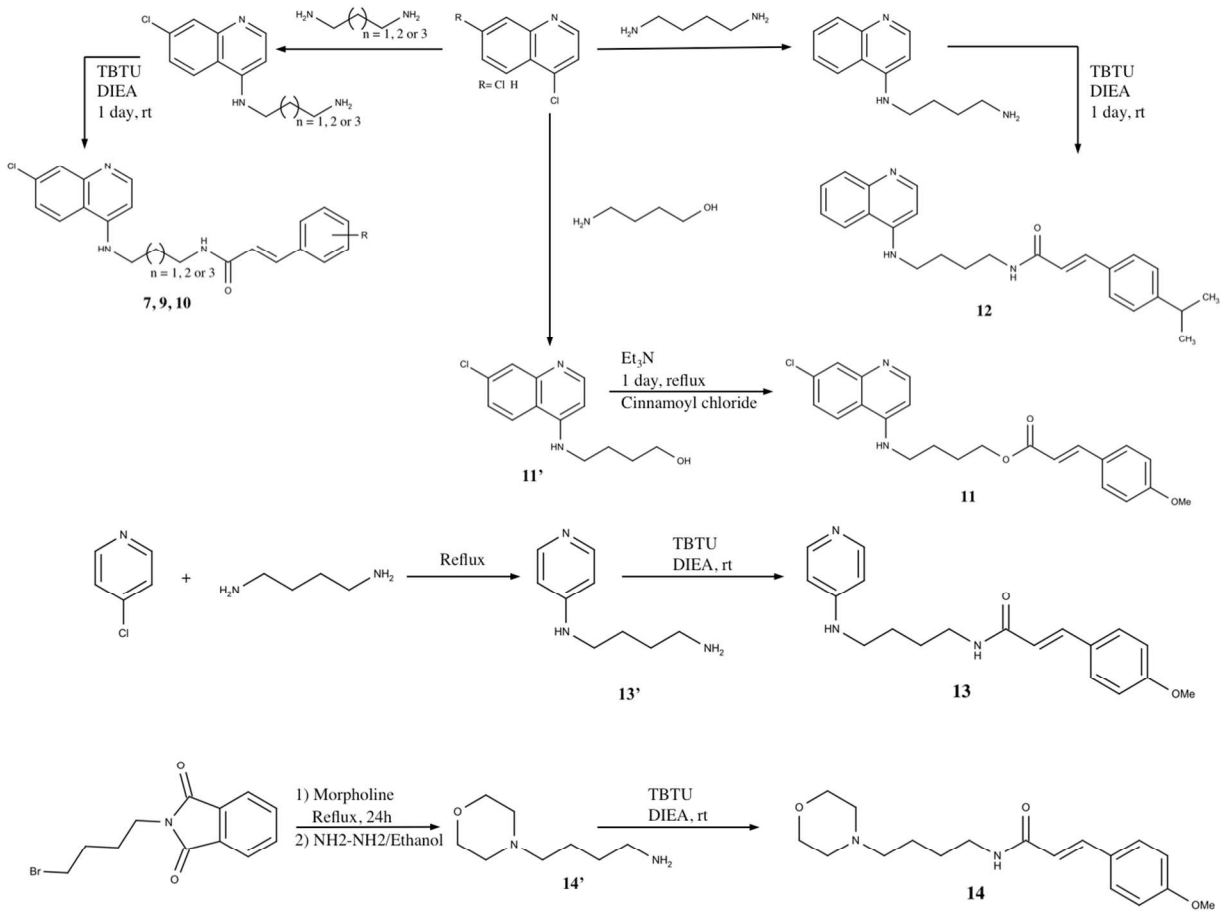


Figure 1: Chemical structures of chloroquine (**1**), artemisinin (**2**), primaquine (**3**), first- (**4** and **5**) and second- (**7** and **8**) generation heterocycle-cinnamic acid conjugates.

Results and Discussion

Chemistry. For the past decade we have been working on the design, synthesis and evaluation of potential antimalarial candidates, bearing in mind that synthetic pathways should be kept as cheap and straightforward as possible. The HEFLECINs reported here are examples of how simple chemistry can yield promising antimalarial leads (Scheme 1). Compound **8d** was obtained through a single amide coupling step between the parent drug PQ (**3**) and the desired cinnamic acid, using previously reported standard peptide coupling conditions[12], namely, activation of the cinnamic acid with *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) in the presence of *N*-ethyl-*N,N*-diisopropylamine (DIEA), followed by addition of PQ. Compounds **7**[13] and **9-13** were obtained in two steps, starting from (i) a classical nucleophilic aromatic substitution reaction between the appropriate diaminoalkane (1,3-diaminopropane, 1,4-diaminobutane or 1,5-diaminopentane) or 4-aminobutan-1-ol (in the synthesis of **11**) and the desired chlorinated heterocycle (4,7-dichoroquinoline, 4-chloroquinoline or 4-chloropyridine), followed by (ii) coupling the resulting compounds to the appropriate cinnamic acids, again conveniently activated by TBTU/DIEA. The synthesis of **14** was the only one requiring three steps, as (i) morpholine was first reacted with 1-(*N*-phthaloyl)amino-4-bromobutane, followed by (ii) hydrazinolysis of the phthaloyl amino-protecting group, to afford the free amine for subsequent (iii) PyBOP/DIEA-mediated coupling to the appropriate cinnamic acid.

Although not all synthesis yields were high, the rather small number of synthesis steps, cheap starting materials and straightforward purification techniques (cf. Experimental Section) largely compensate for low yields. All the target compounds where obtained in high purity, as determined by high performance liquid chromatography with diode array detection (HPLC-DAD), and their structures were confirmed by proton (^{1}H-) and carbon-13 (^{13}C-) nuclear magnetic resonance (NMR), as well as by mass spectrometry (MS) analysis with electrospray ionization and ion-trap quadrupole detection (ESI-IT); relevant data are presented in the Experimental Section.



Scheme 1. Synthetic pathways towards heterocycle-cinnamic acid conjugates **7, 9-12**.

Biology. Our recent discovery of the potent *in vitro* activity of CQ analogues **7** against blood-stage CQ-resistant *P. falciparum* parasites unveiled the key role of the cinnamoyl moiety in reversing parasite resistance to CQ.[13] We have now expanded the family of heterocyclic-cinnamic acid conjugates (HEFLECINs) and tested their *in vitro* activity against blood-stage *P. falciparum* (Table 1), in order to (i) establish relevant SAR (Figure 2), and (ii) select the most promising candidates for *in vivo* challenge in the *P. berghei* rodent malaria model. For some of the compounds we compared activity against chloroquine-sensitive (3D7) and -resistant (W2) *P. falciparum* parasites. Furthermore, given their structural analogy to CQ, which acts via inhibition of β-hematin formation, selected compounds were evaluated as *in vitro* inhibitors of this process (Table 1).[14, 15] Finally, based on our latest disclosure of primaquine-cinnamic acid conjugates (PRIMACINs) with improved *in vitro* activity against liver-stage *Plasmodia*,[12] we also assessed the *in vitro* activity of a subset of the HEFLECINs against liver-stage *P. berghei* (Figure 3, Table 1).

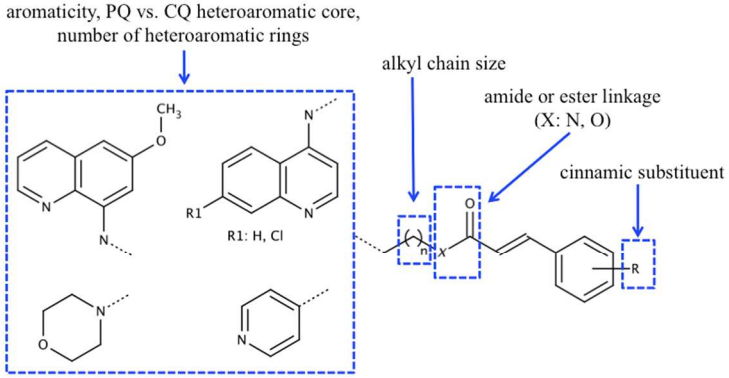


Figure 2: Structural parameters considered in the SAR analysis of the heterocycle-cinnamic acid conjugates.

***In vitro* activity against intraerythrocytic *P. falciparum* parasites**. Blood-stage activity of HEFLECINs was determined by methods previously reported by us.[16, 17] Close inspection of our data (Table 1, columns 8 and 9) shows the following.

(i) Remarkably, most of the test compounds assayed against both strains were more active against the CQ-resistant (W2) strain than against the CQ-sensitive (3D7) strain.

(ii) Compounds from the **7**, **9** and **10** series with a single substituent (R) on the cinnamoyl group (i.e., all except **7p**) were highly active, with IC_{50}s in the range of 11-79 nM. These series differ only in the length of the polymethylene spacer between the chloroquinoline and cinnamoyl cores. Comparison between homologues from series **7**, **9** and **10** (i.e., **7c** *vs.* **9c** *vs.* **10a**, or **7h** *vs.* **9h** *vs.* **10c**, or **7d** *vs.* **9d** *vs.* **10b**) shows that a butyl spacer is preferred over pentyl and propyl spacers.

