Synthesis and biological evaluation of benzhydryl-based antiplasmodial agents possessing Plasmodium falciparum chloroquine resistance transporter (PfCRT) inhibitory activity

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Synthesis and Biological Evaluation of Benzhydryl-based Antiplasmodial Agents Possessing Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT) Inhibitory Activity

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Abstract:	Due to the surge in resistance to common therapies, malaria remains a significant concern to human health worldwide. In chloroquine (CQ)-resistant (CQ-R) strains of Plasmodium falciparum , CQ and related drugs are effluxed from the parasite's digestive vacuole (DV). This process is mediated by mutant isoforms of a protein called CQ resistance transporter (PfCRT). CQ-R strains can be partially re-sensitized to CQ by verapamil (VP), primaquine (PQ) and other compounds, and this has been shown to be due to the ability of these molecules to inhibit drug transport via PfCRT. We have previously developed a series of clotrimazole (CLT)-based antimalarial agents that possess inhibitory activity against PfCRT (4a,b). In our endeavor to develop novel PfCRT inhibitors, and to perform a structure-activity relationship analysis, we synthesized a new library of analogues. When the benzhydryl system was linked to a 4-aminoquinoline group (5a-f) the resulting compounds exhibited good cytotoxicity against both CQ-R and CQ-S strains of P. falciparum . The most potent inhibitory activity against the PfCRT-mediated transport of CQ was obtained with compound 5k . When compared to the reference compound, benzhydryl analogues of PQ (5i,j) showed a similar activity against blood-stage parasites, and a stronger in vitro potency against liver-stage parasites. Unfortunately, in the in vivo transmission blocking assays, 5i,j were inactive against gametocytes.

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DIPARTIMENTO DI BIOTECNOLOGIE, CHIMICA E FARMACIA

Siena, 21st January 2021

Submission of the revised versione of the manuscript "**Synthesis and Biological Evaluation of Benzhydryl-based Antiplasmodial Agents Possessing *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) Inhibitory Activity**" quoted EJMECH-D-20-03447R1 (corresponding author: Sandra Gemma (gemma@unisi.it)

Dear Prof. Zhen-Ming Liu:

Please find here enclosed the revised version of the manuscript titled **Synthesis and Biological Evaluation of Benzhydryl-based Antiplasmodial Agents Possessing *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) Inhibitory Activity**".
The Authors thank the Editor and the Reviewer for positive evaluation of the manuscript. All modifications requested by the Reviewer have been taken into consideration

Sincerely yours

Sandra Gemma

The Authors would like to thank the reviewer for positive evaluation of the manuscript and for helpful suggestions. All reviewer's comments have been taken into consideration as detailed below.

1. In scheme 1, correction w.r.t super scripting of numerals is not carefully done.

The super scripts were carefully checked

2. In the block depicting IC50 values similar (see 1.) changes are still needed.

Superscripts were introduced in the table heading

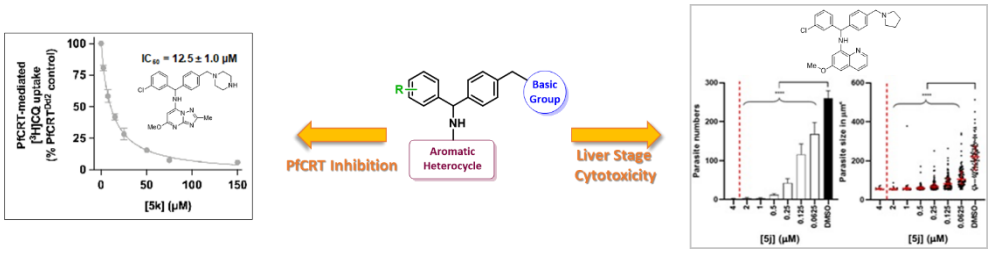
3. If the above (Sr. 2)is a Table, I don't see anywhere its identification as Table 1.

The block has been named Table 1 and a title has been added

4. Also change Cpd to Compound.

The modification has been made

Graphical Abstract



Highlights

- Design and synthesis of novel antiplasmodial compounds as potential PfCRT inhibitors
- **5c,d** were the most potent compounds against CQ-R and CQ-S strains of *P. falciparum*
- **5k** was the most potent inhibitor of CQ transport via PfCRT
- **5i,j** showed an interesting activity against liver-stage parasites

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Synthesis and Biological Evaluation of Benzhydryl-based Antiplasmodial Agents Possessing *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) Inhibitory Activity

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Abstract

Due to the surge in resistance to common therapies, malaria remains a significant concern to human health worldwide. In chloroquine (CQ)-resistant (CQ-R) strains of *Plasmodium falciparum*, CQ and related drugs are effluxed from the parasite's digestive vacuole (DV). This process is mediated by mutant isoforms of a protein called CQ resistance transporter (PfCRT). CQ-R strains can be partially re-sensitized to CQ by verapamil (VP), primaquine (PQ) and other compounds, and this has been shown to be due to the ability of these molecules to inhibit drug transport via PfCRT. We have previously developed a series of clotrimazole (CLT)-based antimalarial agents that possess inhibitory activity against PfCRT (**4a,b**). In our endeavor to develop novel PfCRT inhibitors, and to perform a structure-activity relationship analysis, we synthesized a new library of analogues. When the benzhydryl system was linked to a 4-aminoquinoline group (**5a-f**) the resulting compounds exhibited good cytotoxicity against both CQ-R and CQ-S strains of *P. falciparum*. The most potent inhibitory activity against the PfCRT-mediated transport of CQ was obtained with compound **5k**. When compared to the reference compound, benzhydryl analogues of PQ (**5i,j**) showed a similar activity against blood-stage parasites, and a stronger *in vitro* potency against liver-stage parasites. Unfortunately, in the *in vivo* transmission blocking assays, **5i,j** were inactive against gametocytes.

Keywords

Malaria, *Plasmodium falciparum*, chloroquine resistance, PfCRT, chemosensitization, liver-stage antimalarial, *Xenopus* oocytes

Highlights

- Design and synthesis of novel antiplasmodial compounds as potential PfCRT inhibitors
- **5c,d** were the most potent compounds against CQ-R and CQ-S strains of *P. falciparum*
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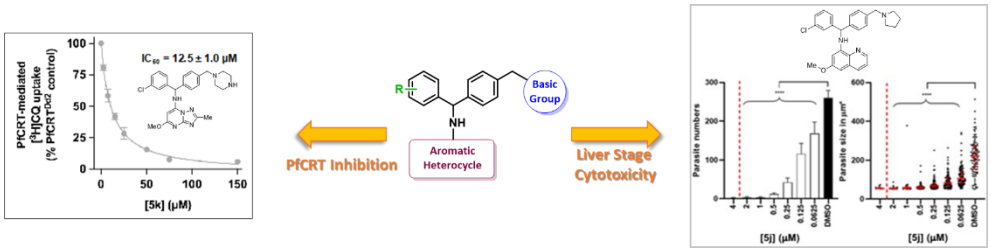
Graphical Abstract



Abbreviations

ACTs, artemisinin-based combination therapies; CP, chlorpheniramine; CQ, chloroquine; CQ-R, CQ-resistant; CQ-S, chloroquine-sensitive; VP, verapamil; DCE, dichloroethane; DCM, dichloromethane; DFA, direct feeding assay; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DV, digestive vacuole; GFP, green fluorescent protein; hpi, hours post infection; i.p., intraperitoneal; i.v., intravenous; MW, microwaves; PetEt, petroleum ether; PfCRT, *P. falciparum* chloroquine-resistance transporter; PQ, primaquine; CLT, clotrimazole; TBAF, tetrabutylammonium fluoride; TBDPSCl, *tert*-butyldiphenylchlorosilane; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TZP, triazolopyrimidine; SAR, structure-activity relationship.

1. Introduction

Despite recent progress in the fight against malaria, it remains a serious problem worldwide with more than 405.000 deaths in 2018 [1]. The current first-line treatments for malaria are the artemisinin-based combination therapies (ACTs), which are characterized by high efficacy together with a fast action and thus reduced potential for the development of resistant parasite strains. However, in recent years some regions of Asia (the Greater Mekong Subregion) have reported a lower vulnerability of the malaria parasite to ACTs [2]. Given the importance of the ACTs for the treatment of drug-resistant malaria, it is necessary to search for new and affordable antiplasmodial drugs that are active against multiple stages of the *Plasmodium* life cycle and/or that are able to rescue older antimalarial drugs. Chloroquine (CQ, **1**, Figure

1) was one of the first safe and effective antimalarials to be widely employed. It is characterized by potent and selective cytotoxic action against *Plasmodium falciparum*, together with a good safety profile and low-cost of the treatment regimen. However, after decades of use, a dramatic spread of CQ-resistant (CQ-R) strains of malaria parasites occurred [3]. The resistance of *P. falciparum* to CQ is related to an increased capacity of the parasite to expel the drug from the digestive vacuole (DV), which is its site of action. CQ-R parasites accumulate much less CQ in their DV than CQ-sensitive (CQ-S) parasites do [4,5], and the presence of a resistance-reverser (e.g., the calcium channel blocker verapamil, VP) increases the accumulation of CQ in CQ-R parasites. The mechanism behind the CQ-efflux from the DV of *P. falciparum* has been established [6]. CQ is transported out of the DV by a mutant isoform of the *P. falciparum* chloroquine-resistance transporter (PfCRT) [7–10], a protein that localizes to the membrane of this organelle [11,12].

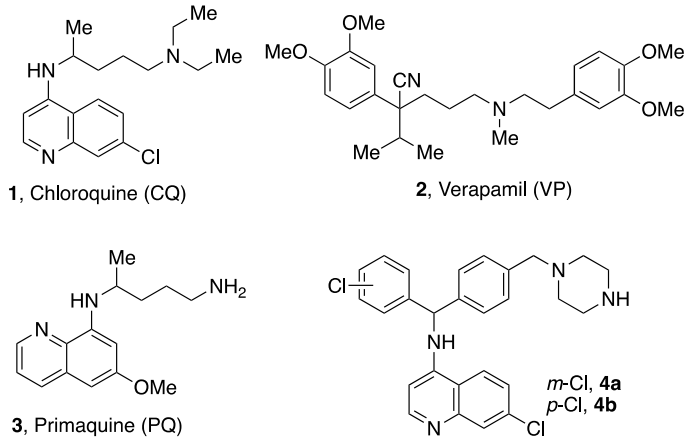


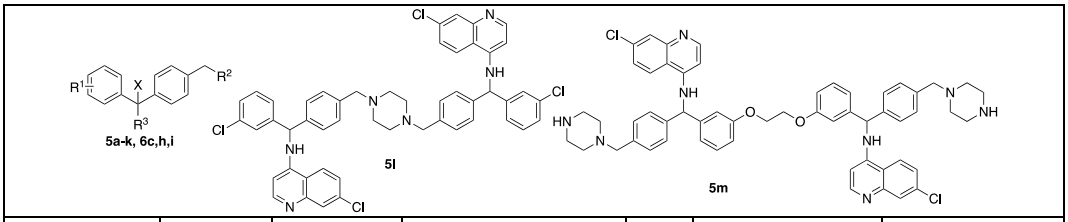
Figure 1. Structure of chloroquine (**1**), verapamil (**2**), primaquine (**3**) and **4a,b**

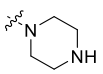
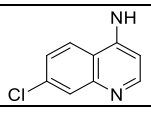
A cryo-electron microscopy structure of PfCRT (49 kDa) complexed with a recombinant PfCRT-specific antigen-binding fragment (50 kDa) has recently been published [7]. PfCRT consists of 424 amino acids, with ten transmembrane domains that cross the DV membrane. Expression of PfCRT at the surface of *Xenopus oocytes* has revealed that the isoforms of the protein associated with CQ resistance, such as the 'Dd2' variant ($PfCRT^{Dd2}$), mediate CQ transport [7,8]. By contrast, wild-type PfCRT ($PfCRT^{3D7}$) lacks significant CQ transport activity. From *in vitro* assays, it has been observed that the sensitivity of CQ-R *P. falciparum* strains to CQ can be partially restored by verapamil (VP, **2**, Figure **1**) as well as by various other compounds, such as the liver-stage antimalarial primaquine (PQ, **3** Figure **1**) [5,13,14]. These CQ resistance-reversers do not usually affect the accumulation of CQ in the DV, of CQ-S strains, and exert little or no intrinsic antiplasmodial activity against the intraerythrocytic stage of *Plasmodium* [15]. Their mode of action arises from the inhibition of CQ transport via the mutant isoforms of PfCRT [14,16–18], thus reinstating the ability of CQ to accumulate inside the DV by a weak-base trapping mechanism [19]. Aside from its roles in the phenomenon of multidrug resistance [6], PfCRT is essential for the survival of the parasite [20]. Its natural function was recently revealed to be the efflux of large host-derived peptides (4-11 residues in length) from the DV, with $PfCRT^{3D7}$

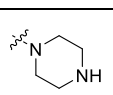
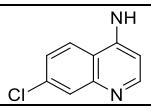
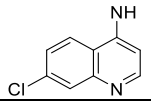
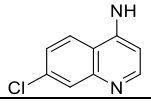
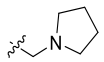
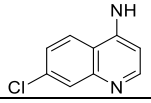
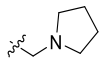
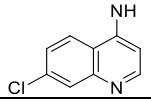
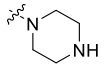
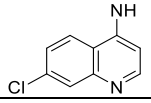
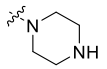
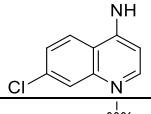
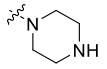
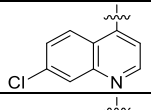
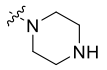
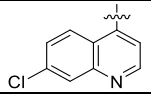
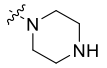
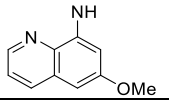
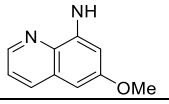
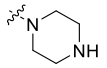
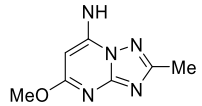
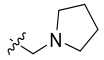
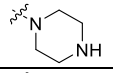
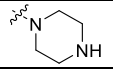
translocating a broader range of peptides and peptide mimics, and at a higher capacity, than almost all of the mutant isoforms (including PfCRT^{Dd2}) [21].

