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Optimization of 4-Aminoquinoline/Clotrimazole-Based Hybrid Antimalarials: Further Structure–Activity Relationships, in Vivo Studies, and Preliminary Toxicity Profiling

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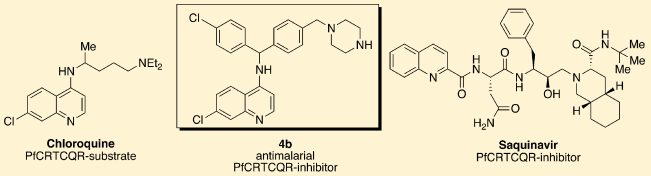
Supporting Information

ABSTRACT: Despite recent progress in the fight against malaria, the emergence and spread of drug-resistant parasites remains a serious obstacle to the treatment of infections. We recently reported the development of a novel antimalarial drug that combines the 4-aminoquinoline pharmacophore of chloroquine with that of clotrimazole-based antimalarials. Here we describe the optimization of this class of hybrid drug through in-depth structure–activity relationship studies. Antiplasmodial properties and mode of action were characterized in vitro and in vivo, and interactions with the parasite's 'chloroquine resistance transporter' were investigated in a *Xenopus laevis* oocyte expression system. These tests indicated that piperazine derivatives **4b** and **4d** may be suitable for coadministration with chloroquine against chloroquine-resistant parasites. The potential for metabolism of the drugs by cytochrome P450 was determined in silico, and the lead compounds were tested for toxicity and mutagenicity. A preliminary pharmacokinetic analysis undertaken in mice indicated that compound **4b** has an optimal half-life.



INTRODUCTION

Malaria morbidity and mortality have decreased in most malaria endemic countries over the past decade.[1] A key factor contributing to this reduction in the malaria burden has been the deployment of artemisinin-based combination therapies (ACTs) against increasingly drug-resistant populations of the parasite. However, the efficacy of these peroxide-based antimalarials is now threatened by the emergence in Southeast Asia of parasites with reduced susceptibility to artemisinin.[2−4] The development and spread of artemisinin-resistant parasites would be disastrous, since no drugs are available to replace the ACTs as the frontline antimalarial treatment.[5] For these reasons, a number of novel antimalarial strategies are currently being explored, including combination therapies that are based on agents which reverse resistance to chloroquine (CQ, **1**, Chart 1).[6−8]

During the intraerythrocytic stage of its life cycle, the parasite takes up host hemoglobin into a specialized acidic organelle called the digestive vacuole (DV). Proteolysis of the hemoglobin results in the release of toxic heme monomers, which are then converted into the inert crystal hemozoin.[9−16] CQ is thought to exert its antimalarial effect by inhibiting this biocrystallization

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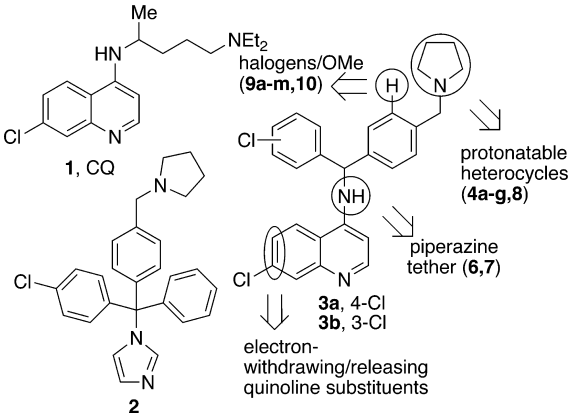
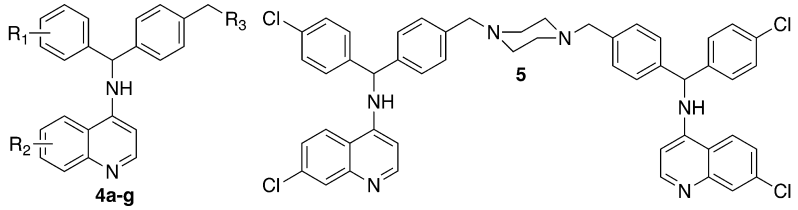
Chart 1. Structures of Chloroquine (1), CLT-Based Antimalarials (2), Hybrid Derivatives (3a,b), and Site of Modifications to the Title Compounds (4a−g, 5−8, 9a−m, 10)