(iii) Removal of the 7-chlorine substituent from the chloroquinoline ring leads to a decrease in antimalarial activity of about one order of magnitude, as shown for compounds **7c** (IC_{50} 11 nM) and its dechlorinated analogue **12** (115 nM).

(iv) Replacement of the amide bond in **7d** (IC_{50} 20 nM) by its ester isostere, to afford **11**, leads to a six-fold decrease in activity (122 nM).

(v) Substitution of the chloroquinoline ring (e.g. **7d**, IC_{50} ~ 20 nM) by another heteroaromatic ring, either quinolinic (8-amino-6-methoxyquinoline ring in **8d**), pyridinic (single heteroaromatic pyridine ring in **13**) or non-aromatic (morpholine ring in **14**), leads to complete loss of activity.

(vi) Comparison of IC_{50} values within a given series, e.g. compounds **7** or **9**, shows that the cinnamoyl substituent R has some effect on activity, but that this effect is not dramatic. The electrodonating/withdrawing character of R seems to have no significant role in activity, whereas the *para* position is clearly preferred over the *ortho* or *meta* positions, as shown for **7k-l** and **9k-l**.

(vii) Increase in compound lipophilicity and/or the bulkiness of R within a series seems to enhance *in vitro* activity. We previously showed that IC_{50} values for compounds **7** showed good correlation with both clogP values and Charton parameters.[13] For the new **9** series, we observed a good correlation with Charton parameters, but not clogP values (cf. Table S1 provided as Supporting Information). Series **10** was too small to allow drawing definitive conclusions in this regard.

(viii) Finally, HEFLECINs bearing the chloroquinoline core were globally more active than CQ, whereas the two most active compounds, **7c** and **7h**, had activities comparable to that of artemisinin. Therefore, these two compounds, as well as **7d**, the most active compound within the series satisfying the lead-likeness,[18] Lipinski[19] and Veber[20] filters, were selected for *in vivo* studies with *P. berghei*.

***In vitro* activity against β-hematin formation**. The ability of HEFLECINs to inhibit β-hematin formation to hemozoin, the main mechanism attributed to CQ and related antimalarials, was assessed by previously reported methods.[11] This activity was not fully correlated with *in vitro* antimalarial activities (Table 1), suggesting that HEFLECINs owe their antimalarial properties to mechanisms in addition to inhibition of β-hematin crystallization. Nonetheless, we believe that inhibition of β-hematin formation contributes to activity because (i) variation of β-hematin inhibition potency amongst homologues, e.g., **7c**, **9c** and **10a** follows the same trend as variation of activities against blood-stage parasites and (ii) the significant decrease in antimalarial activity upon removal of the 7-chlorine substituent (**7c** vs. **12**) may be explained by the prior observation that this chlorine atom is essential to ensure effective β-hematin inhibition by CQ and related structures.[21, 22]

Based on previous and current findings, it is concluded that HEFLECINs do not act against blood-stage parasites *via* inhibition of the cysteine protease falcipains.[13] Activity may include impairment of the New Permeation Pathways (NPP) created by *Plasmodia* in the infected red blood cell (RBC)[13] and inhibition of β-hematin crystallization.

***In vivo* treatment of *P. berghei*-infected mice.** For the 3 most promising HEFLECINS, *in vivo* efficacy was evaluated using a standard *P. berghei* mouse model. Compounds chosen for in vivo analysis were **7c** and **7h** due to their potency against cultured blood-stage *P. falciparum* and **7d** due to its better drug-likeliness. Mice were infected intraperitoneally (ip) with *P. berghei* and then treated twice daily with ip injections of 10, 30 and 100 mg/kg. Chloroquine was used as a positive control at 3, 10 and 30 mg/kg ip twice daily. All mice died due to malaria or toxicity by day 17 at the doses administered. In order to compare compound efficacies, survival over time after the initiation of treatment was compared.

Close inspection of the data leads to the following observations (Figure 3). Compounds **7c** and **7h** were highly toxic at 100 mg/kg, and no mice survived until the last day of treatment. At lower dosages, **7h** extended survival compared to untreated controls by 2-7 days at 30 mg/kg and by 2 days at 10 mg/kg. Compound **7c** did not improve survival compared to untreated controls. Compound **7d** did not appear to be toxic at any tested dosage. It extended survival by 2 days compared to untreated controls at the highest dosage, but offered no benefit over untreated controls at the lower dosages.

Studies with the rodent malaria model confirmed that **7d** and **7h** have *in vivo* antimalarial activity. The lack of *in vivo* activity of **7c** can be attributed to its high lipophilicity (clogP > 5, see Table S1 on SI),[19] but compounds **7d** and **7h** are only slightly less lipophilic (clogP ~5, see Table S1 on SI) than **7c**. However, *in vivo* activities for **7d** and **7h** were lower than might be expected based on their *in vitro* activities, possibly due to limitations in bioavailability. Additionally, relatively poor performance of the compounds *in vivo* might be explained by biological differences between *P. berghei* and *P. falciparum* and by the high hydrophobicity of the compounds, which might result in extensive binding to plasma proteins.[23]

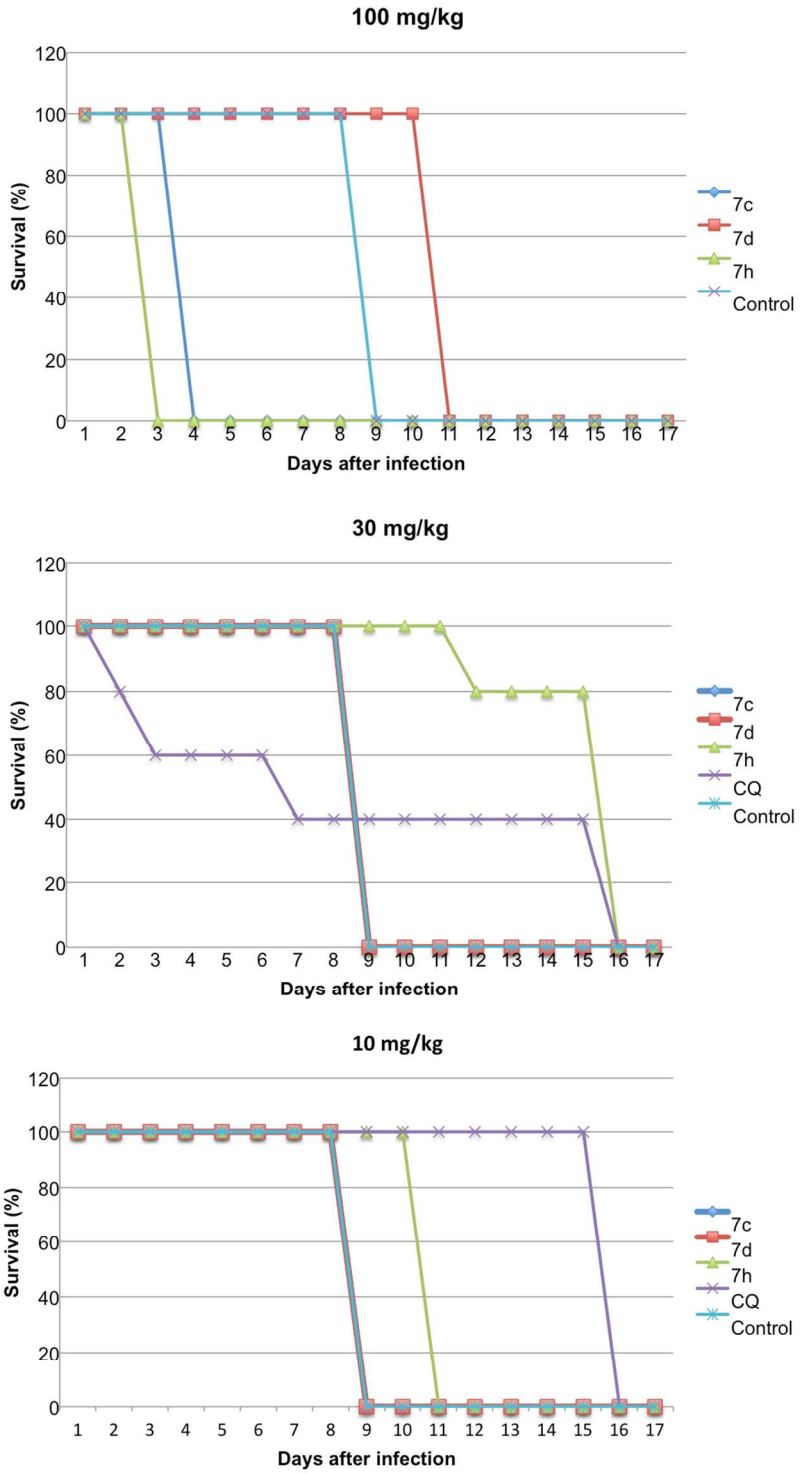
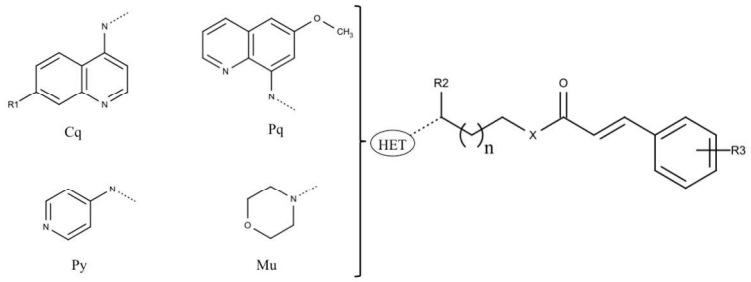


Figure 3: Survival curves for *P.berghei*-infected mice treated with compounds **7c**, **7d** and **7h**, and CQ.