We have been pursuing the design and development of new antiplasmodial drugs endowed with various modes of actions [22,23]. These included clotrimazole (CLT)-based antiplasmodials agents as a novel scaffold able to interfere with heme metabolism inside the DV, as well as a new class of CQ-CLT-hybrid compounds [24–26]. Benzhydryl-derivatives belonging to the latter class, typified by compounds **4a**,**b (**Figure **1**), showed an excellent antiplasmodial activity profile against both CQ-S and CQ-R strains of *P. falciparum in vitro* and *in vivo*. These molecules share all or most of the following features: a weak basic heme-complexing group, a quinoline moiety (to provide the antiplasmodial effect), and a benzhydryl system presenting a basic group (to enable accumulation of the compound inside the DV via weak-base trapping). Most of these molecules showed good antiplasmodial potency against the CQ-R strains, which led us to explore the mechanisms involved in their activity. It was observed that both **4a** and **4b** were able to inhibit CQ transport via PfCRT^{Dd2} in the *Xenopus* oocyte expression system [24]. Encouraged by this discovery and the growing interest in PfCRT as a promising drug target [21,27–29], we investigated a series of new analogues of **4** in order to improve their activity against PfCRT^{Dd2} and to explore the structural features required for the inhibition of PfCRT^{Dd2}-mediated CQ transport, whilst maintaining the intrinsic antiplasmodial properties. Here, we designed and tested analogues **5a-k**, and **6c**,**h**,**i** (Table 1) to evaluate the effect of substitutions at (1) the benzhydryl moiety and (2) the quinoline ring on activity against PfCRT^{Dd2}-mediated CQ transport as well as on antiplasmodial activity. In addition, compounds **5l**,**m** were evaluated in an attempt to improve the inhibitory activity against PfCRT^{Dd2}.

Table 1. Antiplasmodial Activity of Compounds **5a-l**, **6c,h,i** and of Reference Compounds CQ (**1**), PQ (**3**) and **4a,b**.



Compound	R^1	R^2	R^3	X	IC_{50} (µM) D10	IC_{50} (µM) W2
1 **CQ**	-	-	-	-	0.022	0.280
3 **PQ**	-	-	-	-	5.7	4.4
4a[24]	*m*-Cl			H	0.025	0.033

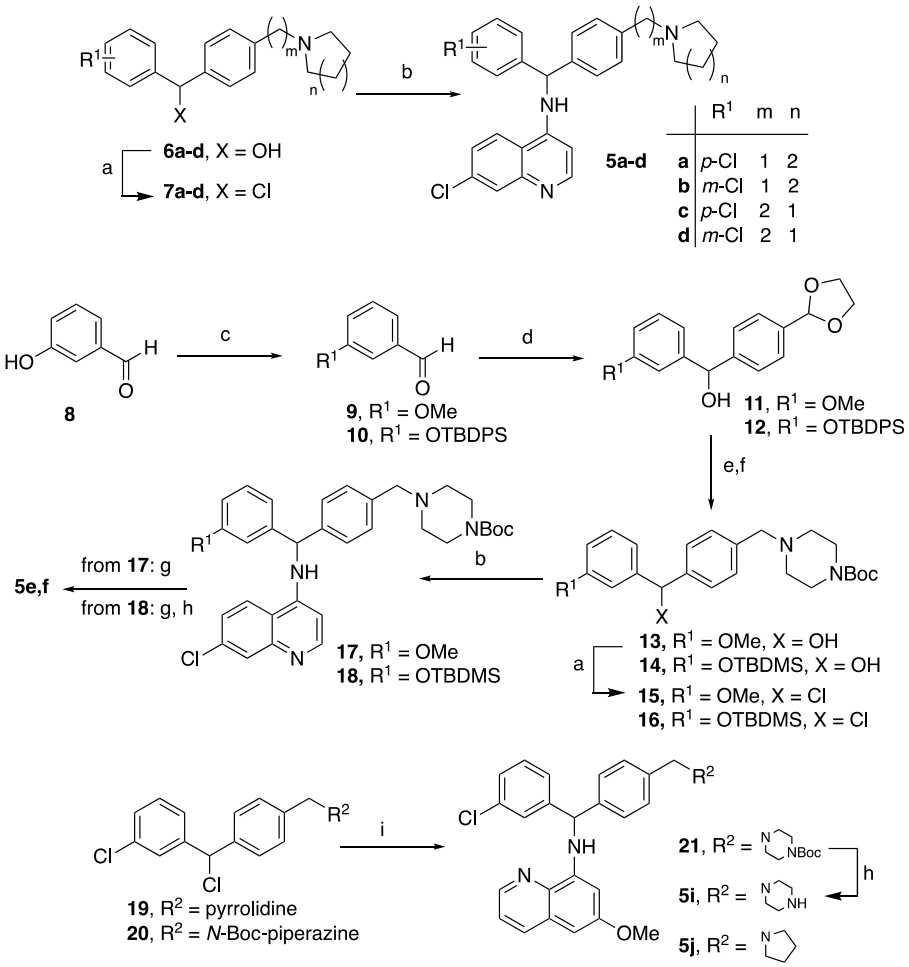
4b[24]	*p*-Cl			H	0.062	0.058
5a	*p*-Cl			H	0.065	0.069
5b	*m*-Cl			H	0.084	0.088
5c	*p*-Cl			H	0.019	0.027
5d	*m*-Cl			H	0.023	0.022
5e	*m*-OMe			H	0.040	0.075
5f	*m*-OH			H	0.179	0.475
5g	*p*-Cl			H	3.70	2.70
5h	*p*-Cl			OH	2.00	2.00
5i	*m*-Cl			H	2.80	2.30
5j	*m*-Cl			H	4.50	3.70
5k	*m*-Cl			H	3.70	0.92
5l	-	-	-	-	0.595	0.460
6c	*p*-Cl		-OH	H	>15	>15
6h	*p*-Cl		=O	-	>15	8.6
6i	*p*-Cl		-OH	H	>15	11

2. Chemistry

Compounds **5a-k, 5m** were synthesized as described in Schemes 1-4, while the synthesis of **5l** and **6a-i** is reported in the Supporting Information file (Schemes S1-5). For the synthesis of compounds **5a-f** and **5i**,**j** an approach that parallels our previously described synthetic pathway was followed (Scheme 1). Starting from the appropriate benzhydrols **6a-d**, the corresponding benzhydryl chlorides were obtained by treatment with $SOCl_2$ in dry DCM. These chlorides were used for the alkylation of the 7-chloro-4-aminoquinoline obtaining compounds **5a-d**. For the synthesis of compounds **5e**,**f**, benzhydrols intermediates **11** and **12** were prepared starting from aldehydes **8** and **9**. For the synthesis of intermediate **10**, the free OH group of **8** was

protected to the corresponding silyl-derivative. Aldehydes **9** and **10** were then subjected to a lithium halogen exchange reaction to afford compounds **11** and **12**, respectively. Deprotection of the cyclic ketal of both compounds released the free aldehyde that was then submitted to a reductive amination protocol to afford the tertiary amines **13** and **14**. Starting from these latter compounds, the subsequent steps, namely chlorination to **15**, **16**, and reaction with 7-chloro-4-aminoquinolines, afforded protected derivatives **17** and **18**. Their deprotection afforded the final compounds **5e**,**f**. Following a similar reaction pathway, alkylation of 8-amino-6-methoxyquinoline with benzhydryl chlorides **19** and **20** (synthesized as reported [25,30]) afforded **21** and **5j**, respectively. Starting from **21**, final compound **5h** was obtained by acid-promoted *N*-Boc deprotection.

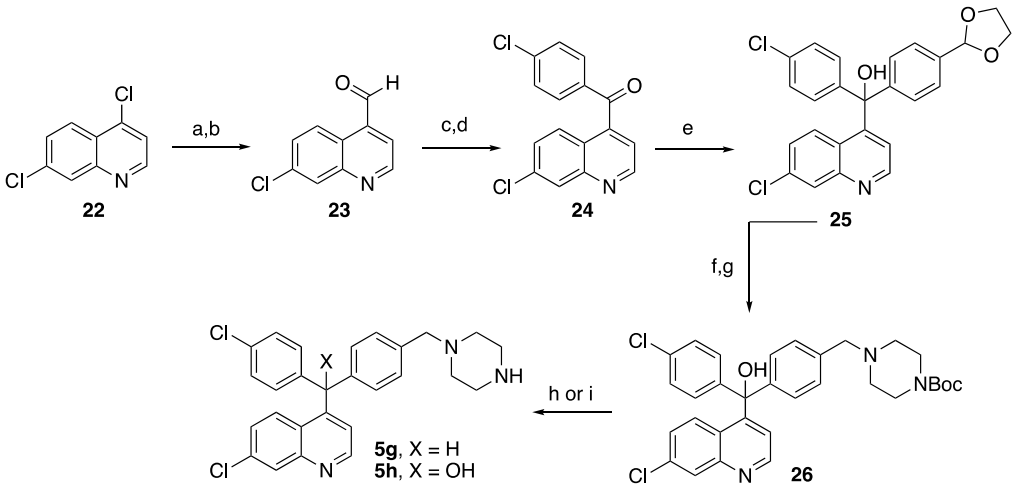
Scheme 1



Reagents and Conditions: a) $SOCl_2$, DCM, 50 °C, 2 h; b) 4-amino-7-chloroquinoline CH_3CN, TEA, 80 °C, 12 h; c) TBDPSCl, imidazole, THF, 0 to 25 °C, 12 h; d) 2-(4-bromophenyl)-1,3-dioxolane, *n*-BuLi, THF, -70 °C, 2 h; e) HCl, 1 N, THF, 4 h, 25 °C; f) 1-Boc-piperazine, $NaBH_3CN$, 1% AcOH in EtOH, 12 h, 25 °C; g) 1 N HCl in MeOH, 40 °C, 30 min; h) TBAF, THF, 0 to 25 °C, 12 h; i) 8-amino-6-methoxy quinoline, CH_3CN, TEA, 80 °C, 12 h.

In Scheme **2** the synthesis of the final compounds **5g**,**h** is reported. 4,7-Dichloroquinoline (**22**) was converted to 7-chloro-4-methylquinoline by treatment with methylmagnesium bromide, in the presence of [1,2-*bis*(diphenylphosphino)ethane]dichloronickel (II) as catalyst [31]. The methyl group was oxidized to the corresponding aldehyde through a reaction carried out in presence of *tert*-butyl iodide, DMSO, AcOH and $FeCl_2$ obtaining compound **23** [32]. This intermediate was used in the next Grignard reaction with 4-chlorophenylmagnesium bromide obtaining an alcohol intermediate that was oxidized to the ketone **24** with MnO_2. This latter compound was submitted to a reaction with *n*-BuLi and 2-(4-bromophenyl)-1,3-dioxolane affording compound **25**. The dioxolane group was converted to aldehyde through a treatment with a mixture of water/acetone under microwave irradiation [33]. The obtained aldehyde was used in the following reductive amination reaction with 1-Boc-piperazine giving compound **26**. From this intermediate, the two final compounds **5g**,**h** were obtained after Boc deprotection and a dehydroxylation procedure involving H_3PO_2 and I_2 (**5h**) [34].

Scheme 2

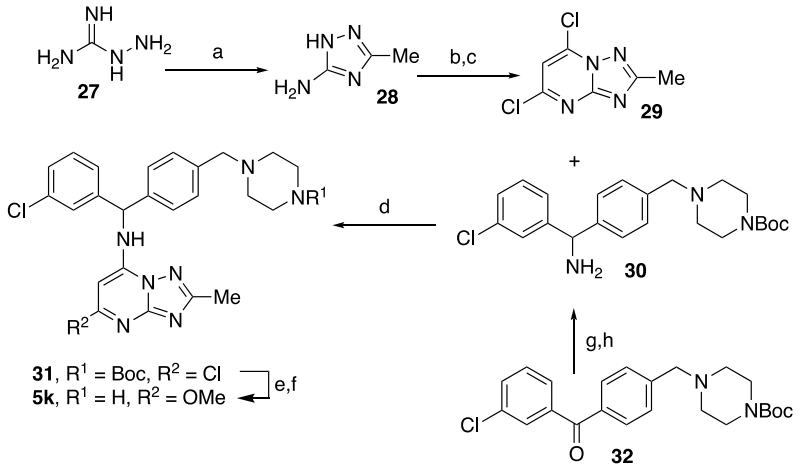


Reagents and Conditions: a) Methylmagnesium bromide, [1,2-bis(diphenylphosphino)ethane]dichloronickel (II), Et_2O, 0 to 25 °C, 12 h; b) *tert*-Butyl iodide, I_2, $FeCl_2$, TFA, DMSO, 80 °C, 6 h; c) 4-Chlorophenylmagnesium bromide, THF, 0 to 25 °C, 6 h; d) MnO_2, $CHCl_3$, 25 °C, 3 h; e) 2-(4-Bromophenyl)-1,3-dioxolane, *n*-BuLi, THF, -70 °C, 2 h; f) Acetone, H_2O, 140 W, 100 °C, 30 min; g) 1-Boc-piperazine, $NaBH_3CN$, EtOH, AcOH, 25 °C, 12 h; h) TFA, DCM, 25 °C, 1.5 h; i) H_3PO_2, I_2, AcOH, H_2O, 60 °C, 12 h.

For the synthesis of **5k** (Scheme 3), aminoguanidine **27** was cyclized to triazole **28** by treatment with AcOH [35], then this compound was converted to the 2-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-5,7-diol by treatment with diethyl malonate in presence of NaOEt [36], afterward the resulting intermediate was converted to the dichloro-derivative **29** through a reaction conducted in $POCl_3$ [37,38]. Intermediate **29** was then submitted to an aromatic substitution with the benzhydrylamine **30** and subsequently treated with a methanolic solution of NaOMe under MW irradiation, to afford compound **31**. After deprotection of the Boc-group, the final compound **5k** was isolated. Amine **30** was in turn prepared by treatment of

ketone **32** (obtained as previously reported [24,25]) with hydroxylamine and successive reduction of the oxime with Zn in acidic medium.

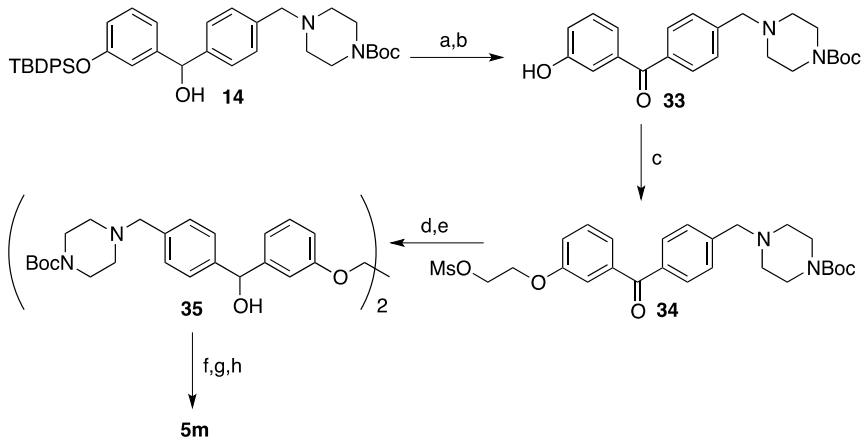
Scheme 3



Reagents and Conditions: a) AcOH, Toluene, 110 °C, 12 h; b) Diethyl malonate, EtONa, EtOH, 0 to 80 °C, 12 h; c) $POCl_3$, 110 °C, 12 h; d) EtOH, 25 °C, 12 h; e) NaOMe, MeOH, 100 W, 85 °C, 30 min; f) 1 N HCl in MeOH, 40 °C, 30 min; g) $NH_2OH{\cdot}HCl$, $BaCO_3$, MeOH, 65 °C, 12 h; h) Zn, AcOH, NH_4Cl, 50 °C, 12h.