process, resulting in the build up of monomeric heme.[9,14−16] This mode of action is highly effective, and because the target cannot be mutated, the development of resistance is not a feat easily achieved by the parasite. The molecular determinants of CQ resistance have been identified and the mechanism of resistance characterized.[7,17] CQ-resistant (CQR) parasites accumulate much less CQ in their DV than CQ-sensitive (CQS) parasites, and there is now a general consensus that this decrease in CQ accumulation is due to the efflux of the drug via mutant forms of the *Plasmodium falciparum* 'chloroquine resistance transporter' (PfCRT; located on the DV membrane).[18,19] The wild-type form of this protein does not transport CQ.[17,18] Compounds that compete with CQ transport via mutant PfCRT, or that block PfCRT-mediated transport, partially restore the parasite's sensitivity to CQ in vitro. This phenomenon of "CQ resistance reversal" provides a rational basis for the study and implementation of CQ-based combination therapies for the treatment of drug-resistant malaria.[20−22]

Table 1. Ionic Forms and Antiplasmodial Activity of Compounds 4a−g, 5, 3a,b, and CQ (1)



compd	R_1	R_2	R_3	% Ionic forms[a] pH 7.4	% Ionic forms[a] pH 5.5	D10[b] IC_{50} (nM)[f]	W2[c] IC_{50} (nM)[f]	RI[d]	NF54[b] IC_{50} (nM)[f]	K1[c] IC_{50} (nM)[f]	RI[e]
4a	4-Cl	7-Cl		DP 1 P 20 N 79	DP 70 P 28 N 2	30	57	1.9	18	22	1.2
4b	4-Cl	7-Cl		DP 4 P 94 N 2	DP 75 P 25 N 0	62	58	0.93	17	22	1.3
4c	4-Cl	6-OMe		DP 59 P 39 N 1	DP 99 P 1 N 0	67	163	2.4	21	58	2.8
4d	3-Cl	7-Cl		DP 3 P 94 N 3	DP 74 P 26 N 0	25	33	1.4	19	23	1.2
4e	4-Cl	7-Cl		DP 2 P 64 N 34	DP 74 P 26 N 0	21	42	2.0	nt	nt	-
4f	4-Cl	7-Cl		DP 2 P 36 N 62	DP 72 P 27 N 1	163	790	4.8	nt	nt	-
4g	4-Cl	7-Cl		nc	nc	93	33	0.35	nt	nt	-
5	-	-	-	TP 0 DP 2 P 31 N 67	TP 53 DP 39 P 8 N 0	190	98	0.5	89	56	0.6
3a	-	-	-	DP 3 P 96 N 1	DP 73 P 27 N 0	46	72	1.5	13	14	1.1
3b	-	-	-	DP 3 P 96 N 1	DP 73 P 27 N 0	21	22	1.0	12	16	1.3
1	-	-	-	DP 8 P 92	DP 87 P 12	22	280	12	7.0	154	22

[a]Percentage of ionic form in parentheses. DP = diprotonated form. P = monoprotonated form. N = neutral form (ACD/p*K*a DB, version 11.00, software, Advanced Chemistry Development, Inc., Toronto, Canada). [b]CQS clone. [c]CQR clone. [d]Resistance index = W2-IC_{50}/D10-IC_{50}. [e]Resistance index = K1-IC_{50}/NF54-IC_{50}. [f]IC_{50} values are the mean of at least three determinations. Standard errors were all within 10% of the mean.

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Table 2. Antiplasmodial Activity of 6−8 and CQ (1)

	IC_{50} (nM)[c]			
compd	D10[a]	W2[b]	NF54[a]	K1[b]
6	631	735	88	156
7	277	582	91	92
8	104	58	83	10
1	22	280	7.0	154

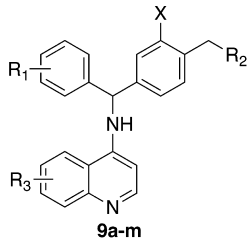
[a]CQS clone. [b]CQR clone. [c]IC_{50} values are the mean of at least three determinations. Standard errors were all within 10% of the mean.