Table 1. *In vitro* data on test compounds for β-hematin inhibition, and blood and liver stage activity.



Compound	HET	n	X	R2	R3	β-Hematin inhibition[a]	*Pf* W2 IC_{50} (nM)[b]	*Pf* 3D7 IC_{50} (nM)[b]	Liver Stage IC_{50} (μM)[c]
7a	Cq R1 = Cl	2	N	H	H	NA	46.6 ± 5.5	62.85±3.5	1.06 ± 0.1
7b					*p*-Me	NA	16.9 ± 1.2	24.23±0.2	2.36 ± 0.5
7c					*p*-^{i}Pr[c]	+	11.0 ± 6.2	15.63±5.4	2.50 ± 0.2
7d					*p*-OMe[c]	+	20.0 ± 2.6	30.63±9.5	2.86 ± 0.4
7e					*p*-NH_2	NA	58.8 ± 1.5	78.12±2.2	ND[g]
7f					*m*-F	+	34.1 ± 4.2	50.92±1.0	1.13 ± 0.2
7g					*p*-F	+	19.7 ± 0.4	39.85±3.5	1.42 ± 0.2
7h					*p*-Cl [d]	+	11.6 ± 0.4	26.21±2.0	1.44 ± 0.3
7i					*p*-Br	++	18.2 ± 2.8	28.75±6.3	2.28 ± 0.6
7j					*o*-NO_2	+	38.3 ± 4.2	56.91±5.1	1.09 ± 0.2
7k					*m*-NO_2	NA	26.2 ± 5.0	42.06±7.1	ND
7l					*p*-NO_2	++	23.5 ± 1.3	48.94±3.2	ND
7o					*p*-NMe_2	+	25.8 ± 2.1	ND	ND
7p					*m,p*-diOMe	NA	110.8 ± 13.0	ND	4.05 ± 0.2
8d	Pq	2	N	CH_3	*p*-OMe		>10000[h]	-	2.35 ± 0.19[h]
9a	Cq R1 = Cl	1	N	H	H	NA	74.8 ± 2.0	141.3±9.3	ND
9b					*p*-Me	NA	47.4 ± 1.2	55.85±5.1	ND
9c					*p*-iPr	NA	38.0 ± 11.2	50.27±1.0	ND
9d					*p*-OMe	NA	55.1 ± 2.3	49.51±5.8	4.02 ± 0.6
9e					*p*-NH_2	NA	79.8 ± 2.0	134.85±5.4	ND
9f					*m*-F	NA	73.3 ± 6.8	137.95±1.2	ND
9g					*p*-F	NA	46.7 ± 3.3	47.73±1.3	ND
9h					*p*-Cl	+	41.6 ± 2.9	46.47±4.5	ND
9i					*p*-Br	NA	38.2 ± 6.9	49.96±3.5	ND
9j					*o*-NO_2	+	52.1 ± 4.0	64.15±6.7	ND
9k					*m*-NO_2	+	50.3 ± 1.2	52.73±5.3	ND
9l					*p*-NO_2	+	23.7 ± 2.1	52.53±10.8	ND
10a	Cq R1 = Cl	3	N	H	*p*-^{i}Pr	+	31.5 ± 3.8	ND	ND
10b					*p*-OMe	NA	50.3 ± 2.6	ND	2.27 ± 0.6
10c					*p*-Cl	NA	25.1 ± 0.2	ND	1.62 ± 0.1
11	Cq R1 = Cl	2	O	H	*p*-OMe	+	121.9 ± 5.5	ND	6.47 ± 0.9
12	Cq R1 = H	2	N	H	*p*-^{i}Pr	NA	114.9 ± 3.0	ND	ND
13	Py	2	N	H	*p*-OMe	NA	>10000	ND	>10
14	Mu	2	N	H	*p*-^{i}Pr	NA	>10000	ND	>10
ART						-	9.5 ± 1.9	23.5±0.8	ND
CQ						++	138[e]	52.45	15.9 ± 0.0
PQ							3300[f]		7.5[h]

[a] ability of the test compounds to inhibit hemozoin formation *in vitro* was calculated as a % of the inhibitory effect displayed by reference drug CQ in the same experiment; test compounds were ranked as follows: <50%, not active (-); between 50 and 75%, moderately active (+); ≥75%, highly active (++); [b] blood-stage antiplasmodial activity was determined against the CQ-resistant *P. falciparum* strain W2 and strain 3D7, using artemisinin (ART) and CQ as reference drugs;[c] IC_{50} of the most active compounds against liver stage;[d] also tested *in vivo*;[e] Value taken from Ref [24]; [f] Value taken from Ref. [17], [g] ND, not determined, [h] Value taken from Ref. [12]

***In vitro* activity against liver-stage *P. berghei* parasites**. Recent findings from our group, demonstrating that the liver-stage activity of PQ is significantly enhanced by conjugation with cinnamic acids, prompted us to investigate whether HEFLECINs would also exhibit liver stage activity, measured using *P. berghei* as previously reported.[12, 17, 25] We found that HEFLECINs bearing the chloroquinoline core of CQ (**7**, **9**, **10**) were all active against liver stage parasites. This result was surprising, as CQ is not active against liver-stage malaria[26]. In view of these results, the activities of compounds selected as most promising based on activity and cytotoxicity, were compared quantitatively (Figure 4, Table 1). Remarkably, activities were much higher than those observed for either CQ or PQ. Specifically, IC_{50} values were 5-15 times lower than those for CQ, and 3-7 times lower than those for the reference drug PQ. This is, to our knowledge, the first report of 4-amino-7-chloroquinolinic (CQ-related) structures displaying *in vitro* activity against liver-stage *Plasmodia* greater than that of PQ. This finding, together with the previous observation that *N*-cinnamoylation of PQ also enhances its *in vitro* activity against liver-stage *Plasmodia*,[12] strongly suggests that the *N*-cinnamoyl moiety is a relevant pharmacophore to boost antiplasmodial activity in both 8- and 4-aminoquinolines. Some SAR could be devised from the liver-stage assays, as follows:

(i) As in blood-stage activity assays, a butyl spacer between the chloroquinoline and the cinnamic cores is generally preferred over propyl and pentyl spacers, both in terms of activity and toxicity, as inferred from comparison of homologues from series **7**, **9** and **10**.

(ii) Unlike what was observed in blood-stage activity assays, substitution of the cinnamoyl aromatic ring in series **7** led to slightly decreased activity, i.e., the most active compound was **7a**, where R = H.

(iii) The presence of electrodonating R groups in the *para* position led to a decrease in activity.

(iv) An increase of the number of OMe substituents on the cinnamoyl core from one (**7d**) to two (**7p**) led to a decrease in toxicity, but also in activity.

(v) Removal of the 7-chlorine atom from the chloroquinoline ring led, as observed for blood-stage activity, to a decrease in liver-stage activity.

(vi) Also as observed against blood-stage *Plasmodia*, replacement of the chloroquinoline ring by pyridine or morpholine led to loss of activity against liver-stage parasites.

As is the case for PQ, the mechanism of HEFLECINs against liver-stage *Plasmodia* remains to be elucidated.[12] Studies are under way to establish the target(s) of HEFLECINs against liver- and blood-stage parasites. The discovery that HEFLECINs bearing the CQ heterocyclic core, unlike their PQ analogues (PRIMACINs[12]), combine significant liver-stage activity with potent blood-stage activity, is of great interest. To our knowledge, this is the first example of CQ-related molecules emerging as multi-stage antimalarial leads.