The synthesis of the dimeric derivative **5m** is reported in Scheme 4. Alcohol **14** was oxidized with MnO_2 to the corresponding ketone, and upon deprotection of the silyl group, intermediate **33** was obtained. Compound **33** was then reacted with ethylene glycol dimesylate in the presence of sodium hydride. The monoalkylated derivative **34** was the major product of the reaction, while only traces of **35** were collected from the reaction mixture. The reaction between **34** and another equivalent of **33** afforded the *bis*-alkylated-derivative **35** in reasonable yield. Compound **5m** was finally obtained from **35** through chlorination, reaction with 7-chloro-4-aminoquinoline and *N*-Boc deprotection.

Scheme 4



Reagents and Conditions: a) MnO_2, DCE, 80 °C, 6 h; b) TBAF, THF, 2 h, 25 °C; c) Ethylene glycol dimesylate, NaH, DMF, 2 h, 80 °C; d) **33**, NaH, DMF, 2 h, 80 °C; e) $NaBH_4$, THF, 12 h, 25 °C; f) $SOCl_2$, 3 h, 80 °C; g) 4-Amino-7-chloroquinoline, TEA, CH_3CN, 12 h, 80 °C; h) 1 N HCl in MeOH, 40 °C, 30 min.

3. Results and Structure-activity Relationship (SAR) Analysis

All synthesized compounds were evaluated for their *in vitro* antiplasmodial activity against the CQ-S strain D10 (which carries $PfCRT^{3D7}$) and the CQ-R resistant strain W2 (which carries $PfCRT^{Dd2}$) and for their ability to inhibit the $PfCRT^{Dd2}$-mediated transport of CQ (Table 1 and Figure 2). *P. falciparum* viability was assessed using the activity of the parasite lactate hydrogenase as a marker, and inhibitory activity against $PfCRT^{Dd2}$ was assessed in the *Xenopus* oocyte system. A subset of 15 compounds were screened for the ability to inhibit the transport of [^{3}H]CQ via $PfCRT^{Dd2}$ when present at 100 µM in the extracellular solution. Treatments containing 100 µM of a known inhibitor of $PfCRT^{Dd2}$ – VP, chlorpheniramine (CP), or PQ – were included for comparison, and non-expressing oocytes were used as a negative control. In all cases, the lack of an effect in the non-expressing oocytes indicated that none of the compounds affect the simple diffusion of [^{3}H]CQ into the oocytes (Figure 2A). Beginning with the two reference compounds **4a**,**b**, we explored small modifications to their chemical structures to understand the key substitutions of our molecules necessary for the inhibition of $PfCRT^{Dd2}$. First, we verified the importance of the piperazine nitrogens by isosteric replacement. Removal of the distal nitrogen gave rise to the isosteric piperidines **5a**,**b** or pyrrolidines **5c**,**d** through ring contraction. In all four cases, a reduction in the ability to inhibit $PfCRT^{Dd2}$ was observed, but this effect was more evident with the piperidines (**5a**,**b**). Regarding the antiplasmodial activity, only modest changes in the IC_{50} values against CQ-S and CQ-R strains were observed, with compounds **5c**,**d** exhibiting the lowest IC_{50} values (27 and 22 nM, respectively) against the CQ-R strain. Comparisons between **5a**,**b** and **5c**,**d** revealed that the position of the chloride on the benzene ring did not affect inhibitory activity against $PfCRT^{Dd2}$. The replacement of the *m*-Cl with a *m*-OMe yielded compound **5e**, which inhibited $PfCRT^{Dd2}$ to a similar extent as compounds **4a**,**b** and produced IC_{50} values of 40 and 75 nM against the CQ-S and CQ-R strains, respectively. Since the phenolic analog **5f** showed a marked decrease in antiplasmodial activity, especially against the CQ-R strain (IC_{50} of 475 nM), coupled with a moderate cross-resistance to CQ, its activity against $PfCRT^{Dd2}$ was not evaluated.

To confirm the importance of the 4-aminoquinoline moiety on biological activity, a further set of compounds (**5g**,**h** and **6c**,**h**,**i**) was designed, synthesized, and tested for inhibitory activity against $PfCRT^{Dd2}$-mediated transport and *P. falciparum* growth. Compounds **6c**,**h**,**i** lack the 4-aminoquinoline moiety, whereas compounds **5g**,**h** lack the exocyclic nitrogen – which affects both the distance of the endocyclic quinoline nitrogen from the benzhydryl system and the basicity of the resulting quinoline. All of these compounds exhibited reduced activity against $PfCRT^{Dd2}$ (relative to **4a**,**b**) and a substantial decrease in antiplasmodial activity against both the CQ-R and CQ-S strains. Compounds **6c**,**h**,**i** were the weakest inhibitors of $PfCRT^{Dd2}$-mediated CQ transport (inhibition of only 22-43% when present at 100 µM). These results confirmed the importance of the 4-aminoquinoline moiety and of its basicity to get a potent antiplasmodial activity and for inhibitory activity against $PfCRT^{Dd2}$.

Dimers of **4a** were also synthesized in an attempt to increase the inhibitory activity against $PfCRT^{Dd2}$ [29]. Two different approaches were used to obtain those compounds: (i) the piperazine moiety was used as a dimerizing unit to produce compound **5l**; (ii) an ethyl glycol linker was used to connect two piperazine moieties, yielding compound **5m**. However, when compared with **4a**, both **5l** and **5m** exhibited reduced inhibitory activities against $PfCRT^{Dd2}$ that could be ascribed to a non-correct orientation of the quinoline/protonatable chain moieties in the transporter induced by the linkers.

Interestingly, the derivatives bearing the 6-methoxy-8-aminoquinoline system characteristic of primaquine (compounds **5i**,**j**) retained appreciable activity against $PfCRT^{Dd2}$ (~65% inhibition at 100 µM), whilst also exhibiting poor antiplasmodial activity, which is slightly higher than PQ. The final modification to the quinoline group was the substitution of the 6-methoxy-8-aminoquinoline moiety with a bioisosteric heterocycle (**5k**). PQ and its metabolic derivatives have an important role in the toxicity associated with this drug. Indeed, PQ and several of its metabolites, the most representative of which is 5-hydroxyprimaquine, induce the formation of methemoglobinemia at a rate and extension higher than usual, leading to the occurrence of hemolytic anemia [39]. Based on these observations, we replaced the quinoline group of **5i,j** with a triazolopyrimidine (TZP) as a bioisostere of the quinoline moiety of CQ [36,40–43]. Relative to compounds **5i**,**j**, **5k** was considerably more potent against the CQ-R strain, with a >10-fold reduction in the IC_{50} (920 nM), but showed a similar level of activity against the CQ-S strain (3.70 µM). Furthermore, amongst the compounds tested in this study, **5k** emerged as the most potent inhibitor of CQ transport via $PfCRT^{Dd2}$ (~98% inhibition at 100 µM and an IC_{50} of 12.5 µM).

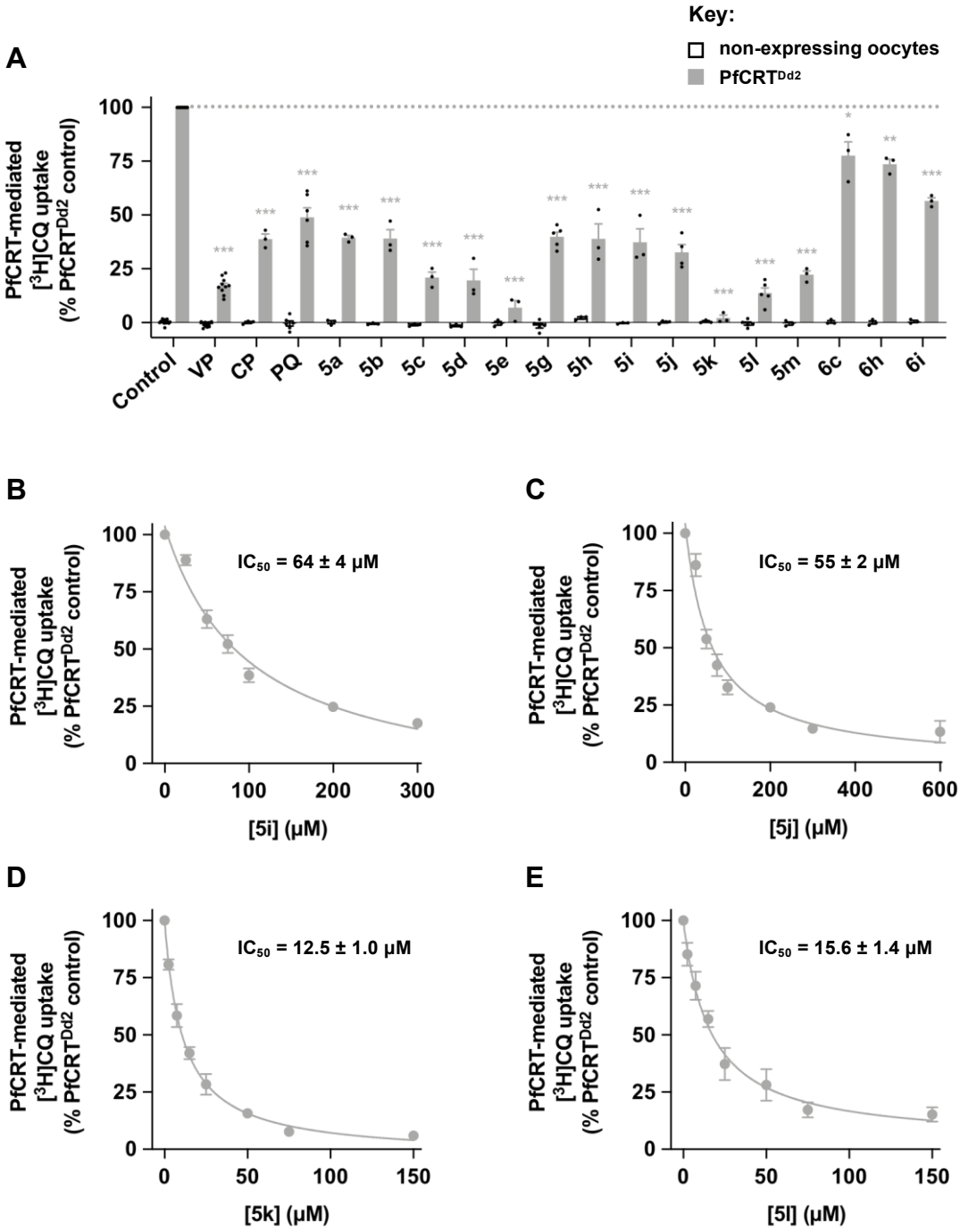


Figure 2. Effect of compounds **5a-m** and **6c**,**h**,**i** on the PfCRT^{Dd2}-mediated transport of CQ into *Xenopus* oocytes. **(A)** The uptake of [^{3}H]CQ into non-expressing oocytes (white bars) and oocytes expressing PfCRT^{Dd2} (grey bars) was measured in the absence or presence of 100 µM of the test compound. The reference drugs were VP, CP, and PQ, all of which partially reverse CQ resistance *in vitro* by inhibiting the transport of CQ via PfCRT^{Dd2} [14,17]. The component of transport attributable to PfCRT^{Dd2} (i.e. PfCRT-mediated transport) was calculated by subtracting the level of uptake measured in non-expressing oocytes from that measured in oocytes expressing PfCRT^{Dd2} (see Figure S1). Within each experiment, measurements were made from 10 oocytes per treatment and uptake was expressed relative to that measured in the PfCRT^{Dd2}-expressing oocytes under control conditions. The data are the mean of *n* = 3-11 independent experiments (each yielding similar results and overlaid as individual data points) and are shown ± s.e.m. The grey asterisks denote a significant difference (***P < 0.001, **P < 0.01, and *P < 0.005; one-way ANOVA)

in [^{3}H]CQ accumulation between the control PfCRT^{Dd2} treatment and that measured in the presence of a test compound. Note that non-expressing oocytes take up [^{3}H]CQ to a low level via simple diffusion of the neutral species of the drug; this represents the "background" level of CQ accumulation in oocytes [7]. The concentration-dependence of the effects of **5i** (**B**), **5j** (**C**), **5k** (**D**), and **5l** (**E**) on [^{3}H]CQ uptake into oocytes expressing PfCRT^{Dd2} (see Figure S1). PfCRT^{Dd2}-mediated transport (grey circles) was calculated by subtracting the level of uptake measured in non-expressing oocytes from that measured in oocytes expressing PfCRT^{Dd2}. The IC_{50}s derived from these data were obtained by a least-squares fit of the equation $Y = Y_{min} + [(Y_{max} - Y_{min})/(1 + ([inhibitor]/IC_{50})^C]$, where Y is the component of CQ uptake attributable to PfCRT^{Dd2}, Y_{min} and Y_{max} are the minimum and maximum values of Y, and C is a constant. Uptake is shown as the mean ± s.e.m. from four (D and E) or six (B and C) independent experiments, within which measurements were made from 10 oocytes per treatment. Where not shown, error bars fall within the symbols.

The poor antiplasmodial activity of the primaquine derivatives **5i**,**j** against asexual blood-stage *P. falciparum* parasites can be readily explained by the mode of action of PQ and its derivative, which target exoerythrocytic forms of the parasites such as gametocytes and hepatic schizonts via a P450-dependent bioactivation mechanism [44]. On the other hand, metabolic and/or non-enzymatic formation of PQ hydroxylated metabolites also play an important role in the toxicity of this drug [45]. Based on the above datasets, we sought to measure the activity of our PQ analogues against the exoerythrocytic forms of *Plasmodium*. A preliminary evaluation of the cytotoxicity of compounds **5i-k** revealed that **5i**,**j** exhibited a moderate level of cytotoxicity in the NIH3T3 cell line (TC_{50} = 19 and 28 μM, respectively), whereas compound **5k** produced a TC_{50} value lower than 3.5 μM. Hence, only compounds **5i**,**j** were progressed to assays with exoerythrocytic forms of *Plasmodium*.