Our approach to the development of novel antimalarials has been to design compounds that interact with the iron center of free heme within the acidic environment of the DV (pH ~5.5) and which produce putative radical species that are selectively toxic to the parasite.[23−28] A further requirement is the ability to accumulate within the DV via weak-base trapping (the mechanism by which CQ and other quinoline antimalarials reach high concentrations inside this compartment). Following this rationale, we previously developed a novel class of antimalarials based on the structure of clotrimazole (CLT), typified by the trityl derivative **2** (Chart 1).[27,28] This class of CLT-based antimalarial was further optimized using a pharmacophore hybridization approach in which the polyarylmethyl system of **2** was hybridized with the 4-aminoquinoline iron-complexing moiety of CQ, leading to **3a,b** as the representative compounds of this class.[26] The in vitro and in vivo antimalarial activities of **3a,b** as well as their mode of action, toxicity, and preliminary pharmacokinetic properties have been evaluated with promising results.[26] Here we extend our previous findings by characterizing this novel class of drug in greater detail. A set of **3a,b** analogues were synthesized by modifying the protonatable heterocycles at the benzhydryl moiety (**4a−g**, **5**, **8**, Chart 1 and Tables 1,2) and the secondary amine tether (**6**, **7**, Table 2) and by introducing electron-withdrawing or -releasing groups at the benzhydryl aromatic ring (in ortho position relative to the protonatable side chain; **9a−m**, **10**, Table 3). The antiplasmodial activities of the resulting compounds were measured in both CQS and CQR parasites. The most promising compounds were then evaluated in a mouse malaria model and tested for the ability to inhibit PfCRT-mediated CQ transport in a heterologous expression system. The toxicity profile of the lead compounds was explored and a preliminary pharmacokinetic evaluation of **4b** undertaken.

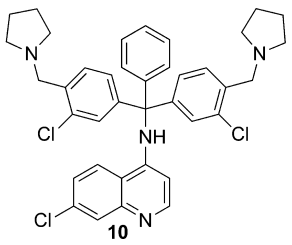
CHEMISTRY

The synthesis of compounds **4a−g**, bearing different protonatable heterocycles at the benzhydryl system is reported in Scheme 1.

Table 3. Ionic Forms and Antiplasmodial Activity of Compounds 9a−m, 10, 3a,b, and CQ (1)



9a-m



10

compd	R_1	R_2	R_3	X	% Ionic forms[a] pH 7.4	% Ionic forms[a] pH 5.5	D10[b]	W2[c]	NF54[b]	K1[c]
								IC_{50} (nM)[d]		
9a	3-Cl		7-Cl	Cl	DP 3 P 96 N 1	DP 69 P 31 N 0	57	50	13	19
9b	3-Cl		7-Cl	F	DP 3 P 96 N 1	DP 69 P 31 N 0	9.0	25	nt	nt
9c	3-Cl		7-Cl	Cl	DP 0 P 7 N 93	DP 54 P 40 N 6	442	277	47	107
9d	3-Cl		6-OMe	Cl	DP 53 P 46 N 1	DP 99 P 1 N 0	56	90	27	34
9e	3-Cl		6-OMe	F	DP 53 P 46 N 1	DP 99 P 1 N 0	41	101	nt	nt
9f	3-Cl		6-OMe	Cl	DP 2 P 55 N 43	DP 77 P 23 N 0	198	229	81	114
9g	3-Cl		7-CF_3	Cl	DP 0 P 98 N 2	DP 26 P 74 N 0	74	111	34	46
9h	4-Cl		7-Cl	Cl	DP 3 P 96 N 1	DP 70 P 30 N 0	8.4	28	nt	nt
9i	4-Cl		7-Cl	F	DP 3 P 96 N 1	DP 70 P 30 N 0	59	45	13	20
9j	4-Cl		6-OMe	Cl	DP 54 P 45 N 1	DP 99 P 1 N 0	24	76	nt	nt
9k	4-Cl		6-OMe	F	DP 54 P 45 N 1	DP 99 P 1 N 0	58	69	25	33
9l	4-Cl		7-CF_3	F	DP 0 P 98 N 2	DP 99 P 1 N 0	51	76	20	33
9m	4-Cl		7-Cl	OMe	DP 3 P 96 N 1	DP 73 P 26 N 0	31	48	nt	nt
3a	-	-	-	-	DP 3 P 96 N 1	DP 73 P 27 N 0	46	72	13	14
3b	-	-	-	-	DP 3 P 96 N 1	DP 73 P 27 N 0	21	22	12	16
10	-	-	-	-	TP 1 DP 96 P 3	TP 47 DP 53 P 0	26	52	9.5	8.4
1	-	-	-				22	280	7.0	154