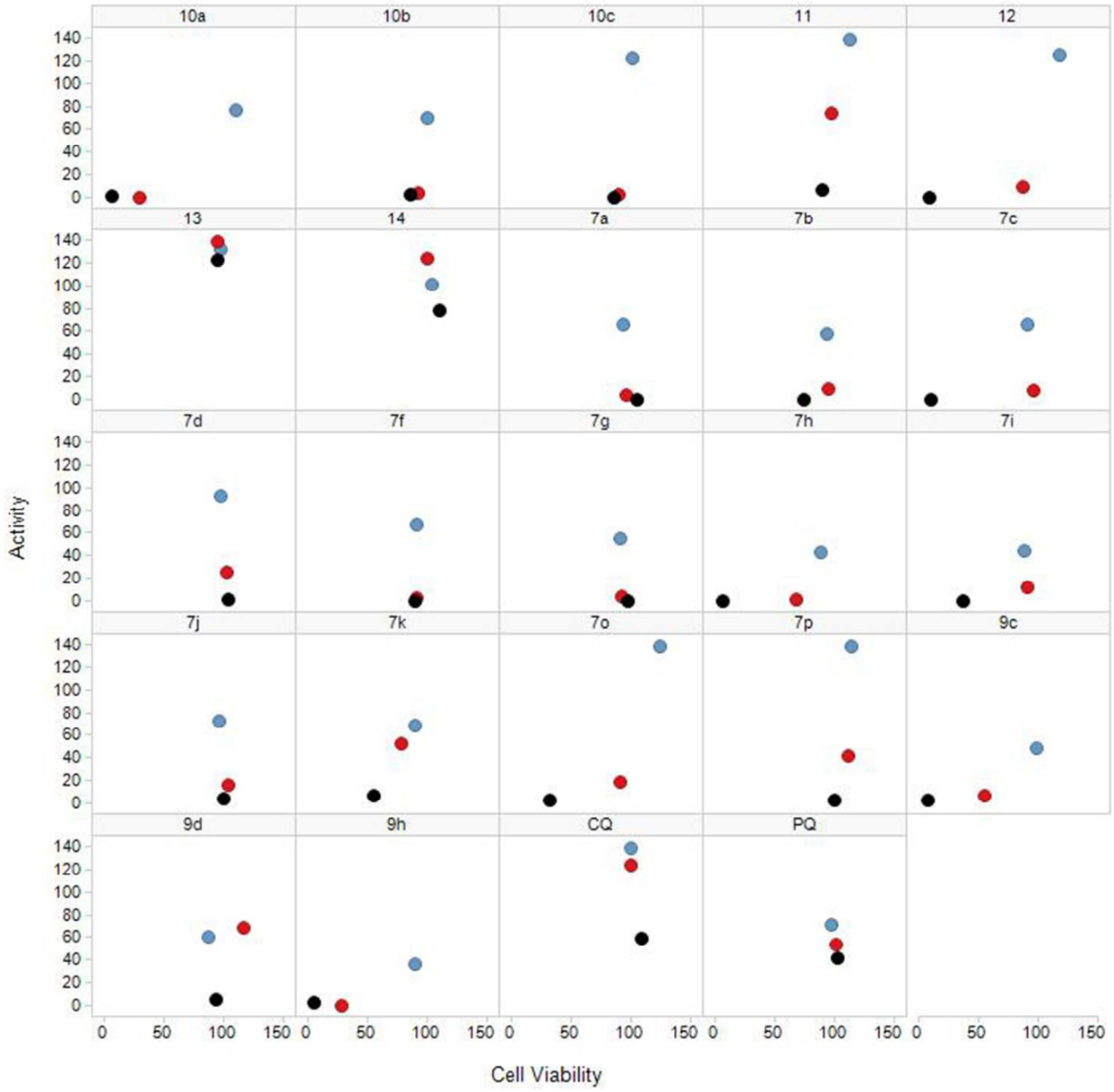


Figure 4: Activity of HEFLECINS against *P. berghei* liver stages. Anti-infective activity (infection scale, y-axis) and toxicity to hepatoma cells (cell confluency scale, x-axis) are shown. Primaquine (PQ) and chloroquine (CQ) were included for comparison. The black, red and blue circles represent results for the tested compounds at 10, 5 and 1 μM, respectively. 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.[25]

Concluding remarks

A new family of chloroquine analogues, HEFLECINS, as dual-stage antimalarial leads, was found. These compounds display potent *in vitro* activity against both liver- and blood-stage *Plasmodia*, including chloroquine-resistant blood-stage *P. falciparum* W2 parasites; all of them performed better than chloroquine itself *in vitro* on both stages, and the best couple of compounds, **7c** and **7h**, were equipotent to artemisinin on blood-stage parasites. Further, all but **11**, **13** and **14** were better than primaquine on liver-stage parasites. Relevantly, both blood- and liver-stage activities were lost upon replacement of the 4-amino-7-chloroquinoline ring with either a non-aromatic (i.e., morpholine) or an aromatic (i.e., 4-aminopyridine) heterocycle, while replacement with the primaquine's 8-amino-6-methoxyquinoline moiety only eliminated blood-stage activity. This demonstrates that the 4-amino-7-chloroquinoline motif has a critical role for the display of dual-stage antimalarial activity. Two of the most promising compounds were confirmed to be active against the murine model of malarial infection, even though with modest *in vivo* performances as compared to *in vitro* ones; this is possibly due to bioavailability issues requiring future optimization of the reported antimalarial leads. Studies on the possible mechanism(s) underlying dual-stage activity displayed by the *N*-cinnamoylated chloroquine analogues are under way; falcipain inhibition has been ruled out, whereas blocking of β-hematin formation cannot fully account for the activity levels observed. The role of these antimalarial leads as blockers of ion channels specific to infected host cells, like the so-called NPP created in *P. falciparum*-infected RBC, is presently under investigation.

Overall, data show that *N*-cinnamoylation of chloroquine enhances the parent drug's activity against both blood- and liver-stage *Plasmodia*, which is the first example of chloroquine-derived molecules with multi-stage antimalarial activity. This finding, along with the recent disclosure of an apparent re-susceptibilization of *P. falciparum* to chloroquine in Nigeria,[29] may have relevant impact on the reactivation of chloroquine manufacturers, especially in African countries like Ghana, in which chloroquine production has been halted due to the well-known parasite resistance to this drug.[30]

Experimental Section

Chemistry

Chemicals and instrumentation. All solvents and common chemicals were from Sigma-Aldrich (Spain), whereas Boc-protected amino acids were from NovaBiochem (VWR International, Portugal) and 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 or deuterated dimethylsulfoxide (DMSO-d_6) containing tetramethylsilane as internal reference. Mass spectroscopy (MS) analyses were run on a Thermo Finnigan LCQ Deca XP Max LC/MS^n 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 conditions: 30-100% of B in A (A = H_2O with 0.05% of trifluoroacetic acid; B=acetonitrile) in 22 minutes with a flow rate of 1 mL/min on a Merck-Hitachi Lachrom Elite instrument equipped with a diode-array detector (DAD) and thermostated (Peltier effect) autosampler, using a Purospher STAR RP-18e column (150 × 4.0 mm; particle size, 5 μM).

Synthesis of compounds 7-10. Compounds **7a-l** and **8** were prepared by previously described methods, and their analytical and structural data were in perfect agreement with formerly reported data.[12, 13]. Compounds **7o,p** were prepared identically to **7a-l** and their spectroscopic data were compatible with the respective structures, as follows.

4-[*N*-(*p*-dimethylamino)cinnamoylaminobutyl]amino-7-chloroquinoline (7o): White solid (14 mg, 8%); mp=176-180°C; R_F (DCM/MeOH 8:2) 0.44; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.6Hz, 2H), 8.32 (d, J=8.8Hz, 1H), 7.95 (m, 1H), 7.79 (d, J=2Hz, 1H), 7.62 (m, 1H), 7.47 (dd, J=8.8, 2.4Hz, 1H), 7.36 (d, J=8.8Hz, 2H), 7.29 (d, J=16Hz, 1H), 6.70 (d, J=8.8Hz, 2H), 6.53 (d, J=5.6Hz, 1H), 6.35 (d, J=16Hz, 1H), 3.32 (m, 2H), 3.22 (m, 2H), 2.94 (s, 6H), 1.68 (m, 2H), 1.57 (m, 2H); δ_C (100 MHz, DMSO-d_6) 165.6, 150.9, 150.7, 150.6, 147.6, 138.8, 133.9, 128.7, 126.3, 124.3, 122.3, 117.1, 116.7, 111.9, 98.6, 42.1, 38.1,

26.9, 25.1; ESI-IT MS: $^{m}/_{z}$ ($M+H^+$) 423.47, M^+ ($C_{24}H_{27}ClN_4O$) requires 422.19; HPLC-DAD: rt = 11.7 min, % area = 96.0.

4-[*N*-(*m,p*-dimethoxy)cinnamoylaminopropyl]amino-7-chloroquinoline (7p): White solid (29 mg, 17%); mp=168-172°C; R_F (DCM/MeOH 8:2) 0.44; δ_H (400 MHz, DMSO-d_6) 8.41 (d, J=5.6Hz, 1H), 8.36 (d, J=9.2Hz, 1H), 8.06 (m, 1H), 7.81 (m, 2H); 7.50 (dd, J=9.4Hz, 2.4Hz, 1H), 7.34 (d, J=16Hz, 1H), 7.10 (m, 2H), 6.96 (d, J=8.4Hz, 1H), 6.57 (d, J=5.6Hz, 1H), 6.51 (d, J=16Hz, 1H), 3.78 (s, 3H), 3.77 (s, 3H), 3.35 (m, 2H), 3.23 (m, 2H), 1.69 (m, 2H), 1.57 (m, 2H); δ_C (100 MHz, DMSO-d_6) 165.2, 151.2, 150.0, 149.8, 148.8, 146.6,138.4,134.3, 127.6, 125.5, 124.5, 124.4, 121.2, 119.9, 116.9, 111.7, 109.9, 98.6, 55.4, 55.3, 42.2, 38.2, 26.7, 25.1; ESI-IT MS: $^{m}/_{z}$ (M+H), M^+ ($C_{24}H_{26}ClN_3O_3$) requires 439.17; HPLC-DAD: rt = 12.5 min, % area = 100.