3.1 *In vitro* evaluation of 5i and 5j against liver-stage parasites

Since PQ is the most important commercially available drug against the liver-stage of malaria, the PQ derivatives **5i** and **5j** were also tested against the liver-stage of *P. berghei*. Infected HepG2 cells were treated from 2 hpi onwards with different doses of PQ, **5i** or **5j**. At 48 hpi, parasite number (Figure 3A) and size (Figure 3B) were evaluated by automated microscopy. All the three drugs tested showed a dose-response effect on the number of parasites. PQ was the least active (8 μM) to reduce the parasite numbers to about 50%. Compound **5i** reached this level at doses as low as 0.5 μM and compound **5j** even lower at 0.125 μM. Not only the number of parasites was reduced but also their size. Whereas PQ reduced the average parasite size to about half at 2 μM, compound **5i** showed a 50% reduction at 125 nM, and compound **5j** at 62.5 nM. These data demonstrate that the two PQ derivatives, compared with PQ, are more potent inhibitors against liver-stage parasites of *P. berghei*.

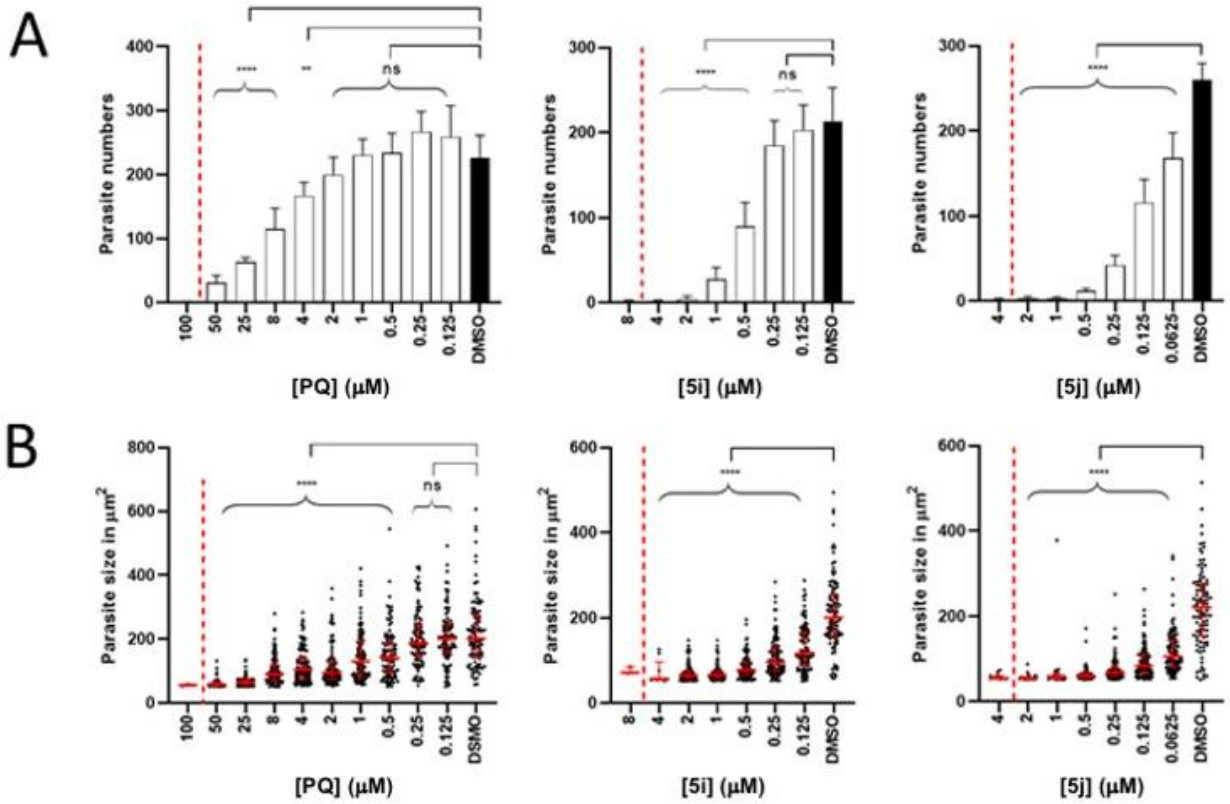


Figure 3. Effects of compounds **5i** and **5j** on liver-stage growth and development compared to primaquine. HepG2 cells infected with mCherry expressing *Plasmodium berghei* sporozoites were exposed to different drug concentrations from 2 hpi. At 48 h the cells were fixed, and parasite size and count were evaluated by automated microscopy (INCell analyzer 2000, GE healthcare). The dotted red line indicates the host cell growth inhibiting concentration. DMSO was used as solvent control at the highest concentration used (e.g. for **5i**, same as for 8 µM concentration). In A) mean parasite numbers (6 replicates) with SD are shown for the individual compound concentrations. In B) median parasite sizes with interquartile range are shown for each concentration. The number of parasite size measurements were limited to 100 parasites for illustrative purposes. Statistical evaluations (One-way ANOVA with Dunnett's multiple comparisons) were done using Prism 8 (GraphPad) (ns: not significant, **: P<0.01,****: P<0.0001).

3.2 *In vivo* evaluation of 5i and 5j as transmission blocking compounds

The effects of the PQ analogues **5i** and **5j** on transmissible stages of *Plasmodium* were assessed with the murine malaria parasite *P. berghei* (ANKA strain GFP-con) using BALB/c mice and *Anopheles stephensi* mosquitoes. Neither **5i** nor **5j** showed any impact on transmissible stages. Mosquitoes fed on gametocytaemic mice, previously treated with **5i** or **5j** at 10 µmol/kg resulted in being highly infected (**Figure 4**). Mean oocyst numbers amounted to 454 in the **5i** group and 491 in **5j** treated mosquitoes. For comparison, a similarly high number of oocysts (357) was counted in solvent control mosquitoes whereas in the positive reference group, in which mice were treated with PQ at the same dosage (10 µmol/kg), the mean oocyst number was markedly decreased (17). Since experimental mice have received 2 treatments, the first on day 3 of infection at the moment of gametocyte maturation and the second on day 4 just 1.5 h

before mosquito feeding, it can be concluded that **5i** and **5j** did not interfere neither with gametocytes nor with the development of early sporogonic stages in the mosquito midgut.

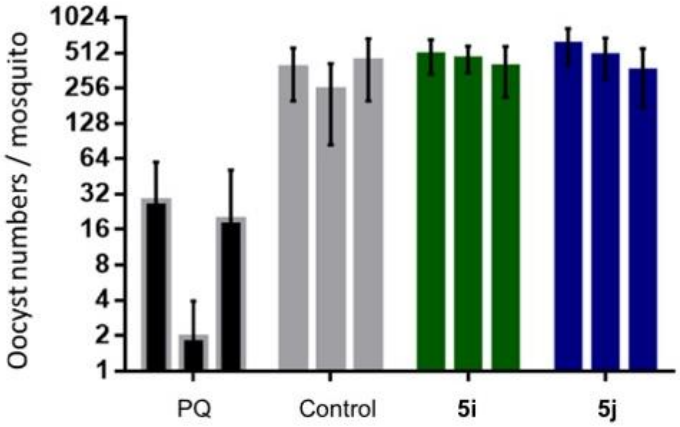


Figure 4. Effects of **5i** and **5j** on transmissible stages of *P. berghei in vivo*. Gametocytaemic mice were treated with **5i** and **5j** at 10 µmol/kg twice (3 days after mouse infection and 1.5 h before mosquito feeds). PQ (10 µmol/kg) served as positive reference and PBS as solvent control. For each treatment 3 mouse - mosquito cage replicates were performed. Oocyst numbers were assessed on 20 - 30 mosquitoes (minimum 8) of each replicate cage by gut dissection on day 8 after infection. Columns represent geometric mean numbers of oocysts from positive mosquitoes of 3 cages per treatment. Vertical bars depict the SD. Mean oocyst numbers of the **5i** and **5j** treatments were not different from solvent control replicates (2-tailed t-test: **5i** vs control: p=0.2024; **5j** versus control: p=0.2118).

4. Conclusions

In this work we describe the discovery of a new library of antimalarial compounds endowed with an inhibitory activity against the Dd2 isoform of PfCRT. The synthesis of compounds **5a**,**b**,**i-l** was performed by applying procedures previously described by us, while the other final products were obtained following newly developed synthetic schemes. The SAR analysis revealed that the presence of the 4-aminoquinoline moiety is crucial for retaining strong potency against both CQ-S and CQ-R strains of *P. falciparum* (**5a-f**). *N*-methylenpyrrolidine as a basic substituent yielded the most potent activity against the *P. falciparum* strains. No particular differences in potency were highlighted by *p*- or *m*- substitution on the aromatic moiety. In this context, a chloride or a methoxy group generated the compounds with the highest activities. Among these compounds, **5d** and **5e** exhibited potent inhibition of CQ transport via $PfCRT^{Dd2}$. However, within all of the synthesized products, **5k** was the most potent against $PfCRT^{Dd2}$, and this property correlates with the marked increase in its cytotoxicity against the CQ-R *P. falciparum* strain (IC_{50} = 0.92 µM) versus its activity against the CQ-S strain (IC_{50} = 3.70 µM). Compounds **5l** and **5m**, which are dimers of compound **4a,** were also synthesized in an attempt to increase the inhibitory activity against PfCRT. However, when compared to **4a**,**b**, both **5l** and **5m** exhibited reduced inhibitory and cytotoxicity profiles. Compounds **5i** and **5j**, linked to an 8-aminoquinoline moiety, showed a similar activity to their parental compound (PQ) against blood-stage parasites. Interestingly, in the *in vitro* assays, these compounds were up to 64 times

more active against the liver-stage than PQ. These findings will be the subject of further studies aimed at defining their mode of action. These compounds showed an inhibitory activity against PfCRT in line with the other analogues described in this work. From the cytotoxicity assays against murine fibroblasts, compounds **5i** and **5j** showed a safe profile. Given that PQ has a cytotoxic action against the sexual stages of malaria, and that **5i** and **5j** were more active than PQ against the liver-stage parasites, we investigated their activity in an *in vivo* transmission blocking assay. Unfortunately, they did not interfere with the development of either gametocytes or the early sporogonic stages in the mosquito midgut. We believe that this work may pave the way for the development of potent antimalarial agents, active against liver- and blood-stage CQ-S and CQ-R parasites, that are also potent blockers of PfCRT. Additional optimization of these compounds may be useful to identify potential lead compounds for further biological investigation.

5. Experimental section

5.1 General Information

Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by TLC using silica gel 60 F254 (0.040–0.063 mm) with detection by UV. Silica gel 60 (0.040–0.063 mm) was used for column chromatography. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 300 MHz spectrometer or a Bruker 400 MHz spectrometer by using the residual signal of the deuterated solvent as internal standard. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q) and broad (br); the values of chemical shifts (δ) are given in ppm and coupling constants (J) in Hertz (Hz). HPLC analysis were performed with a Shimadzu Prominence apparatus equipped with a scanning absorbance UV-VIS detector (Diode Array SPD-M20A) also equipped with a thermostatic chamber or with Agilent 1100 Series equipped with UV-VIS detector or LaPrep P130 apparatus using a P311 UV detector. Purity of final products (>95%) was determined by analytical HPLC Merk Purospher® STAR RP-18e (5 µm) LiChroCART® 250-4 column; detection at 254 nm; flow rate = 1.0 mL/min; mobile phase A, 0.01% TFA (v/v) in water; mobile B, methanol; gradient, 90/10–10/90 A/B in 20 min. The gradient was optimized based on the compound polarity. Unless otherwise indicated, retention times (RT) refer to the above-described conditions. ESI-MS spectra were performed by an Agilent 1100 Series LC/MSD spectrometer. Melting points were detected by a BÜCHI melting point B-450 and reported as °C. The yields are referred to purified products and are not optimized. All moisture-sensitive reactions were performed under argon atmosphere using oven-dried glassware and anhydrous solvents.

5.2. Chemistry

*5.2.1. 1-(4-(Chloro(4-chlorophenyl)methyl)benzyl)piperidine (**7a**)*

To a solution of **6a** (0.20 g, 0.63 mmol) in dry DCM (10 mL) $SOCl_2$ (274 µL, 3.78 mmol) was added and the reaction was stirred at 45 °C for 1 h. The solvent was evaporated, and crude **7a** (0.21 g, quantitative yield) was used in the next step without further purifications. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.66 (d, 2H,

J = 7.9 Hz), 7.43 (d, 2H, *J* = 7.9 Hz), 7.29-7.14 (m, 4H), 6.06 (s, 1H), 4.11 (s, 2H), 3.41 (d, 2H, *J* = 11 Hz) 2.64-2.53 (m, 2H), 2.34-2.25 (m, 2H), 1.88-1.77 (m, 2H), 1.37-1.32 (m, 2H). ESI MS m/z: $[M + H]^+$ 334.

*5.2.2. 1-(4-(Chloro(3-chlorophenyl)methyl)benzyl)piperidine (**7b**)*

Starting from **6b** (0.18 g, 0.6 mmol) the title compound was prepared following the procedure of **6a**. Crude was used in the next reaction without any further purification. **7b** (0.20 g, quantitative yield) was obtained as a brown oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.68-7.65 (m, 2H), 7.38-7.09 (m, 6H), 6.00 (s, 1H), 4.15 (s, 2H), 3.35 (br, 2H), 2.72-2.67 (m, 2H), 2.18-2.12 (m, 2H), 1.77-172 (m, 2H), 1.36- 1.31 (m, 2H). ESI MS m/z: $[M + H]^+$ 334.

*5.2.3. 1-(4-(Chloro(4-chlorophenyl)methyl)phenethyl)pyrrolidine (**7c**)*

Starting from **6c** (0.18 g, 0.6 mmol) the title compound was prepared following the procedure of **7a**. Crude was used in the next reaction without any further purification. **7c** (0.20 g, quantitative yield) was obtained as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 – 7.29 (m, 6H), 7.28 – 7.22 (m, 2H), 6.05 (s, 1H), 3.81 (br, 2H), 3.24 (s, 4H), 2.78 (br, 2H), 2.24 (br, 2H), 2.06 (br, 2H). MS (ESI) *m/z* 334 $[M+H]^+$.

*5.2.4. 1-(4-(Chloro(3-chlorophenyl)methyl)phenethyl)pyrrolidine (**7d**)*

Starting from **6d** (0.18 g, 0.6 mmol) the title compound was prepared following the procedure of **7a**. Crude was used in the next reaction without any further purification. **7d** (0.191 g, quantitative yield) was obtained as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 – 6.98 (m, 8H), 5.99 (s, 1H), 3.73 (br, 2H), 3.21 (br, 4H), 2.81 (br, 2H), 2.26 – 1.84 (m, 4H). MS (ESI) *m/z* 334 $[M+H]^+$.