[a]Percentage of ionic form in parentheses. DP = diprotonated form. P = protonated form. N = neutral form (ACD/p*K*a DB, version 11.00, software, Advanced Chemistry Development, Inc., Toronto, Canada). [b]CQS clone. [c]CQR clone. [d]IC_{50} values are the mean of at least three determinations. Standard errors were all within 10% of the mean.

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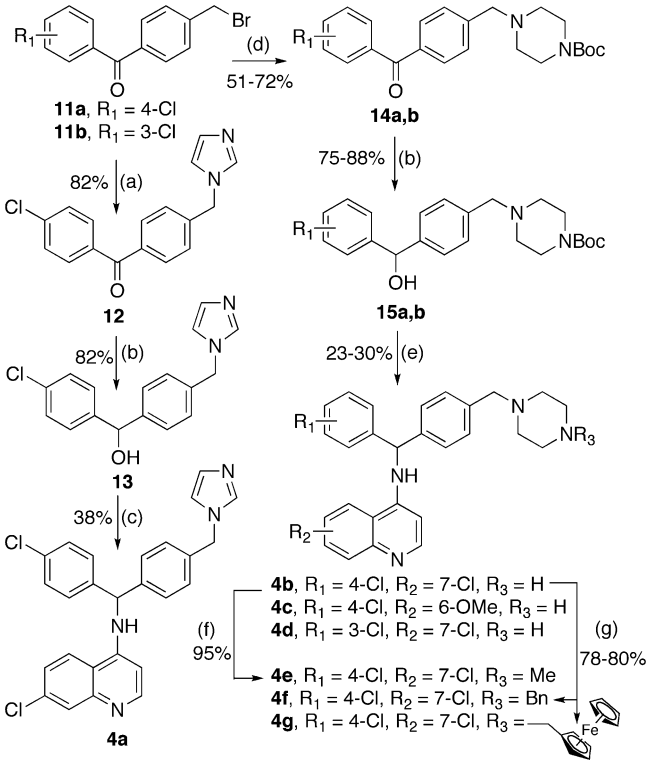
Scheme 1. Synthesis of Compounds 4a−g[a]



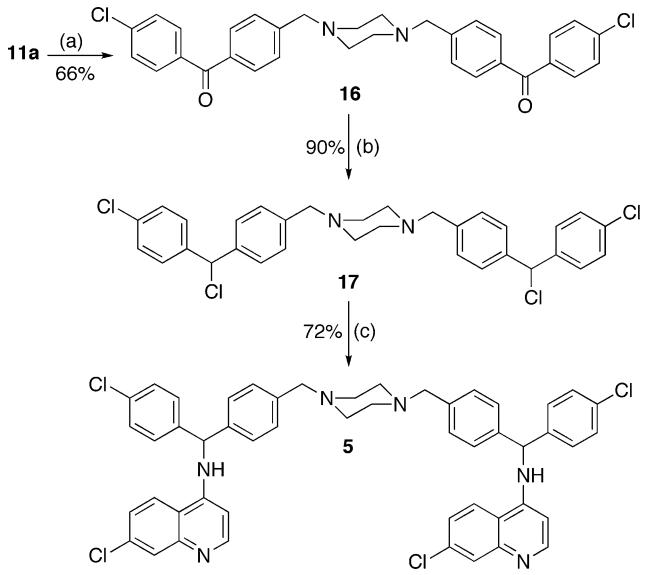
[a]Reagents and conditions: (a) NaH, imidazole, *N,N*-DMF, 25 °C, 1 h, then **11a**, 25 °C, 1 h; (b) $NaBH_4$, EtOH, 0 °C, 1 h; (c) i. $SOCl_2$, CH_2Cl_2, 0 °C 20 min, then 45 °C, 4 h; ii. 7-chloro-4-aminoquinoline, Et_3N, MeCN, 80 °C, 6 h; (d) *N*-(*tert*-butoxycarbonyl)piperazine, Et_3N, 0 °C, 1 h; (e) i. $SOCl_2$, CH_2Cl_2, 0 °C, 20 min, then 45 °C, 4 h; ii. 7-chloro-4-aminoquinoline or 6-methoxy-4-aminoquinoline, Et_3N, MeCN, 80 °C, 6 h; iii. AcCl, MeOH, 25 °C 2 h; (f) from **4b**, CH_2O 37% in water, 1% AcOH, EtOH, 25 °C, 6 h; (g) from **4b**, benzaldehyde (for **4f**) or ferrocenyl aldehyde (for **4g**), 1% AcOH, EtOH, 25 °C, 6 h.