Compounds **9** and **10** were synthesized by methods similar to those previously described for compounds **7**;[13] briefly, 4,7-dichloroquinoline (1 eq, 1.2 mmol) was reacted with the appropriate diaminoalkane (10 eq, 12 mmol) at 100 ºC for 3 h; the mixture was brought to room temperature and then diluted with dichlorometane (DCM), and the resulting solution was washed with 5% aqueous Na_2CO_3. The organic layer was isolated and dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to afford the desired 4-(*N*-aminoalkyl)amino-7-chloroquinoline (**6**, Scheme 1); each compound **6** was then coupled to the respective cinnamic acid in *N,N*-dimethylformamide (DMF), using *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) and *N,N*-diethyl-*N*-isopropylamine (DIEA) (Scheme 1): a solution of cinnamic acid (1.1 eq, 0.22 mmol), TBTU (1.1 eq, 0.22 mmol), and 2.2eq of DIEA (68µL) in DMF was stirred at 0 ºC for 10 min; a solution of the appropriate compound **6** (1 eq, 0.2 mmol) in DMF was then added, and the reaction allowed to proceed at room temperature for 24 h; the mixture was diluted with DCM and the resulting solution was washed with 5% aqueous Na_2CO_3; the organic layer was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, and the crude product was purified by liquid chromatography on silica using EtOAc/MeOH, 8:2 (v/v) to give the desired compounds **9**, **10**. In the particular case of **9e**, 4-(*N*-aminopropyl)amino-7-chloroquinoline was first coupled to *p*-(*N*-

tert-butoxycarbonyl)aminocinnamic acid as above, to yield the *N*-*tert*-butoxycarbonyl (Boc) protected precursor (**9e'**), followed by removal of Boc through acidolysis with neat trifluoroacetic acid (TFA). Spectral/analytical data on compounds **9,10** follow.

4-(*N*-cinnamoylaminopropyl)amino-7-chloroquinoline (9a). White solid (32 mg, 42%); mp 174-192^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.6Hz, 1H), 8.27 (m, 2H), 7.79 (d, J=2.4Hz, 1H), 7.46 (m, 8H), 6.67 (d, J=15.6Hz, 1H), 6.49 (d, J=5.6Hz, 1H), 3.32 (m, 4H), 1.89 (m, 2H); δ_C (100 MHz, DMSO-d_6) 172.0, 165.1, 151.9, 150.0, 149.0, 138.5, 134.8, 133.3, 129.3, 128.8, 127.4, 127.4, 124.0, 122.1, 117.4, 98.6, 40.0, 36.6, 27.8; ESI-IT MS: $^{m}/_{z}$ 366.47 ($M+H^+$), M^+ ($C_{21}H_{20}ClN_3O$) requires 365.13; HPLC-DAD: rt = 13.0 min, % area = 100.

4-[*N*-(*p*-methyl)cinnamoylaminopropyl]amino-7-chloroquinoline (9b): White solid (53 mg, 68%); mp 204-209^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.6Hz, 1H), 8.27 (d, J=9.2Hz, =1H), 8.22 (m, 1H), 7.79 (d, J=2.4Hz, 1H), 7.40 (m, 5H), 7.20 (d, J=8Hz, 2H), 6.60 (d, J=16Hz, 1H), 6.48 (d, J=5.6Hz, 1H), 3.32 (m, 4H), 2.30 (s, 3H), 1.87 (m, 2H); δ_C (100 MHz, DMSO-d_6) 165.2, 151.8, 149.9, 149.0, 139.1, 138.5, 133.3, 132.1, 129.4, 127.4, 127.4, 124.0, 121.0, 117.4, 98.6, 39.4, 36.6, 27.8, 20.8; ESI-IT MS: $^{m}/_{z}$ 380.4 ($M+H^+$) M^+ ($C_{22}H_{22}ClN_3O$) requires 379.15; HPLC-DAD: rt = 14.0 min, % area =100.

4-[*N*-(*p*-isopropyl)cinnamoylaminopropyl]amino-7-chloroquinoline (9c). White solid (28.7 mg, 33%); mp 173-178^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.2Hz, 1H), 8.27 (d, J=9.2Hz, =1H), 8.22 (m, 1H), 7.79 (d, J=2.4Hz, 1H), 7.40 (m, 5H), 7.27 (d, J=8Hz, 2H), 6.59 (d, J=15.6Hz, 1H), 6.48 (d, J=5.6Hz, 1H), 3.32 (m, 4H), 2.89 (m,1H), 1.86 (m, 2H), 1.19 (d, J=6.8Hz, 6H); δ_C (100 MHz, DMSO-d_6) 165.2, 151.8, 150.0, 149.9, 149.0, 138.5, 133.3, 132.5, 127.5, 127.5, 126.8, 124.0, 121.1, 117.4, 98.6, 40.0, 36.6, 33.2, 27.8, 23.6; ESI-IT: $^{m}/_{z}$ 408.47 ($M+H^+$), M^+ ($C_{24}H_{26}ClN_3O$) requires 407.18; HPLC-DAD: rt = 16.0 min, % area =94.9.

4-[*N*-(*p*-methoxy)cinnamoylaminopropyl]amino-7-chloroquinoline (9d). White solid (15 mg, 7%); mp 178-180°C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.6Hz, 1H), 8.28 (d, J=9.2Hz, =1H), 8.18 (m, 1H), 7.79 (d, J=2.4Hz, 1H), 7.44 (m, 5H), 6.97 (d, J=8.8Hz, =2H), 6.51 (m, 2H), 3.78 (s, 3H), 3.32 (m, 4H), 1.85 (m, 2H); δ_C (100 MHz, DMSO-d_6) 172.0, 165.4, 160.2, 151.6, 150.1, 148.7, 138.2, 133.4, 129.0, 127.4, 127.2, 124.0, 119.6, 117.4, 114.2, 98.6, 55.2, 40.0, 36.5, 27.8; ESI-IT MS: $^m/_z$ 396.53 ($M+H^+$), M^+ ($C_{22}H_{22}ClN_3O_2$) requires 395.14; HPLC-DAD: rt = 13.1 min, % area = 98.0.

4-{*N*-[*p*-(*N*-*tert*-butoxycarbonyl)amino]cinnamoylaminopropyl}amino-7-chloroquinoline (9e'). White solid (124.5 mg, 41%); mp 218-220°C; R_F (EtOAc/MeOH 8:2) 0.1; δ_H (400 MHz, DMSO-d_6) 9.54 (s, 1H), 8.39 (d, J=5.6Hz, 1H), 8.27 (d, J=8.8Hz, =1H), 8.15 (m, 1H), 7.78 (d, J=2.4Hz, 1H), 7.46 (m, 5H), 7.34 (m, 2H), 6.49 (m, 2H), 3.32 (m, 4H), 1.85(m, 2H), 1.48 (s, 9H); δ_C (100 MHz, DMSO-d_6) 165.3, 152.5, 151.8, 149.9, 149.0, 140.7, 138.3, 133.3, 128.5, 128.1, 127.4, 124.0, 123.9, 119.9, 118.0, 98.6, 79.3, 40.0, 36.5, 28.0, 27.8; ESI-IT: $^m/_z$ 481.33 ($M+H^+$), M^+ ($C_{26}H_{29}ClN_4O_3$) requires 480.19; HPLC-DAD: rt = 15.0 min, % area = 100.

4-[*N*-(*p*-amino)cinnamoylaminopropyl]amino-7-chloroquinoline (9e). White solid (28.8 mg, 73%); mp 103-105°C; R_F (EtOAc/MeOH 8:2) 0.1; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.2Hz, 1H), 8.27 (d, J=9.2Hz, =1H), 8.0 (m, 1H), 7.79 (d, J=2Hz, 1H), 7.44 (dd, J=8.8Hz, 2Hz, 1H), 7.29 (m, 4H), 6.56 (d, J=8.4Hz, 2H), 6.48 (d, J=5.6Hz, 1H), 6.29 (d, J=15.6Hz, 1H), 5.55 (b, 2H), 3.29 (m, 4H), 1.84 (m, 2H); δ_C (100 MHz, DMSO-d_6) 166.0, 151.8, 150.4, 150.0, 149.0, 139.4, 133.3, 129.0, 127.4, 124.0, 122.1, 117.4, 115.7, 113.6, 98.6, 40.0, 36.5, 27.9; ESI-IT MS: $^m/_z$ 381.40 ($M+H^+$), M^+ ($C_{21}H_{21}ClN_4O$) requires 380.14; HPLC-DAD: rt = 8.65 min, % area = 96.0.