*5.2.5. 7-Chloro-N-((4-chlorophenyl)(4-(piperidin-1-ylmethyl)phenyl)methyl)quinolin-4-amine (**5a**)*

To a solution of **7a** (0.84 g, 2.52 mmol) in MeCN (5 mL) TEA (1.05 mL, 7.56 mmol) and 4-Amino-7-chloroquinoline (0.54 g, 3.024 mmol) were added. The reaction was stirred at 80 °C for 12 h, then the solvent was evaporated, water was added, and the mixture was extracted with DCM. The crude material was purified by chromatography on silica gel (2% of MeOH in DCM) obtaining **5a** (0.406 g, 34%) as a white solid. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.40 (d, 1H, *J* = 5.3 Hz), 7.95 (d, 1H, *J* = 2.1 Hz), 7.74 (d, 1H, *J* = 9.1 Hz), 7.36-7.23 (m, 8H), 6.22 (d, 1H, *J* = 5.6 Hz), 5.69 (d, 1H, *J* = 4.1 Hz), 5.50 (d, 1H, *J* = 4.4 Hz), 3.44 (s, 2H), 2.36 (br, 4H), 1.60-1.52 (m, 4H), 1.44-1.42 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.0, 149.0, 148.2, 139.3, 135.0, 133.7, 130.0, 129.2, 129.0, 128.6, 127.3, 125.7, 120.8, 117.1, 110.0, 101.3, 63.3, 61.5, 54.5, 25.8, 24.3; HRMS-ESI *m/z*: calcd for $C_{28}H_{28}Cl_2N_3^+$ $[M+H]^+$: 476.1655; found: 476.1651; ESI MS *m/z*: 476 $[M +H]^+$ m.p.: 200 - 202 °C; HPCL RT: 11.2 min.

*5.2.6. 7-Chloro-N-((3-chlorophenyl)(4-(piperidin-1-ylmethyl)phenyl)methyl)quinolin-4-amine (**5b**)*

Starting from **7c** (0.12 g, 0.36 mmol) the title compound was prepared following the procedure of **5a**. The crude residue was purified by flash column chromatography on alumina (5% MeOH in DCM) obtaining **5c**

(0.50 g, 29%) as a white solid. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.44 (d, 1H, *J* = 5.3 Hz), 7.98 (d, 1H, *J* = 2.3 Hz), 7.73 (d, 1H, *J* = 8.8 Hz) 7.40-7.25 (m, 8H), 6.24 (d, 1H, *J* = 5.3 Hz), 5.68 (d, 1H, *J* = 4.4 Hz), 5.42 (d, 1H, *J* = 4.1 Hz), 3.46 (s, 2H), 2.38 (br, 4H), 1.61-1.54 (m, 4H), 1.47-1.44 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.9, 149.0, 148.2, 142.8, 139.2, 138.4, 135.0 (2C), 130.4, 130.2, 129.0, 128.2, 127.4, 127.3, 125.7, 125.3, 120.9, 117.1, 101.3, 63.1, 61.7, 54.5, 25.7, 24.1; HRMS-ESI *m/z*: calcd for $C_{28}H_{28}Cl_2N_3^+$ $[M+H]^+$: 476.1655; found: 476.1660; ESI MS *m/z*: 476 $[M + H]^+$; Melting point 204 – 206 °C; HPLC RT: 11.0 min.

*5.2.7. 7-Chloro-N-((4-chlorophenyl)(4-(2-(pyrrolidin-1-yl)ethyl)phenyl)methyl)quinolin-4-amine (**5c**)*

Starting from **7c** (0.20 g, 0.6 mmol) the title compound was prepared following the procedure of **5a**. The crude residue was purified by flash column chromatography on alumina (5% MeOH in DCM) obtaining **5c** (0.05 g, 18%) as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.41 (d, *J* = 5.2 Hz, 1H), 7.96 (s, 1H), 7.71 (d, *J* = 8.9 Hz, 1H), 7.42 – 7.12 (m, 9H), 6.21 (d, *J* = 5.2 Hz, 1H), 5.67 (d, *J* = 3.8 Hz, 1H), 5.42 (d, *J* = 3.1 Hz, 1H), 2.93 – 2.76 (m, 2H), 2.76 – 2.62 (m, 2H), 2.57 (br, 4H), 1.80 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.9, 149.0, 148.2, 140.3, 139.3, 138.5, 135.0, 133.7, 129.5, 129.2, 128.6, 127.6, 125.7, 121.0, 117.1, 101.4, 61.4, 57.9, 54.1, 35.0, 23.5; MS (ESI) *m/z* 476 $[M+H]^+$; HRMS-ESI *m/z*: calcd for $C_{28}H_{28}Cl_2N_3^+$ $[M+H]^+$: 476.1655; found: 476.1663; HPLC RT: 11.4 min.

*5.2.8. 7-Chloro-N-((3-chlorophenyl)(4-(2-(pyrrolidin-1-yl)ethyl)phenyl)methyl)quinolin-4-amine (**5d**)*

Starting from **7d** (0.191 g, 0.6 mmol) the title compound was prepared following the procedure of **5a**. The crude residue was purified by flash column chromatography on alumina (5% MeOH in DCM) obtaining **5d** (0.117 g, 43%) as a pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (d, *J* = 5.1 Hz, 1H), 7.96 (s, 1H), 7.70 (d, *J* = 8.9 Hz, 1H), 7.44 – 7.29 (m, 2H), 7.29 – 7.04 (m, 7H), 6.21 (d, *J* = 5.2 Hz, 1H), 5.65 (d, *J* = 3.9 Hz, 1H), 5.42 (d, *J* = 3.6 Hz, 1H), 2.87 – 2.74 (m, *J* = 8.9 Hz, 2H), 2.74 – 2.63 (m, 2H), 2.56 (br, 4H), 1.78 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.4, 148.6, 148.5, 142.7, 139.0, 137.6, 135.2, 135.0, 130.4, 129.5, 128.4, 128.2, 128.1, 127.4, 125.8, 125.5, 121.5, 117.4, 101.2, 61.5, 57.0, 53.9, 32.8, 23.4; MS (ESI) *m/z* 476 $[M+H]^+$; HRMS-ESI *m/z*: calcd for $C_{28}H_{28}Cl_2N_3^+$ $[M+H]^+$: 476,1655; found: 476.1660; HPLC RT: 11.1 min

*5.2.9. 3-((tert-Butyldiphenylsilyl)oxy)benzaldehyde (**10**)*

To a solution of **8** (2.0 g, 16.4 mmol) in dry THF (100 mL) cooled at 0 °C, imidazole (1.3 g, 19.7 mmol) and TBDPSCl (5.1 mL, 19.7 mmol) were added. Reaction was stirred at 25 °C for 12 h, then water was added, solvent was evaporated, and the residue was extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (5% EtOAc in PetEt) obtaining **10** (5.3 g, 90%) as colorless oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 9.82 (s, 1H), 7.76 – 7.66 (m, 4H), 7.47 – 7.34 (m, 7H), 7.30 – 7.27 (m, 1H), 7.24 – 7.18 (m, 1H), 6.99 – 6.91 (m, 1H), 1.12 (s, 9H); MS (ESI) *m/z*: 361 $[M + H]^+$.

*5.2.10. (4-(1,3-Dioxolan-2-yl)phenyl)(3-methoxyphenyl)methanol (**11**)*

Starting from **9** (585 µL, 4.8 mmol) and 2-(4-bromophenyl)-1,3-dioxolane (1.0 g, 4.4 mmol) the title compound was prepared following the procedure of **25**. The crude residue was purified by flash column chromatography on silica gel (25% EtOAc in PetEt) obtaining **11** (0.505 g, 44%) as colorless oil. ^{1}H NMR: (300 MHz, Acetone) δ 7.50 – 7.34 (m, Hz, 4H), 7.20 (t, *J* = 7.9 Hz, 1H), 7.03 (s, 1H), 6.96 (d, *J* = 6.3 Hz, 1H), 6.77 (dd, *J* = 8.2, 2.6 Hz, 1H), 5.81 (d, *J* = 3.9 Hz, 1H), 5.70 (s, 1H), 4.85 (d, *J* = 3.9 Hz, 1H), 4.18 – 3.87 (m, 4H), 3.76 (s, 3H); MS (ESI) *m/z*: 287 $[M + H]^+$.

*5.2.11. (4-(1,3-Dioxolan-2-yl)phenyl)(3-((tert-butyldiphenylsilyl)oxy)phenyl)methanol (**12**)*

Starting from **10** (2.2 g, 6.0 mmol) and 2-(4-bromophenyl)-1,3-dioxolane (1.3 g, 5.4 mmol) the title compound was prepared following the procedure of **25**. The crude residue was purified by flash column chromatography on silica gel (25% EtOAc in PetEt) obtaining **12** (1.5 g, 54%) as colorless oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.67 (d, *J* = 6.7 Hz, 4H), 7.49 – 7.30 (m, 8H), 7.19 (d, *J* = 8.1 Hz, 2H), 7.02 (t, *J* = 7.9 Hz, 1H), 6.82 (d, *J* = 7.6 Hz, 1H), 6.77 (s, 1H), 6.64 (d, *J* = 9.5 Hz, 1H), 5.79 (s, 1H), 5.62 (s, 1H), 4.16 – 3.97 (m, 4H), 1.08 (s, 9H); MS (ESI) *m/z*: 533 $[M + Na]^+$.

*5.2.12. tert-Butyl 4-(4-(hydroxy(3-methoxyphenyl)methyl)benzyl)piperazine-1-carboxylate (**13**)*

Step e: To a solution of **11** (0.40 g, 1.4 mmol) in THF (8 mL) a 1 N solution of HCl was added (4 mL). Mixture was stirred at 25 °C for 4 h; $NaHCO_3$ was added and THF was evaporated. Residue was extracted with EtOAc and the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*; the crude residue was used in the next reaction without any further purification. Compound *4-(Hydroxy(3-methoxyphenyl)methyl)benzaldehyde* (0.38 g, quantitative yield) was obtained as a colorless oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 9.91 (s, 1H), 7.80 (d, *J* = 6.7 Hz, 2H), 7.54 (d, *J* = 8.1 Hz, 2H), 7.24 (t, *J* = 7.3 Hz, 1H), 6.95 – 6.86 (m, 2H), 6.80 (d, *J* = 7.5 Hz, 1H), 5.81 (s, 1H), 3.75 (s, 3H), 3.06 (br, 1H); MS (ESI) *m/z*: 241 $[M - H]^-$.

Step f: Compound **13** was obtained starting from the above-obtained compound (0.35 g, 1.4 mmol) following the procedure used for **26g**. Crude compound was purified by chromatography on silica gel (40% PetEt in EtOAc) obtaining **13** (0.578 g, 89%) as a pale yellow oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.35 – 7.11 (m, 5H), 6.91 (d, *J* = 8.9 Hz, 2H), 6.74 (s, 1H), 5.68 (s, 1H), 3.70 (s, 3H), 3.40 (s, 2H), 3.32 (br, 4H), 2.28 (br, 4H), 1.42 (s, 9H); MS (ESI) *m/z*: 413 $[M + H]^+$.

*5.2.13. tert-Butyl (4-(4-((3-((tert-butyldiphenylsilyl)oxy)phenyl)(hydroxy)methyl)benzyl)piperazine-1-carboxylate (**14**)*

Starting from **12** (1.5 g, 2.9 mmol) the title compound was prepared following the procedure of **13**. The crude residue was purified by flash column chromatography on silica gel (25% EtOAc in PetEt) obtaining **14** (1.5 g, 70%) as colorless oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.72 – 7.62 (m, 4H), 7.47 – 7.28 (m, 6H),

7.24 – 7.10 (m, 4H), 7.04 (t, *J* = 7.9 Hz, 1H), 6.85 (d, *J* = 7.2 Hz, 1H), 6.79 (s, 1H), 6.66 (d, *J* = 8.1 Hz, 1H), 5.61 (s, 1H), 3.46 (s, 2H), 3.41 (br, 4H), 2.36 (br, 4H), 1.45 (s, 9H), 1.08 (s, 9H); MS (ESI) *m/z*: 637 $[M + H]^+$.

*5.2.14. tert-butyl 4-(4-(((7-chloroquinolin-4-yl)amino)(3-methoxyphenyl)methyl)benzyl)piperaz-ine-1-carboxylate (**17**)*

Step e: Starting from **13** (0.124 g, 0.3 mmol), **15** was prepared following the procedure used for compound **7a**. **15** (0.10 g, 76%) was obtained as a pale yellow oil and was used in the next step without any further purification. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.41 – 7.28 (m, 4H), 7.26 – 7.14 (m, 1H), 7.05 – 6.96 (m, 2H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.09 (s, 1H), 3.80 (s, 3H), 3.49 (s, 2H), 3.43 (br, 4H), 2.38 (br, 4H), 1.46 (s, 9H); MS (ESI) *m/z*: 431 $[M + H]^+$.

Starting from **15** (0.10 g, 0.2 mmol), the title compound **17** (0.034 g, 26%) was obtained following the procedure used for compound **5a**. **17** was obtained as a pale-yellow oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.38 (d, *J* = 5.3 Hz, 1H), 7.93 (d, *J* = 2.1 Hz, 1H), 7.73 (d, *J* = 9.0 Hz, 1H), 7.37 – 7.19 (m, 6H), 6.93 (d, *J* = 7.7 Hz, 1H), 6.90 – 6.87 (m, 1H), 6.84 – 6.78 (m, 1H), 6.25 (d, *J* = 5.4 Hz, 1H), 5.67 (d, *J* = 4.4 Hz, 1H), 5.55 (d, *J* = 4.5 Hz, 1H), 3.73 (s, 3H), 3.47 (s, 2H), 3.40 (br, 4H), 2.36 (br, 4H), 1.43 (s, 9H); MS (ESI) *m/z*: 573 $[M + H]^+$.

*5.2.15. tert-Butyl 4-(4-((3-((tert-butyldiphenylsilyl)oxy)phenyl)((7-chloroquinolin-4-yl)amino)methyl)benzyl)piperazine-1-carboxylate (**18**)*

Starting from **14** (0.088 g, 0.1 mmol) the title compound was prepared following the procedure of **5a**. The crude residue was purified by flash column chromatography on silica gel (2% MeOH in DCM) obtaining **18** (0.035 g, 34%) as pale-yellow oil. ^{1}H NMR: (300 MHz, $CDCl_3$): δ 8.36 (d, *J* = 5.1 Hz, 1H), 7.97 (s, 1H), 7.69 – 7.60 (m, 2H), 7.57 – 7.50 (m, 2H), 7.45 – 7.09 (m, 11H), 7.00 (d, *J* = 7.0 Hz, 2H), 6.84 (d, *J* = 7.9 Hz, 2H), 6.67 (s, 1H), 6.10 (d, *J* = 5.4 Hz, 1H), 5.44 (d, *J* = 4.0 Hz, 1H), 5.08 (d, *J* = 4.0 Hz, 1H), 3.46 (s, 2H), 3.43 (br, 4H), 2.36 (br, 4H), 1.46 (s, 9H), 1.04 (s, 9H); MS (ESI) *m/z*: 798 $[M + H]^+$.

*5.2.16. 7-Chloro-N-((3-methoxyphenyl)(4-(piperazin-1-ylmethyl)phenyl)methyl)quinolin-4-amine (**5e**)*

17 (0.085 g, 0.2 mmol) was dissolved in MeOH and a 1 M solution of HCl in MeOH was slowly added. The mixture was evaporated at 45 °C under vacuum. This procedure was repeated three times. **5e** was obtained as a colorless oil (0.07 g, quantitative yield). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.43 (d, *J* = 5.3 Hz, 1H), 7.98 (d, *J* = 2.0 Hz, 1H), 7.71 (d, *J* = 9.0 Hz, 2H), 7.43 – 7.26 (m, 5H), 7.01 – 6.79 (m, 3H), 6.27 (d, *J* = 5.4 Hz, 1H), 5.68 (d, *J* = 4.3 Hz, 1H), 5.40 (d, *J* = 4.3 Hz, 1H), 3.77 (s, 3H), 3.48 (s, 2H), 2.88 (br, 4H), 2.41 (br, 4H); ^{13}C NMR: (75 MHz, $CDCl_3$) δ 159.1, 151.0, 148.0, 147.4, 141.6, 138.7, 137.1, 133.9, 129.1, 128.8, 127.9, 126.3, 124.5, 120.0, 118.5, 116.1, 112.4, 111.7, 100.2, 62.1, 61.0, 54.2, 53.3, 44.9; MS (ESI) *m/z*: 473 $[M + H]^+$; HRMS-ESI *m/z*: calcd for $C_{28}H_{30}ClN_4O^+$ $[M+H]^+$: 473.2103; found: 473.2110; HPLC RT: 8.9 min.

*5.2.17. 3-(((7-Chloroquinolin-4-yl)amino)(4-(piperazin-1-ylmethyl)phenyl)methyl)phenol (**5f**)*

Step g: To a solution of **18** (0.035 g, 0.04 mmol) in dry THF, cooled to 0 °C (5 mL) a 1 M solution of TBAF in THF (130 L, 0.1 mmol) was added. Reaction was allowed to reach 25 °C and stirred for 2 h. Water was added, THF was evaporated and residue was extracted with DCM and the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Crude was purified by flash chromatography on silica gel (MeOH 3% in DCM). Compound *tert-Butyl 4-(4-(((7-chloroquinolin-4-yl)amino)(3-hydroxyphenyl)methyl)benzyl)piperazine-1-carboylate* (0.02 g, 81%) was obtained as pale yellow oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.20 (d, *J* = 5.5 Hz, 1H), 7.92 (d, *J* = 2.0 Hz, 1H), 7.54 (d, *J* = 9.0 Hz, 1H), 7.28 (s, 4H), 7.26 (d, *J* = 0.6 Hz, 1H), 7.25 (d, *J* = 2.0 Hz, 1H), 7.20 (t, *J* = 7.8 Hz, 1H), 6.90 (s, 1H), 6.82 (d, *J* = 8.0 Hz, 1H), 6.74 (d, *J* = 7.8 Hz, 1H), 6.15 (d, *J* = 5.6 Hz, 1H), 5.60 (d, *J* = 4.3 Hz, 1H), 5.48 (d, *J* = 4.5 Hz, 1H), 3.48 (s, 2H), 3.42 (br, 4H), 2.38 (br, 4H), 1.45 (s, 9H); MS (ESI) *m/z*: 559 $[M + H]^+$.

Step h: Starting from the above obtained compound (0.02 g, 0.04 mmol), the title compound was prepared following the procedure of **5e**. **5f** was obtained as a colorless oil (0.017 g, quantitative yield). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.26 (d, *J* = 5.4 Hz, 1H), 7.92 (d, *J* = 2.1 Hz, 1H), 7.59 (d, *J* = 9.1 Hz, 1H), 7.33 – 7.17 (m, 6H), 6.90 – 6.69 (m, 3H), 6.19 (d, *J* = 5.6 Hz, 1H), 5.63 (d, *J* = 4.4 Hz, 1H), 5.44 (d, *J* = 4.5 Hz, 1H), 3.44 (s, 2H), 2.84 (br, 4H), 2.38 (br, 4H); ^{13}C NMR: (75 MHz, $CDCl_3$) δ 158.1, 151.5, 148.9, 148.3, 142.1, 140.1, 137.9, 135.3, 130.6, 130.2, 128.2, 127.7, 125.9, 121.1, 119.0, 117.0, 115.8, 114.1, 101.3, 63.2, 61.9, 54.1, 45.8; MS (ESI) *m/z*: 459 $[M + H]^+$. HPLC RT: 7.3 min.

*5.2.18. tert-Butyl 4-(4-((3-chlorophenyl)((6-methoxyquinolin-8-yl)amino)methyl)benzyl)piperazine-1-carboxylate (**21**)*

To a solution of **20** (0.104 g, 0.2 mmol) in dry CH_3CN (5 mL) DIPEA (110 µL, 0.6 mmol) and **SI19** (0.05 g, 0.3 mmol) were added. Solution was stirred at 80 °C for 12 h, then water was added, solvent was evaporated, and residue was extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Crude compound **21** was purified by chromatography on silica gel (50% EtOAc in PetEt), and it was obtained as a pale-yellow oil (0.05 g, 30%). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 4.2 Hz, 1H), 7.94 (d, *J* = 8.3 Hz, 1H), 7.41 (s, 1H), 7.36 – 7.22 (m, 8H), 6.73 (d, *J* = 4.8 Hz, 1H), 6.38 (d, *J* = 2.3 Hz, 1H), 6.10 (d, *J* = 2.3 Hz, 1H), 5.59 (d, *J* = 4.9 Hz, 1H), 3.77 (s, 3H), 3.48 (s, 2H), 3.43 (br, 4H), 2.38 (br, 4H), 1.45 (s, 9H); MS (ESI) *m/z*: 573 $[M + H]^+$.

*5.2.19. N-((3-chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)methyl)-6-methoxyquinolin-8-amine (**5i**)*

Compound **5i** was obtained starting from **21** (0.017 g, 0.03 mmol) following the procedure described before to obtain compound **5e**. Crude **5i** was purified by chromatography on silica gel (3% MeOH in DCM) and was obtained as a pale yellow oil (0.012 g, 86%). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 4.1 Hz, 1H), 7.94 (d, *J* = 8.3 Hz, 1H), 7.41 (s, 1H), 7.39 – 7.16 (m, 8H), 6.73 (d, *J* = 4.8 Hz, 1H), 6.38 (d, *J* = 2.1 Hz, 1H), 6.11 (d, *J* = 2.0 Hz, 1H), 5.59 (d, *J* = 4.8 Hz, 1H), 3.80 (s, 3H), 3.46 (s, 2H), 2.88 (br, 4H), 2.41 (br,

4H); ^{13}C NMR: (75 MHz, $CDCl_3$) δ 159.3, 144.9, 144.8, 144.7, 140.9, 137.9, 135.6, 135.0, 134.9, 130.3, 129.9, 129.8 (2C), 127.8. 127.7, 125.8, 122.2, 99.0, 93.2, 63.5, 62.3, 55.4, 54.6, 46.2; MS (ESI) *m/z*: 473 $[M + H]^+$. HPLC (gradient, 95/5–55/45 A/B in 12 min) RT: 9.5 min.

*5.2.20. N-((3-Chlorophenyl)(4-(pyrrolidin-1-ylmethyl)phenyl)methyl)-6-methoxyquinolin-8-amine (**5j**)*

Starting from **19** (0.10 g, 0.3 mmol) and **SI19** (0.065 g, 0.4 mmol) the title compound was prepared following the procedure of **21**. The crude material was purified by flash chromatography on silica gel (2% MeOH in DCM) to give **5j** as a pale yellow oil (0.091 g, 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, *J* = 4.2 Hz, 1H), 7.94 (d, *J* = 8.3 Hz, 1H), 7.51 – 7.13 (m, 8H), 6.73 (d, *J* = 4.8 Hz, 1H), 6.38 (s, 1H), 6.11 (s, 1H), 5.59 (d, *J* = 4.9 Hz, 1H), 3.80 (s, 3H), 3.60 (s, 2H), 2.51 (br, 4H), 1.78 (br, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.3, 144.9, 144.8, 144.8, 140.7, 139.2, 137.5, 135.6, 135.0, 134.9, 130.3, 129.8, 129.7, 127.8, 127.7, 125.8, 122.2, 99.0, 93.2, 62.3, 60.6, 55.4, 54.5, 23.7; ESI MS *m/z*: 458 $[M + H]^+$; HRMS-ESI *m/z*: calcd for $C_{28}H_{29}ClN_3O^+$ $[M+H]^+$: 458,1994; found: 458.1998; HPLC RT: 14.6 min.

*5.2.21. 7-Chloroquinoline-4-carbaldehyde (**23**)*

To a solution of 4,7-dichloroquinoline (1.5 g, 7.6 mmol) in dry Et_2O, [1,2-bis(diphenylphosphino)-ethane]dichloronickel (II) (0.04 g, 0.07 mmol) was added. Suspension was cooled to 0 °C and a 3.0 M solution of methylmagnesium bromide (2.6 mL, 7.6 mmol) in Et_2O was added. Reaction was stirred at 25 °C for 12h. A saturated solution of NH_4Cl was added to the reaction and mixture was extracted with Et_2O. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (5% of EtOAc in PetEt) obtaining *7-Chloro-4-methylquinoline* (1.9 g, 70%) as a low melting point white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.77 (d, *J* = 4.4 Hz, 1H), 8.10 (s, 1H), 7.93 (d, *J* = 8.9 Hz, 1H), 7.52 (d, *J* = 8.9 Hz, 1H), 7.23 (d, *J* = 4.5 Hz, 1H), 2.70 (s, 3H); MS (ESI) *m/z* 178 $[M+H]^+$.

To a solution of *7-Chloro-4-methylquinoline* (0.50 g, 2.8 mmol) in DMSO (15 mL) TFA (274 µL, 3.6 mmol), *tert*-butyliodide (342 µL, 2.9 mmol), iodine (0.75 g, 3.0 mmol) and $FeCl_2$ (0.13 g, 0.7 mmol) were added in this order and mixture was heated at 80 °C for 6 h. Thereafter a saturated solution of $Na_2S_2O_3$ and a saturated solution of $NaHCO_3$ were added. Mixture was extracted with EtOAc and the combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (20% of EtOAc in PetEt) obtaining **23** (0.468 g, 87%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.45 (s, 1H), 9.21 (d, *J* = 4.2 Hz, 1H), 9.01 (d, *J* = 9.1 Hz, 1H), 8.22 (d, *J* = 2.1 Hz, 1H), 7.79 (d, *J* = 4.2 Hz, 1H), 7.68 (dd, *J* = 9.1, 2.1 Hz, 1H); MS (ESI) *m/z* 192 $[M+H]^+$.

*5.2.22. (4-Chlorophenyl)(7-chloroquinolin-4-yl)methanone (**24**)*

Step c: To a solution of **23** (1.0 g, 5.2 mmol) in dry THF (5 mL) cooled at 0 °C, a 1 M solution of 4-chlorophenylmagnesium bromide (5.7 mL, 5.7 mmol) in Et_2O was added. Reaction was stirred at 25 °C for 6 h. The reaction was quenched with a saturated solution of NH_4Cl, THF was evaporated and residue was

extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (33% of EtOAc in PetEt) obtaining *(4-chlorophenyl)(7-chloroquinolin-4-yl)methanol* (1.3 g, 82%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.84 (d, *J* = 4.3 Hz, 1H), 8.04 (s, 1H), 7.82 (d, *J* = 9.0 Hz, 1H), 7.63 (d, *J* = 4.4 Hz, 1H), 7.39 (d, *J* = 9.0 Hz, 1H), 7.33 – 7.21 (m, 4H), 6.40 (s, 1H); MS (ESI) *m/z* 304 $[M+H]^+$.

Step d: To a solution of the above obtained compound (1.3 g, 4.3 mmol) in $CHCl_3$ (100 mL) MnO_2 (1.5 g, 17.1 mmol) was added. Suspension was stirred for 3 h at 25 °C and reaction was filtered through paper and solvent evaporated. Crude was purified by flash chromatography on silica gel (20% of EtOAc in PetEt) obtaining **24** (1.2 g, 92%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.03 (d, *J* = 4.3 Hz, 1H), 8.21 (d, *J* = 2.1 Hz, 1H), 7.82 (s, 1H), 7.81 – 7.72 (m, 2H), 7.52 (d, *J* = 2.2 Hz, 1H), 7.51 – 7.43 (m, 2H), 7.38 (d, *J* = 4.3 Hz, 1H). MS (ESI) *m/z* 302 $[M+H]^+$.

*5.2.23. (4-(1,3-Dioxolan-2-yl)phenyl)(4-chlorophenyl)(7-chloroquinolin-4-yl)methanol (**25**)*

To a solution of 2-(4-bromophenyl)-1,3-dioxolane (0.186 g, 0.8 mmol) in dry THF (10 mL) cooled at -70 °C a solution of *n*-BuLi (485 µL, 1.0 mmol) was added. Mixture was stirred at -70 °C for 30 min, then a solution of **24** (0.27 g, 0.9 mmol) in dry THF (5 mL) was added, and reaction was stirred for another 1.5 h at -70 °C. Thereafter, the reaction was allowed to reach 25 °C, water was added and THF was evaporated. Residue was extracted with EtOAc and the combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (20% of EtOAc in PetEt) obtaining **25** (0.287 g, 78%) as amorphous white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.57 (d, *J* = 4.5 Hz, 1H), 8.11 – 7.84 (m, 2H), 7.44 (d, *J* = 8.0 Hz, 2H), 7.37 – 7.02 (m, 7H), 6.73 (d, *J* = 4.5 Hz, 1H), 5.77 (s, 1H), 4.31 – 3.79 (m, 5H); MS (ESI) *m/z* 452 $[M+H]^+$.