4-[*N*-(*m*-fluoro)cinnamoylaminopropyl]amino-7-chloroquinoline (9f). White solid (36 mg, 45%); mp 185-190°C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.2Hz, 1H), 8.27 (m, 2H), 7.79 (d, J=2Hz, 1H), 7.41 (m, 6H), 7.20 (m, 1H), 6.67 (d, J=16Hz, 1H), 6.48 (d, J=5.6Hz, 1H), 3.33 (m, 4H), 1.87 (m, 2H); δ_C (100 MHz, DMSO-d_6) 172.7, 164.8, 163.6, 161.2, 151.8, 150.0, 149.0, 137.4,

137.2, 133.3, 130.7, 127.4, 124.0, 123.6, 117.4, 115.9, 113.8, 98.6, 40.0, 36.7, 27.7; ESI-IT MS: $^{m}/_{z}$ 384.40 ($M+H^+$), M^+ ($C_{21}H_{19}ClFN_3O$) requires 383.12; HPLC-DAD: rt = 13.4 min, % area = 100.

4-[*N*-(*p*-fluoro)cinnamoylaminopropyl]amino-7-chloroquinoline (9g). White solid (27 mg, 34%); mp 187-193^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.2Hz, 1H), 8.28 (m, 2H), 7.78 (d, J=2.4Hz, 1H), 7.61 (m, 2H), 7.42 (m, 2H), 7.23 (m, 2H), 6.59 (d, J=16Hz, 1H), 6.48 (d, J=4.8Hz, 1H), 3.32 (m, 4H), 1.87 (m, 2H); δ_C (100 MHz, DMSO-d_6) 172.1, 165.0, 163.8, 161.3, 151.8, 150.0, 149.0, 137.4, 133.3, 129.6, 127.4, 124.0, 122.0, 117.4, 115.9, 98.6, 40.0, 36.6, 27.8; ESI-IT MS: $^{m}/_{z}$ 384.47 ($M+H^+$) M^+ ($C_{21}H_{19}ClFN_3O$) requires 383.12; HPLC-DAD: rt = 13.3 min, % area = 100.

4-[*N*-(*p*-chloro)cinnamoylaminopropyl]amino-7-chloroquinoline (9h). White solid (39 mg, 46%); mp 191-195^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.6Hz, 1H), 8.28 (m, 2H), 7.78 (d, J=2.4Hz, 1H), 7.58 (m, 2H), 7.44 (m, 5H), 6.63 (d, J=15.6Hz, 1H), 6.49 (d, J=5.6Hz, 1H), 3.32 (m, 4H), 1.87 (m, 2H); δ_C (100 MHz, DMSO-d_6) 164.9, 151.7, 150.1, 148.8, 137.2, 133.8, 133.4, 129.1, 128.9, 127.3, 124.1, 124.0, 122.9, 117.4, 98.6, 40.0, 36.6, 27.8; ESI-IT MS: $^{m}/_{z}$ 400.40 ($M+H^+$), M^+ ($C_{21}H_{19}Cl_2N_3O$) requires 399.09; HPLC-DAD: rt = 13.4 min, % area = 95.7.

4-[*N*-(*p*-bromo)cinnamoylaminopropyl]amino-7-chloroquinoline (9i). White solid (22 mg, 39%); mp 214-219^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (d, J=5.2Hz, 1H), 8.29 (m, 2H), 7.78 (d, J=2Hz, 1H), 7.60 (m, 2H), 7.51 (m, 2H), 7.40 (m, 3H), 6.65 (d, J=16Hz, 1H), 6.48 (d, J=5.6Hz, 1H), 3.32 (m, 4H), 1.86 (m, 2H); δ_C (100 MHz, DMSO-d_6) 164.9, 151.8, 150.0, 149.0, 137.3, 134.1, 133.3, 131.8, 129.4, 127.4, 124.0, 123.0, 122.5, 117.4, 98.6, 40.0, 36.6, 27.7; ESI-IT MS: $^{m}/_{z}$ 446.20 ($M+H^+$) M^+ ($C_{21}H_{19}ClBrN_3O$) requires 443.04; HPLC-DAD: rt = 14.7, % area = 100.

(4-[*N*-(*o*-nitro)cinnamoylaminopropyl]amino-7-chloroquinoline (9j). White solid (61.7 mg, 72%); mp 190-195^{o}C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (m, 2H), 8.29 (d, J=9.2Hz, 1H), 8.03 (d, J=8Hz, 1H), 7.69 (m, 5H), 7.44 (dd, J= 9Hz, 2Hz, 1H), 7.33 (m, 1H), 6.63 (d, J=15.6Hz, 1H), 6.49 (d, J=5.2Hz, 1H), 3.33 (m, 4H), 1.89 (m, 2H); δ_C (100 MHz, DMSO-d_6) 164.2, 151.8, 149.9, 149.0,

148.2, 133.7, 133.5, 133.3, 130.1, 130.0, 128.6, 127.4, 126.8, 124.5, 124.0, 117.4, 98.6, 40.0, 36.7, 27.7; ESI-IT MS: $^{m}/_{z}$ 411.47 ($M+H^+$), M^+ ($C_{21}H_{19}ClN_4O_3$) requires 410.11; HPLC-DAD: rt = 13.0 min, % area = 100.

4-[*N*-(*m*-nitro)cinnamoylaminopropyl]amino-7-chloroquinoline (9k). Yellow solid (22 mg, 25%); mp 209-214°C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (m, 2H), 8.26 (m, 2H), 8.20 (dd, J=8Hz, 1.6Hz, 1H), 8.01 (d, J=7.6Hz, 1H), 7.77 (d, J=2Hz, 1H), 7.70 (m, 1H), 7.55 (d, J=15.6Hz, 1H), 7.44 (dd, J=8.8Hz, 2.4Hz, 1H), 7.34 (m, 1H), 6.82 (d, J=15.6Hz, 1H), 6.49 (d, J=5.6Hz, 1H), 3.34 (m, 4H), 1.89 (m, 2H); δ_C (100 MHz, DMSO-d_6) 164.5, 151.8, 149.9, 149.0, 148.2, 136.7, 136.2, 133.8, 133.3, 130.4, 127.4, 125.0, 123.8, 123.6, 121.4, 117.4, 98.6, 36.7, 27.7; ESI-IT MS: $^{m}/_{z}$ 411.40 ($M+H^+$), M^+ ($C_{21}H_{19}ClN_4O_3$) requires 410.11; HPLC-DAD: rt = 13.2 min, % area = 95.0.

4-[*N*-(*p*-nitro)cinnamoylaminopropyl]amino-7-chloroquinoline (9l). Yellow solid (42 mg, 49%); mp 200-205°C; R_F (EtOAc/MeOH 8:2) 0.25; δ_H (400 MHz, DMSO-d_6) 8.39 (m, 2H), 8.26 (m, 3H), 7.81 (m, 3H), 7.53 (d, J=15.6Hz, 1H), 7.45 (d, J=9Hz, 2.4Hz, 1H), 7.35 (m, 1H), 6.81 (d, J=16Hz, 1H), 6.49 (d, J=5.6Hz, 1H), 3.34 (m, 4H), 1.88 (m, 2H); δ_C (100 MHz, DMSO-d_6) 164.4, 151.7, 150.0, 149.0, 147.4, 141.5, 136.2, 133.4, 128.5, 127.3, 126.4, 124.0, 124.0, 117.4, 98.6, 36.8, 27.8; ESI-IT MS: $^{m}/_{z}$ 411.40 ($M+H^+$) M^+ ($C_{21}H_{19}ClN_4O_3$) requires 410.11; HPLC-DAD: rt = 13.2 min, % area = 96.0.

4-[*N*-(*p*-isopropyl)cinnamoylaminopentyl]amino-7-chloroquinoline (10a). White solid (190 mg, 50%); mp=152-155°C; R_F (DCM/MeOH 8:2) 0.58; δ_H (400 MHz, DMSO-d_6) 8.38 (d, J=5.6Hz, 1H), 8.28 (d, J=9.5Hz, 1H), 8.07 (m, 1H), 7.77 (d, J=2Hz, 1H), 7.36 (m, 7H), 6.56 (d, J=16Hz, 1H), 6.46 (d, J=5.6Hz, 1H), 3.22 (m, 4H), 2.89 (m, 1H), 1.69 (m, 2H), 1.46 (m, 4H), 1.19 (d, J=6.8Hz, 6H); δ_C (100 MHz, DMSO-d_6) 164.9, 151.8, 150.0, 149.8, 148.9, 138.2, 133.3, 132.5, 127.4, 127.3, 126.8, 124.0, 123.9, 121.3, 117.3, 98.5, 42.3, 38.5, 33.2, 28.9, 27.4, 24.0, 23.6; ESI-IT MS: $^{m}/_{z}$ ($M+H^+$) 436.47, M^+ ($C_{26}H_{30}ClN_3O$) requires 435.21; HPLC-DAD: rt = 16.3 min, % area = 98.0.