*5.2.24. tert-Butyl 4-(4-((4-chlorophenyl)(7-chloroquinolin-4-yl)(hydroxy)methyl)benzyl)piperazine -1-carboxylate (**26**)*

Step f: Compound **25** (0.05 g, 0.1 mmol) was dissolved in water (2 mL) and the minimum amount of acetone necessary to have a limpid solution was added. Mixture was submitted under MW irradiation at 140 W, 100 °C for 30 min. Mixture was extracted with EtOAc and the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Residue *4-((4-chlorophenyl)(7-chloroquinolin-4-yl)(hydroxy)methyl)benzaldehyde* (quantitative yield) was used in the next reaction without any further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.02 (s, 1H), 8.72 (d, *J* = 4.6 Hz, 1H), 8.08 (s, 1H), 7.95 (d, *J* = 9.2 Hz, 1H), 7.87 (d, *J* = 8.0 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.33 – 7.23 (m, 1H), 7.19 (d, *J* = 8.4 Hz, 2H), 6.76 (d, *J* = 4.4 Hz, 1H); MS (ESI) *m/z* 408 $[M+H]^+$.

Step g: The above obtained compound (0.05 g, 0.12 mmol) was dissolved in a 1% solution of AcOH in EtOH (5 mL) and 1-Boc-piperazine (0.022 g, 0.1 mmol) and $NaBH_3CN$ (0.015 g, 0.3 mmol) were added. Reaction was stirred at 25 °C for 12 h, then a saturated solution of $NaHCO_3$ was added, and EtOH was evaporated. Residue was extracted with DCM and the combined organic layers were dried over Na_2SO_4

and concentrated *in vacuo*. The crude material was purified by chromatography on silica gel (1% of MeOH in DCM) obtaining **26** (0.06 g, 87%) as amorphous white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.59 (d, *J* = 4.6 Hz, 1H), 8.05 (d, *J* = 9.3 Hz, 1H), 7.98 (d, *J* = 2.1 Hz, 1H), 7.39 – 7.08 (m, 9H), 6.75 (d, *J* = 4.6 Hz, 1H), 4.44 (br, 1H), 3.49 (s, 2H), 3.39 (br, 4H), 2.36 (br, 4H), 1.42 (s, 9H); MS (ESI) *m/z* 600 $[M+Na]^+$.

*5.2.25. 7-Chloro-4-((4-chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)methyl)quinoline (**5g**)*

Compound **26** (0.025 g, 0.04 mmol) was dissolved in AcOH (5 mL) then a solution of H_3PO_4 (123 µL, 0.19 mmol) 50% in water and a catalytic amount of iodine were added. Reaction was stirred at 60 °C for 12 h and water was added. AcOH was evaporated under reduced pressure; residue was neutralized with $NaHCO_3$ and extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by chromatography on alumina (5% of MeOH in DCM) obtaining **28** (0.015 g, 80%) as amorphous white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (d, *J* = 4.5 Hz, 1H), 8.10 (d, *J* = 1.9 Hz, 1H), 7.81 (d, *J* = 9.0 Hz, 1H), 7.37 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.33 – 7.19 (m, 5H), 6.98 (dd, *J* = 8.1, 3.7 Hz, 3H), 6.81 (d, *J* = 4.5 Hz, 1H), 6.10 (s, 1H), 3.45 (s, 2H), 2.86 (br, 4H), 2.39 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.3, 149.1, 149.0, 140.4, 140.2, 136.3, 135.0, 133.0, 130.7, 129.5, 129.4, 129.2, 129.0, 127.8, 125.5, 122.1, 62.3, 52.9, 51.8, 50.9, 44.2; HRMS-ESI *m/z*: calcd for $C_{27}H_{26}Cl_2N_3^+$ $[M+H]^+$: 462.1498; found: 462.1504; MS (ESI) *m/z* 462 $[M+H]^+$. HPLC RT: 10.2 min.

*5.2.26. (4-Chlorophenyl)(7-chloroquinolin-4-yl)(4-(piperazin-1-ylmethyl)phenyl)methanol (**5h**)*

To a solution of **26** (0.035 g, 0.06) in dry DCM (3 mL) TFA (250 µL) was added and mixture was stirred at 1.5 h at 25 °C. Reaction was quenched with $NaHCO_3$ and extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude material was purified by chromatography on alumina (2% of MeOH in DCM) obtaining **5h** (0.02 g, 68%) as amorphous white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, *J* = 4.6 Hz, 1H), 8.15 – 7.88 (m, 2H), 7.42 – 7.03 (m, 9H), 6.77 (d, *J* = 4.6 Hz, 1H), 3.43 (s, 2H), 2.73 (br, 4H), 2.33 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.0, 150.5, 150.0, 144.1, 143.8, 137.3, 134.8, 133.8, 129.5, 129.1, 128.9, 128.5, 127.5, 127.1, 124.7, 121.8, 82.1, 62.5, 52.0 (2C), 44.7; HRMS-ESI *m/z*: calcd for $C_{27}H_{26}Cl_2N_3O^+$ $[M+H]^+$: 478.1447; found: 478.1455; MS (ESI) *m/z* 478 $[M+H]^+$. HPLC RT 9.6 min.

*5.2.27. 3-Methyl-1H-1,2,4-triazol-5-amine (**28**)*

To aminoguanidine bicarbonate (2.0 g, 14.7 mmol) AcOH (2 mL) was added. Mixture was stirred at 25 °C until effervescence had completely vanished, then toluene (100 mL) was added, reaction was linked to a dean-stark system and heated at 110 °C for 12 h. Solvent was evaporated and residue was purified by chromatography on silica gel (11% MeOH, 1.1% NH_4OH in DCM). **28** (1.2, 86%) was obtained as a white solid. ^{1}H NMR: (300 MHz, DMSO) δ 5.47 (br, 2H), 2.03 (s, 3H), 1.85 (s, 1H); ESI MS *m/z*: 99 $[M + H]^+$; m.p. 143-145 °C.

*5.2.28. 5,7-Dichloro-2-methyl-[1,2,4]triazolo[1,5-a]pyrimidine (**29**)*

To 20 mL of dry EtOH Na^0 (0.32 g, 13.9 mmol) was added at 0 °C; after complete Na solubilization compound **28** (1.1 g, 11.6 mmol) and diethyl malonate (2 mL, 13.2 mmol) were added. The reaction was heated at 80 °C for 12 h. A white precipitate was obtained and collected, then it was washed with EtOH, solubilized in water, and precipitated again with HCl 37%. The white solid obtained was collected, obtaining *2-Methyl-[1,2,4]triazolo[1,5-a]pyrimidine-5,7-diol* (1.2 g, 62%). ^{1}H NMR: (300 MHz, DMSO) δ 12.24 (br, 2H), 5.04 (s, 1H), 2.33 (s, 3H); ESI MS *m/z*: 167 $[M + H]^+$; m.p. decomposition at 220 °C.

The above obtained compound ((1.2 g, 7.22 mmol) was suspended in $POCl_3$ (20 mL) and stirred at 110 °C for 12 h. $POCl_3$ was evaporated and the residue was neutralized with a saturated solution of $NaHCO_3$, and extracted with DCM. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Crude compound was purified by chromatography on silica gel (2% MeOH in DCM) obtaining **29** (0.883 g, 60%) as a white solid. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.17 (s, 1H), 2.67 (s, 3H); MS (ESI) *m/z*: 202 $[M + H]^+$; m.p. 222 - 224 °C.

*5.2.29. tert-Butyl 4-(4-(amino(3-chlorophenyl)methyl)benzyl)piperazine-1-carboxylate (**30**)*

Step g: To a solution of **32** (0.44 g, 1.1 mmol) in EtOH (20 mL) $BaCO_3$ (0.481 g, 2.4 mmol) and $NH_2OH{\cdot}HCl$ (0.157 g, 2.4 mmol) were added. Reaction was stirred at 65 °C for 12 h, then was filtered through celite and solvent was evaporated. *(E,Z)-tert-butyl 4-(4-((3-chlorophenyl)(hydroxyimino)-methyl)benzyl)piperazine-1-carboxylate* (0.46 g, quantitative yield) was obtained as an amorphous white solid and was used in the next step without any further purification. ^{1}H NMR: (400 MHz, $CDCl_3$) δ 8.34 (br, 1H), 7.51 – 7.19 (m, 8H), 3.52 (br, 6H), 2.31 (br, 4H), 1.43 (s, 9H); MS (ESI) *m/z*: 430 $[M + H]^+$.

Stepe h: To a solution of the above-obtained compound (0.46 g, 1.1 mmol) in AcOH (50 mL) NH_4Cl (1.2 g, 22.3 mmol) and Zn^0 (2.7 g, 41.9 mmol) were added. Reaction was stirred at 50 °C for 12 h then was filtered through celite, solvent was evaporated, and residue neutralized with $NaHCO_3$. Mixture was extracted with DCM, and the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Crude **30** (0.30 g, 70%) obtained as a pale-yellow oil, was used in the next step without any further purification. ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.36 (s, 1H), 7.30 – 7.12 (m, 7H), 5.12 (s, 1H), 3.43 (s, 2H), 3.38 (br, 4H), 2.33 (br, 4H), 1.83 (br, 2H), 1.42 (s, 9H); MS (ESI) *m/z*: 416 $[M + H]^+$.

*5.2.30. tert-Butyl 4-(4-(((5-chloro-2-methyl-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)amino)(3-chlorophenyl)methyl)benzyl)piperazine-1-carboxylate (**31**)*

Compound **29** (0.07 g, 0.4 mmol) and **30** (0.216 g, 0.5 mmol) were dissolved in EtOH (5 mL) and the reaction was stirred at 25 °C for 12 h. The solvent was evaporated, and the crude was purified by chromatography on silica gel (3% MeOH in DCM) obtaining **31** (0.20 g, 66%). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.44 – 7.17 (m, 8H), 6.59 (d, *J* = 5.9 Hz, 1H), 5.90 (s, 1H), 5.72 (d, *J* = 6.0 Hz, 1H), 3.48 (s, 2H), 3.42 (br, 4H), 2.52 (s, 3H), 2.37 (br, 4H), 1.43 (s, 9H); MS (ESI) *m/z*: 582 $[M + H]^+$.

*5.2.31. N-((3-chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)methyl)-5-methoxy-2-methyl-[1,2,4]triazolo[1,5-a]pyrimidin-7-amine (**5k**)*

To dry MeOH (2 mL) placed in a microwave tube, and cooled at 0 °C, metallic sodium (0.015 g, 0.70 mmol) was added. After complete dissolution of sodium, compound **31** (0.06 g, 0.10 mmol) was added and the mixture was heated to 85 °C under microwave irradiation 80 W for 30 min. Water was slowly added and the mixture was extracted with DCM; the combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. Crude **5k** was purified by chromatography on alumina (MeOH 5% in DCM) and was obtained as a colorless oil (0.025 g, 54%). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.48 – 7.10 (m, 8H), 6.32 (s, 1H), 5.62 (s, 1H), 5.28 (s, 1H), 3.91 (s, 3H), 3.45 (s, 2H), 2.86 (br, 4H), 2.47 (s, 3H), 2.35 (br, 4H); ^{13}C NMR: (75 MHz, $CDCl_3$) δ 167.4, 164.2, 155.5, 146.8, 141.8, 139.2, 138.0, 135.4, 130.7, 130.2 (2C), 128.8, 127.5 (2C), 125.4, 63.2, 60.9, 54.4, 46.1, 15.1; MS (ESI) *m/z*: 500 $[M + Na]^+$; HRMS-ESI *m/z*: calcd for $C_{25}H_{29}ClN_7O^+$ $[M+H]^+$: 478.2117; found: 478.2121; HPLC RT: 9.2 min.

*5.2.32. tert-Butyl 4-(4-(3-hydroxybenzoyl)benzyl)piperazine-1-carboxylate (**33**)*

Step a: 14 (1.0 g, 1.6 mmol) was dissolved in DCE (50 mL) and 85% MnO_2 (0.655 g, 6.40 mmol) was added. The reaction was stirred at 80 °C for 6 h then it was filtered through paper and the solvent was evaporated. *tert-Butyl 4-(4-(3-((tert-butyldiphenylsilyl)-oxy)-benzoyl)benzyl)piperazine-1-carboxylate* (0.725 g, 70%) was obtained as a pale yellow oil and it was used in the next step without further purifications. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.75 – 7.63 (m, 3H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.47 – 7.24 (m, 10H), 7.22 – 7.12 (m, 2H), 6.99 – 6.91 (m, 1H), 3.55 (s, 2H), 3.45 (br, 4H), 2.40 (br, 4H), 1.46 (s, 9H), 1.10 (s, 9H); MS (ESI) *m/z*: 635 $[M + H]^+$.

Step b: Compound **33** was obtained from the above compound (0.084 g, 0.1 mmol) following the procedure described before for compound **5f step g**. Crude was purified by chromatography on silica gel (3% MeOH, in DCM) obtaining **33** (0.05 g, 97%) as a white solid. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.70 (d, *J* = 8.0 Hz, 2H), 7.37 (d, *J* = 8.1 Hz, 2H), 7.30 – 7.19 (m, 3H), 7.05 (d, *J* = 7.7 Hz, 1H), 3.54 (s, 2H), 3.42 (br, 4H), 2.40 (br, 4H), 1.43 (s, 9H); MS (ESI) *m/z*: $[M + H]^+$ 397; m.p. 157 – 159 °C.

*5.2.33. tert-Butyl 4-(4-(3-(2-((methylsulfonyl)oxy)ethoxy)benzoyl)benzyl)piperazine-1-carboxylate (**34**)*

To a solution of **33** (0.10 g, 0.3 mmol) in dry DMF (5 mL) NaH (0.012 g, 0.5 mmol) was added. The reaction was heated at 80 °C and a solution of ethylene glycol dimesylate (0.065 g, 0.3 mmol) in dry DMF (2 mL) was added. The mixture was stirred at 80 °C for 2 h, then DMF was evaporated and crude was treated with water. The residue was extracted with DCM and the combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. Crude compound was purified by chromatography on silica gel (2% MeOH in DCM). **34** (0.041 g, 27%) was obtained as a pale-yellow oil. ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.1 Hz, 2H), 7.43 (d, *J* = 8.0 Hz, 2H), 7.36 (t, *J* = 9.6 Hz, 3H), 7.12 (d, *J* = 7.2 Hz, 1H), 4.57 (t, *J* = 4.0 Hz, 2H), 4.29 (t, *J* = 4.0 Hz, 2H), 3.56 (s, 2H), 3.42 (br, 4H), 3.05 (s, 3H), 2.39 (br, 4H), 1.44 (s, 9H); MS (ESI) *m/z*: 519 $[M + H]^+$.