4-[*N*-(*p*-methoxy)cinnamoylaminopentyl]amino-7-chloroquinoline (10b). White solid (100 mg, 27%); mp=142.144°C; R_F (DCM/MeOH 8:2) 0.58; δ_H (400 MHz, DMSO-d_6) 8.38 (d, J=5.6Hz, 1H), 8.28 (d, J-9.2Hz, 1H), 8.01 (m, 1H), 7.77 (d, J=2Hz, 1H), 7.39 (m, 4H), 6.96 (d, J=8.8Hz, 2H), 6.47 (m, 2H), 3.78 (s, 3H), 3.22 (m, 4H), 1.69 (m, 2H), 1.47 (m, 4H); δ_C (100 MHz, DMSO-d_6) 165.1, 160.1, 151.7, 150.0, 148.9, 138.0, 133.3, 128.9, 127.4, 127.3, 124.0, 123.9, 119.8, 117.3, 114.3, 98.5, 55.1, 42.3, 38.4, 28.9, 27.4, 24.0; ESI-IT MS: $^m/_z$ ($M+H^+$) 424.40, M^+ ($C_{24}H_{26}ClN_3O_2$) requires 423.17; HPLC-DAD: rt = 13.7 min, % area = 100.

(4-[*N*-(*p*-chloro)cinnamoylaminopentyl]amino-7-chloroquinoline (10c). Yellowish solid (65 mg, 17%); mp=164-168°C; R_F (DCM/MeOH 8:2) 0.58; δ_H (400 MHz, DMSO-d_6) 8.38 (d, J=5.2Hz, 1H), 8.29 (d, J=8.8Hz, 1H), 8.16 (m, 1H), 7.77 (d, J=1Hz, 1H), 7.56 (d, J=8.8Hz, 2H), 7.42 (m, 4H), 7.32 (m, 1H), 6.64 (d, J=16Hz, 1H), 6.44 (d, J=5.2Hz, 1H), 3.22 (m, 4H), 1.68 (m, 2H), 1.51 (m, 2H), 1.41 (m, 2H); δ_C (100 MHz, DMSO-d_6); ESI-IT MS: $^m/_z$ ($M+H^+$) 428.53, M^+ ($C_{23}H_{23}Cl_2N_3O$) requires 427.12; HPLC-DAD: rt = 14.9, % area = 100.

Synthesis of compound 11.

4-[(7-chloroquinolin-4-yl)amino]butan-1-ol (11'). 1 eq of 4,7-dichloroquinoline (0.5g, 2.5mmol) was mixed with 4 eq of 4-aminobutan-1-ol (0.9g, 10mmol) in a round bottom flask, and reaction was allowed to proceed at 100°C for 4 h. Then, DCM (25 mL) was added, followed by addition of 30% aqueous Na_2CO_3 (25 mL), with concomitant precipitation of a light pink solid; the solid was submitted to column chromatography on silica, using DCM:MeOH (8:2 v/v) as eluent, and pure **11'** was obtained as a white solid (0.49 g, 78%); mp=171-174°C; R_F=0.26 (DCM/MeOH 8:2); δ_H (400 MHz, DMSO-d_6); 8.38 (d, J=5.2Hz, 1H), 8.27 (d, J=9.2Hz, 1H), 7.77 (d, J=2.4Hz, 1H), 7.42 (dd, J=9.2Hz, 2.4Hz, 1H), 7.32 (m, 1H), 6.44 (d, J=5.6Hz, 1H), 4.51 (b, 1H), 3.46 (m, 2H), 3.26 (m, 2H), 1.69 (m, 2H), 1.54 (m, 2H); δ_C (100 MHz, DMSO-d_6) 151.8, 150.0, 149.0, 133.2, 127.4, 124.0, 123.9, 117.4, 98.5, 60.4, 42.2, 30.0, 24.4; ESI-IT MS: $^m/_z$ ($M+H^+$) 251.33, M^+ ($C_{13}H_{15}ClN_2O$) requires 250.09.

4-[*N*-(*p*-methoxy)cinnamoyloxypropyl]amino-7-chloroquinoline (11). 1 eq of *p*-methoxycinnamoyl chloride (0.08 g, 0.4 mmol) was reacted with 1 eq of **11'** (0.1 g, 0.4 mmol) in reluxing DCM (2 mL), in the presence of 2 eq of triethylamine (112 μL); the solution turned yellow after 10 min and the reaction ran for 24 h. The organic layer was washed with 5% aqueous Na_2CO_3 (3 × 2 mL), dried with anhydrous Na_2SO_4, filtered, and led to dryness on a rotary evaporator. The residue was submitted to column chromatography on silica, using DCM:Me_2CO (1:1 v/v) as eluent, to yield **11** as a white solid (34 mg, 21%); mp=129-134°C; R_F (DCM/Me_2CO 1:1) 0.26; δ_H (400 MHz, $CDCl_3$) 8.49 (d, J=5.2Hz, 1H), 7.93 (d, J=2Hz, 1H), 7.71 (d, J=9.2Hz, 1H), 7.63 (d, J=16Hz, 1H), 7.43 (d, J=8.4Hz, 2H), 7.31 (d, J=8.8Hz, 1H), 6.88 (d, J=8.4Hz, 2H), 6.39 (d, J=5.2Hz, 1H), 6.28 (d, J=16Hz, 1H), 5.38 (m, 1H), 4.27 (m, 2H), 3.82 (s, 3H), 3.36 (m, 2H), 1.89 (m, 4H); δ_C (100 MHz, $CDCl_3$) 167.3, 161.4, 151.7, 149.8, 148.8, 144.7, 134.9, 129.7, 128.4, 126.9, 125.3, 121.1, 117.1, 115.1, 114.3, 99.0, 63.7, 55.3, 42.9, 26.5, 25.3; ESI-IT MS: $^m/_z$ ($M+H^+$) 411.33, M^+ ($C_{23}H_{23}ClN_2O_3$) requires 410.14; HPLC-DAD: rt = 15.4 min, % area = 97.0.

4-[*N*-(*p*-isopropyl)cinnamoylaminopropyl]aminoquinoline (12). Following the experimental procedure previously described for compounds **9** and **10**, and starting from 4-chloroquinoline (0.25 g, 1.5 mmol) instead of 4,7-dichloroquinoline, **12** was obtained as a beige solid (55 mg, 11%); mp=73-78°C; R_F (DCM/MeOH 8:2) 0.30; δ_H (400 MHz, $CDCl_3$) 8.41 (d, J=5.6Hz, 1H), 8.02 (d, J=8.4Hz, 1H), 7.95 (d, J=8.4Hz, 1H), 7.60 (m, 2H), 7.39 (m, 3H), 7.18 (d, J=8Hz, 2H), 6.52 (m, 1H), 6.46 (d, J=16Hz, 1H), 6.34 (d, J=5.6Hz, 1H), 6.15 (b, 1H), 3.46 (m, 2H), 3.35 (m, 2H), 2.89 (m, 1H), 1.74 (m, 4H), 1.22 (d, J=6.8Hz, 6H) ; δ_C (100 MHz, $CDCl_3$) 166.7, 150.9, 149.2, 146.6, 141.0, 132.4, 129.7, 128.0, 127.9, 126.9, 125.0, 120.5, 119.7, 118.5, 98.4, 43.1, 39.1, 34.0, 27.8, 25.5, 23.8; ESI-IT MS: $^m/_z$ ($M+H^+$) 388.27, M^+ ($C_{25}H_{29}N_3O$) requires 387.23; HPLC-DAD: rt = 15.2 min, % area = 99.0.

Synthesis of compound 13.