*5.2.34. Di-tert-butyl 4,4'-(((((ethane-1,2-diylbis(oxy))bis(3,1-phenylene))bis(hydroxymethylene))-bis(4,1-phenylene))bis(methylene))bis(piperazine-1-carboxylate) (**35**)*

Step d: To a solution of **33** (0.031 g, 0.1 mmol) in dry DMF (2 mL) NaH (0.003 g, 0.1 mmol) was added. The mixture was stirred at 80 °C and **34** (0.034 g, 0.1 mmol) was added; the reaction was stirred at 80 °C for 2 h. Then DMF was evaporated, water was added to the residue and mixture was extracted with DCM. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. Crude compound was purified by chromatography on silica gel (2% MeOH in DCM) obtaining *di-tert-butyl 4,4'-(((3,3'-(ethane-1,2-diylbis(oxy))bis(benzoyl))bis(4,1-phenylene))bis(methylene))bis(piperazine-1-carboxylate* (0.038 g, 71%) as a pale yellow oil. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.9 Hz, 4H), 7.50 – 7.31 (m, 9H), 7.23 – 7.09 (m, 3H), 4.39 (s, 4H), 3.58 (s, 4H), 3.44 (br, 8H), 2.41 (br, 8H), 1.45 (s, 18H); MS (ESI) *m/z*: 819 $[M + H]^+$.

Step e: The above obtained compound (0.098 g, 0.13 mmol) was dissolved in THF (5 mL), cooled at 0 °C, then $NaBH_4$ (0.012 g, 0.33 mmol) was added. The reaction was stirred at 25 °C for 12 h then water was added, and the mixture was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. **35** (0.10 g, quantitative yield) was obtained as a colorless oil and used in the next step without any further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.13 (m, 10H), 7.02 – 6.87 (m, 4H), 6.79 (dd, *J* = 8.3, 2.6 Hz, 2H), 5.74 (s, 2H), 4.24 (s, 4H), 3.43 (s, 4H), 3.35 (t, *J* = 5.0 Hz, 8H), 2.82 (s, 2H), 2.31 (t, *J* = 5.0 Hz, 8H), 1.42 (s, 18H); MS (ESI) *m/z*: 823 $[M + H]^+$.

*5.2.35. N,N'-(((Ethane-1,2-diylbis(oxy))bis(3,1-phenylene))bis((4-(piperazin-1-ylmethyl)phenyl)-methylene))bis(7-chloroquinolin-4-amine) (**5m**)*

Step f: Compound *di-tert-butyl 4,4'-(((((ethane-1,2-diylbis(oxy))bis(3,1-phenylene))-bis(chloro-methylene))bis(4,1-phenylene))bis(methylene))bis(piperazine-1-carboxylate)* (0.103 g, quantitative yield) was obtained as a brown oil, starting from **35** and following the procedure used before to obtain compound **6a**. It was used in the next step without any further purification. ^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.41 – 7.18 (m, 10H), 7.09 – 6.96 (m, 4H), 6.86 (dd, *J* = 7.9, 2.1 Hz, 2H), 6.07 (s, 2H), 4.29 (s, 4H), 3.47 (s, 4H), 3.41 (br, 8H), 2.37 (br, 8H), 1.46 (s, 18H); MS (ESI) *m/z*: 859 $[M + H]^+$.

Step g: Compound *di-tert-butyl 4,4'-(((((ethane-1,2-diylbis(oxy))bis(3,1-phenylene))bis(((7-chloroquinolin-4-yl)amino)methylene))bis(4,1-phenylene))bis(methylene))bis(piperazine-1-carboxylate)* was obtained starting from the above-obtained compound (0.075 g, 0.1 mmol) following the procedure used for compound **5a**. Crude was purified by chromatography obtaining the desired compound as a pale yellow oil (0.017 g, 17%). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.48 – 8.38 (m, 2H), 7.97 (s, 2H), 7.72 (d, *J* = 9.1 Hz, 2H), 7.40 – 7.24 (m, 12H), 7.04 – 6.90 (m, 4H), 6.84 (d, *J* = 8.1 Hz, 2H), 6.25 (d, *J* = 5.4 Hz, 2H), 5.67 (s, 2H), 5.49 (s, 2H), 4.23 (s, 4H), 3.49 (s, 4H), 3.42 (br, 8H), 2.38 (br, 8H), 1.45 (s, 18H); MS (ESI) *m/z*: 572 $[M + 2H]^{2+}$.

Step h: Compound **5m** was obtained starting from the above-obtained compound (0.017 g, 0.02 mmol) following the same procedure used to obtain **5e**. Crude was purified by HPLC equipped with a UV-VIS detector (Diode Array SPD-M20A) and the column used was Chromolith® Semiprep RP-18e (100-10 mm). All runs were performed in gradient mode (flow rate = 2.5 mL/min; gradient, 80/20–5/95 A/B in 10 min; isocratic 5/95 A/B 5 min) at 25 °C with detection at 254 nM. Sample solutions were prepared (approximately 2 mg/mL) by dissolving the analytes in the eluent and filtering through 0.45 µM Acrodisc filters. The injection volume was 50 µL and RT of **5e** was 5.94 min. **5e** (0.006 g, 50%) was obtained as a pale-yellow oil.

^{1}H NMR: (300 MHz, $CDCl_3$) δ 8.41 (d, *J* = 3.1 Hz, 2H), 8.09 (d, *J* = 5.1 Hz, 1H), 7.96 (d, *J* = 1.8 Hz, 1H), 7.71 (d, *J* = 9.0 Hz, 2H), 7.43 – 7.22 (m, 14H), 7.04 – 6.71 (m, 6H), 6.25 (d, *J* = 5.3 Hz, 2H), 5.66 (s, 2H), 5.45 (s, 2H), 4.23 (s, 4H), 3.47 (s, 4H), 2.87 (br, 8H), 2.41 (br, 8H); MS (ESI) *m/z*: 472 $[M + 2H]^{2+}$.

5.3. Expression of PfCRT in Xenopus oocytes and measurements of CQ transport.

Ethical approval of the work performed with the *Xenopus laevis* frogs was obtained from the Australian National University Animal Experimentation Ethics Committee (Animal Ethics Protocol Number A2013/13) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Sections of ovary were surgically removed from adult female frogs (purchased from NASCO) using a procedure outlined in detail elsewhere [46]. Single oocytes were obtained by incubating the ovary sections with collagenase in accordance with a protocol described previously [46] and with the following minor modification: the ovary tissue (weighing 7-14 g) was transferred to a 100 mL Erlenmeyer flask and incubated in the dark in OR^{2-} buffer supplemented with 0.5 M Na_2HPO_4 (Sigma-Aldrich), bovine serum albumin (250 mg/mL, Sigma-Aldrich), 5-10 µg/mL of collagenase (of which 60% was collagenase A and 40% collagenase D; Roche), and 40-100 µg/mL of soybean trypsin inhibitor (type II-S; Sigma-Aldrich). Healthy, de-folliculated oocytes of a suitable size and age (developmental stages V and VI) were selected for microinjection. cRNA encoding $PfCRT^{Dd2}$ was transcribed *in vitro* and microinjected (20 ng per oocyte) into the oocytes as outlined elsewhere [28,46]. The oocytes were stored in the dark at 16-18 °C in OR^{2+} buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, 1 mM $CaCl_2$, and 50 µg/mL gentamicin; pH 7.8), which was replaced daily. The uptake of [^{3}H]CQ (0.25 µM; 20 Ci/mmol; American Radiolabeled Chemicals) was measured 3-4 days post-injection using a MicroBeta^2 microplate liquid scintillation analyzer (PerkinElmer) and a protocol described in detail previously [28]. Briefly, the [^{3}H]CQ influx assays were performed over 1.5 h at 27.5 °C and in a medium that contained 96 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM MES, 10 mM Tris base (pH 5.5), and 15 µM unlabeled CQ. In all cases, at least three independent experiments were performed (on oocytes from different frogs), and in each experiment measurements were made from 10 oocytes per treatment. All errors cited in the text and shown in the figures represent the s.e.m. Statistical comparisons were made using one-way ANOVAs in conjunction with Tukey's multiple comparisons test.

5.4. Transmission blocking assay

5.4.1 Murine malaria model

Plasmodium berghei ANKA GFP-con strain, expressing a green fluorescent protein (GFP) at all life cycle stages was employed in the *in vivo* Direct Feeding Assay (DFA). BALB/c mice were used as vertebrate hosts and *An. stephensi* mosquitoes as experimental vectors.

5.4.2. Mice

Eight to ten-week-old BALB/c mice, weighing 18 to 25 g were used in this study. The mice were reared in the animal house (24 °C, 14 h light/10 h dark cycle and 70% relative humidity) of the University of Camerino and were fed *ad-libitum* with standard mouse food (Mucedola s.r.l., Milano, Italy) and tap water.

5.4.3. Mosquitoes

Anopheles stephensi mosquitoes were maintained at a temperature of 30 ˚C, 12 h light/12 h dark cycle and 75 to 85% relative humidity in the insectary of the University of Camerino. Larvae were raised in the same facility in trays filled with well water and fed with grinded mouse food. Four to five-day old female mosquitoes were used in the experimentations. Mosquito cages were transferred to a 19 ˚C chamber 24 h prior to the experimental blood-meals to allow sporogonic development to be completed which in the case of *P. berghei* requires a temperature between 18 and 20 °C.

5.5. Direct Feeding Assay (DFA)

The transmission blocking activity of the compounds was evaluated in a double *in vivo* assay including both, the mouse, and the mosquito host. The effects of the compounds on gametocytes in the mouse and on early sporogonic development to oocyst formation in mosquitoes was tested as described in detail before [47].

Experimental mice received a standardized i.p. infection with 10^7 *P. berghei* GFP-con infected red blood cells. In the morning of day 3 after mouse infection parasitemia and gametocytemia was determined on Giemsa stained thin films. Mice showing a parasitemia of about 5% (3 – 10%) with the presence of micro- and macrogametocytes were selected for the DFA and allocated randomly to the four treatment groups (3 mice per group), namely PQ derivative **5i** and **5j**, PBS solvent control and PQ as positive reference. In the afternoon of day 3 post infection, i.e. at the time point of *P. berghei* gametocyte maturation, a first treatment was carried out by administering (i.p.) compounds **5i**, **5j** and PQ at a dosage of 10 µmol/kg to the treatment mice and giving plain PBS to controls. On day four post-infection, the treatment was repeated 1.5 h before the exposure of mice to mosquitoes. Experimental animals were anaesthetized and kept on top of cages containing approximately 150 female *A. stephensi* mosquitoes for 1 h. Unfed females were removed the following day. On day 8 after blood-meal, 20 - 30 mosquitoes (minimum 8) from each cage were dissected and oocyst densities assessed by counting the number of fluorescent oocysts on mosquito mid-guts at the fluorescent microscope (Zeiss Axio observer Z1, 60 – 100 x magnification) (Figure S2). Experimental

treatments were affected in triplicates, using for each treatment or control group 3 mice for the infection of 3 mosquito cages. For each mosquito cage, the geometric mean number of oocysts and the standard deviation was assessed on positive mosquitoes and the arithmetic mean calculated for each treatment using the geometric means of the 3 replicates. Differences in mean oocyst numbers between each of the 2 PQ derivative treatment groups and the solvent control were evaluated by t-test (two-tailed).

5.6. Liver-stage analysis

6-12 weeks of age BALB/cJRj (Janvier-laboratories) mice were infected by i.p. injection of PbmCherry$_{hsp70}$ blood stabilities [48]. At around 2% parasitemia (~ day 3) blood was passaged i.v. into naïve mice. When these mice reached parasitemia of about 5% (around day 3) thin blood smears were stained using Wright stain and gametocyte development was examined. Mice necessary to infect mosquitos were anesthetized with a mixture of 50 mg/kg Ketamine and 10 mg/kg

Xylazine in PBS by i.p., route. After reaching deep anesthesia they were placed on a mosquito cage for 30 minutes where mosquitos (female *Anopheles stephensi*) could take a blood meal. Mosquitos were kept at 20.5 °C and 80% humidity (12 h/12 h light-dark cycle). Between 18- and 26-days post feed, salivary glands of *Plasmodium berghei* infected mosquitos were dissected and used to infect HepG2 cells.

Confluent HepG2 cell cultures (60000 cells/96 well seeded the day before) were infected with 20'000 *P. berghei* sporozoites each (PbmCherry$_{hsp70}$,) for 2 h. In total 6 replicates with 3 wells each were infected. The cells of each replicate were detached by the use of accutase (Innovative Cell Technologies) pooled and distributed to 3 times 8 wells containing either primaquine, **5i** or **5j**, each at different concentrations plus control (DMSO same concentration as in highest drug concentration). Media were changed at 24 hpi, and the cells were fixed with 4% PFA/PBS at 48 hpi. Host cell growth was monitored by phase contrast microscopy to evaluate growth inhibiting drug concentrations. Parasite numbers and sizes were determined by automated microscopy (IN Cell analyzer, GE life sciences) monitoring the mCherry signal of PbmCherry$_{hsp70}$ parasites [49]. Statistical evaluations (One-way ANOVA with Dunnett's multiple comparisons) were done using Prism 8 (GraphPad).

5.7 Cytotoxicity assay on NIH3T3 cell line

Cells (5 x 10^4) suspended in 1 mL of complete medium were seeded in each well of a 24 well round multidish and incubated at 37 °C in an atmosphere of 5% CO_2. After 24 hours of culture, the culture medium was discharged and test samples, solubilized in DMSO, were added to each well at different concentration values. For the evaluation of cytotoxicity, three experiments repeated in six replicates were performed and all compounds were tested at increasing concentrations ranging from 8 to 160 µM. The samples were set up in six replicates for each tested concentration. Complete medium was used as negative control. Cell viability and proliferation was evaluated by Neutral Red uptake after 24 hours of incubation with NIH3T3 as follows.

First, the following solutions were prepared in order to determine the percentage of viable cells:

1. Neutral Red (NR) stock solution: 0.33 g NR dye powder in 100 ml sterile H_2O

2. NR medium: 1.0 mL NR stock solution + 99.0 routine culture medium pre-warmed to 37 °C

3. NR desorb solution: 1% glacial acetic acid solution + 50% ethanol + 49% H_2O

At the end of incubation, the routine culture medium was removed from each plate and the cells were carefully rinsed with 1 ml pre-warmed D-PBS 0.1 M. Plates were then gently blotted with paper towels. 1.0 ml NR medium was added to each dish and further incubated at 37 °C, 95% humidity, 5.0% CO_2 for 3 hours. The cells were checked during incubation for NR crystal formation. After incubation, the NR medium was removed, and the cells were carefully rinsed with 1 mL pre-warmed D-PBS 0.1 M. PBS was decanted and blotted from the dishes and exactly 1 mL NR desorb solution was added to each sample. Plates were placed on a shaker for 20-45 minutes to extract NR from the cells and form a homogeneous solution. During this step the samples were covered to protect them from light. Five minutes after removal from the shaker, absorbance was read at 540 nm with a UV/visible spectrophotometer (Varian Cary 1E).

Author contributions

The manuscript was written through contribution of all authors. All authors have given approval to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at xxxx

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