***N*^{1}-(pyridin-4-yl)butane-1,4-diamine (13').** 1 eq of 4-chloropyridine (0.5 g, 3.3 mmol) was reacted with 10 eq of butane-1,4-diamine (2.9 g, 33 mmol) in a round bottom flask put under reflux for 2 hours; after

cooling the reaction mixture to room temperature, 25 mL of distilled water were added, and the desired product was extracted with DCM (3 × 25 mL); the organic layer was dried over anhydrous Na_2SO_4, filtered, and led to dryness on a rotary evaporator; this afforded **13'** as a yellow oil (0.22 g, 40%); ESI-IT MS: $^m/_z$ ($M+H^+$) 166.33, M^+ ($C_9H_{15}O_3$) requires 165.13. Compound **13'** was used without further purification in the synthesis of **13.**

4-[*N*-(*p*-methoxy)cinnamoylbutyl]aminopyridine (13). In a round bottom flask put at 0 ^oC, 1.1 eq of *p*-methoxycinnamic acid (0.29 g, 1.5 mmol) was dissolved in DMF (2.5 mL) and activated by addition of 1.1 eq of TBTU (0.47 g, 1.5 mmol) and 2 eq of DIEA (454 μL); after 10 min, a solution of 1 eq of **13'** (0.22 g, 1.3 mmol) in DMF (2.5 mL) was added to the previous mixture and the reaction allowed to proceed for 24 h. Water (25 mL) was added to the reaction mixture and the desired product was extracted with DCM (3 × 25 mL); the organic extract was then washed with 5% aqueous Na_2CO_3 (3 × 25mL), dried over anhydrous Na_2SO_4, filtered, and taken to dryness on a rotary evaporator; the residue was submitted to column chromatography on silica, using DCM/MeOH 8:2 (v/v) as eluent, to afford **13** as a white solid (0.13 g, 30%); mp=132-136^oC; R_F=0.13 (DCM/MeOH 8:2); δ_H (400 MHz, $CDCl_3$) 8.04 (b, 2H), 7.54 (d, J=16Hz, 1H), 7.33 (d, J=8.8Hz, 2H), 7.28 (m, 1H), 6.77 (d, J=8.8Hz, 2H), 6.36 (m, 3H), 5.14 (m, 1H), 3.73 (s, 3), 3.35 (m, 2H), 3.06 (m, 2H), 1.60 (m, 4H); δ_C (100 MHz, $CDCl_3$) 166.8, 160.6, 153.7, 149.0, 140.0, 129.1, 127.4, 118.5, 114.1, 107.4, 55.2, 42.1, 39.1, 27.2, 26.0; ESI-IT MS: $^m/_z$ ($M+H^+$) 326.33, M^+ ($C_{19}H_{23}N_3O_2$) requires 325.18; HPLC-DAD: rt = 11.6 min, % area = 100.

Synthesis of compound 14.

***N*-(*N*-phthaloyl)aminobutylmorpholine (14'').** 2 eq of morpholine (0.5g, 5.7 mmol) were reacted with 1 eq of 1-(*N*-phthaloyl)amino-4-bromobutane (0.8 g, 2.9 mmol) in refluxing DCM for 24 h, after which the reaction mixture was washed three times with 5% aqueous Na_2CO_3, dried over anhydrous Na_2SO_4, filtered, and led to dryness in a rotary evaporator; the residue was submitted to column chromatography on silica, using as eluent DCM:MeOH (8:2 v/v), to afford **14'** as a white solid (0.53 g, 64%); mp=61-

63°C; R_F=0.67 (DCM/MeOH 8:2); δ_H (400 MHz, $CDCl_3$) 7.80 (m, 2H), 7.70 (m, 2H), 3.66 (m, 6H), 2.39 (m, 6H), 1.68 (m, 2H), 1.51 (m, 2H); δ_C (100 MHz, $CDCl_3$) 168.4, 133.8, 132.1, 123.1, 66.9, 58.3, 53.6, 37.8, 26.4, 23.8; ESI-IT MS: $^m/_z$ ($M+H^+$) 289.40, M^+ ($C_{16}H_{20}N_2O_3$) requires 288.15.

***N*-aminobutylmorpholine (14')**. 1 eq of **14''** (0.1 g, 0.30 mmol) was reacted with 50% ethanolic hydrazine (0.40 mL) for 2 h at room temperature, for removal of the phthaloyl *N*-protecting group; water was then added to the reaction mixture and the desired product was extracted with DCM (3 × 25 mL), the organic layer was dried over anhydrous sodium sulfate, filtered, and led to dryness on the rotary evaporator to afford **14'** as a yellowish oil (25 mg, 45%); ESI-IT MS: $^m/_z$ ($M+H^+$) 159.27, M^+ ($C_8H_{18}N_2O$) requires 158.14. Compound **14'** was used without further purification in the synthesis of **14.**

***N*-(*p*-methoxy)cinnamoylaminobutylmorpholine (14).** 1 eq of *N*-aminobutylmorpholine (0.025 g, 0.16 mmol) was immediately used, without further purification, in the coupling with *p*-methoxycinnamic acid (0.032 g, 0.17 mmol) carried out as above described for **13** but using PyBOP (0.088 g, 0.17 mmol) instead of TBTU as coupling reagent, and DCM as a solvent; this yielded **14** as a white solid (32 mg, 61%); mp=92-94°C; R_F (DCM/MeOH 8:2) 0.52; δ_H (400 MHz, $CDCl_3$) 7.59 (d, J=15.6Hz, 1H), 7.40 (d, J=8.4Hz, 2H), 7.20 (d, J=8.4Hz, 2H), 6.34 (m, 2H), 3.72 (m, 4H), 3.38 (m, 2H), 2.90 (m, 1H), 2.44 (m, 4H), 2.37 (m, 2H), 1,60 (m, 4H), 1.23 (d, J=6.8Hz, 6H); δ_C (100 MHz, $CDCl_3$); 160.1, 150.7, 140.7, 132.5, 127.7, 126.9, 119.8, 66.9, 58.3, 53.6, 39.5, 34.0, 27.4, 24.0, 23.8; ESI-IT MS: $^m/_z$ ($M+H^+$) 331.40, M^+ ($C_{20}H_{30}N_2O_2$) requires 330.23; HPLC-DAD: rt = 13.2 min, % area = 100.

***In vitro* assays**

Blood stage activity assays. The activity of compounds against cultured *P. falciparum* was evaluated as previously reported.[11] Briefly, synchronized ring-stage W2 strain *P. falciparum* parasites were cultured with multiple concentrations of test compounds (added from 1,000× stocks in DMSO) in RPMI 1640 medium supplemented with 0.5% Albumax (Invitrogen, GIBCO) and 100 uM hypoxanthine. After a 48 h incubation, when control cultures contained new rings, parasites were fixed with 1% formaldehyde in

PBS, pH 7.4, for 48 h at room temperature and then labeled with 1 nM YOYO-1 (Molecular Probes) in 0.1% Triton X-100, 100 mM ammonium chloride in PBS. Parasitemias were determined from dot plots (forward scatter *vs.* fluorescence) acquired on a FACSort flow cytometer using CELLQUEST software (Becton Dickinson). IC_{50}s for growth inhibition were determined with GraphPad Prism software from plots of percentages of the level of parasitemia of the control relative to inhibitor concentration. In each case, goodness of curve fit was documented by R^2 values of > 0.95.

Inhibition of β-hematin. The β-hematin inhibition assay was performed as previously described.[27, 28] Different concentrations (0.1 to 1 mM) of test compounds dissolved in DMSO were added in triplicate to 50 μL hemin chloride dissolved in DMSO (5.2 mg/mL). Negative controls were water and DMSO. β-hematin formation was initiated by the addition of acetate buffer 0.2M (100 μL, pH 4.4), plates were incubated at 37ºC for 48 h and they were then centrifuged at 3000 rpm for 15 min (SIGMA 3-30K). After discarding the supernatant, the pellet was washed four times with DMSO (200 μL), and finally dissolved in 0.2 M aq. NaOH (200 μL). The solubilized aggregates were further diluted 1:6 with 0.1 M aq. NaOH and absorbances were recorded at 405 nm (Biotek Powerwave XS with software Gen5 1.07).

Liver stage infection assays. Inhibition of liver stage infection by test compounds was determined by measuring the luminescence intensity in Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc_{con}, as previously described.[29] Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% v/v fetal calf serum, 1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2. For infection assays, Huh-7 cells (1.2×10^4 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, 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.

***In vivo* assays**

Evaluation of the *in vivo* antimalarial effects of HEFLECINS. Female Swiss Webster mice (n = 5) were infected with 10^6 *P. berghei*-infected erythrocytes by ip injection. Two hours later the treatment was initiated with ip injections of 10, 30 and 100 mg/kg of compounds **7c**, **7d**, and **7h** twice daily for 4 days. Chloroquine was used as a positive control at 3, 10 and 30 mg/kg ip twice daily. For all murine malaria experiments, mice were evaluated daily for toxicity and for parasitemia by evaluation of Giemsa-stained blood smears. Animals were sacrificed when significant toxicity was identified or when parasitemias topped 50%.

Acknowledgments

This work was co-funded by Fundação para a Ciência e Tecnologia (FCT ref. PTDC/QUI-QUI/116864/2010 and PTDC/SAU-MII/099118/2008) and by FEDER (European Union), within the frame of the COMPETE programme (ref. FCOMP-01-0124-FEDER-020963). PG and JRBG also thank FCT for funding through strategic projects PEst-C/QUI/UI0081/2011 and PEst-C/CTM/LA0011/2011, respectively. CT and JRBG thank FCT for the post-doctoral fellowship SFRH/BPD/62967/2009 and for Programa Ciência 2007, respectively. BP thanks FCT for doctoral grant SFRH/BD//86166/2012. PJR is a Doris Duke Charitable Foundation Distinguished Clinical Scientist.

Supporting Information Available: Table S1 reporting the physicochemical descriptors for *N*-cinnamoylated CQ analog **7** and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table of Contents Graphic

Heterocycle-cinnamic acid conjugates were synthesized and tested for their antimalarial properties, providing disclosure of new *N*-cinnamoylated chloroquine analogues with potent *in vitro* activity against both blood- and liver-stage malaria parasites. The compounds also displayed *in vivo* activity against rodent malaria.