Benzyl bromides **11a,b**, prepared as previously described,[27,28] were used to alkylate imidazole (pretreated with sodium hydride) to afford **12**, or *N*-Boc-piperazine to afford intermediates **14a,b**. Reduction of **12** and **14a,b** with sodium borohydride was followed by activation of the resulting alcohols **13** and **15a,b** into the corresponding chlorides. These latter compounds were reacted with the appropriate 4- aminoquinoline intermediates to furnish **4a** and, after deprotection with hydrochloric acid in methanol, **4b−d**. Compound **4b** was submitted to a reductive amination protocol using formaldehyde, benzaldehyde, or ferrocenyl aldehyde to afford *N*-alkylpiperazine derivatives **4e,f,g**, respectively.

N,N′-Disubstituted piperazino derivative **5** was prepared as described in Scheme 2. The benzyl bromide **11a** was treated with piperazine to afford dimeric benzophenone intermediate **16**. This latter compound was converted into the corresponding chloride **17** through reduction/chlorination reactions and, following the procedure described in Scheme 1, reacted with 7-chloro-4-aminoquinoline to furnish the final compound **5**.

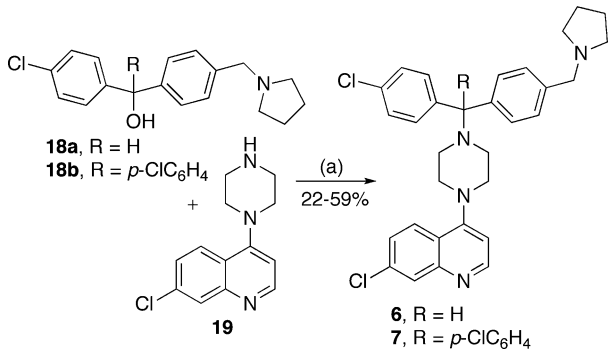
Compounds **6** and **7**, which possess a piperazine tether, were prepared as reported in Scheme 3. Carbinols **18a,b**[26,27] were converted into their corresponding chlorides and immediately reacted with 4-piperazino-7-chloroquinoline **19**,[29] in the presence of triethylamine in acetonitrile heated under reflux to afford the final compounds **6** and **7**.

Scheme 2. Synthesis of Dimeric Derivative 5[a]



[a]Reagents and conditions: (a) piperazine, Et_3N, 0 °C, 1 h; (b) i. $NaBH_4$, EtOH, 0 °C, 1 h; ii. $SOCl_2$, CH_2Cl_2, 0 °C, 20 min, then 45 °C, 4 h; (c) 7-chloro-4-aminoquinoline, Et_3N, MeCN, 80 °C, 6 h.

Scheme 3. Synthesis of Compounds 6 and 7[a]



[a]Reagents and conditions: (a) i. $SOCl_2$, CH_2Cl_2, 0 °C, 20 min, then **19** 45 °C, 4 h; ii. Et_3N, MeCN, 80 °C, 6 h.

The key intermediates for the synthesis of **8** and **9a−m** (Scheme 4) were benzhydrylamines **24a−g**, which were obtained following a modification of the previously reported methodology. Reaction of Grignard's reagents obtained from aryl bromides **21a,b** with excess aldehydes **20a,b** afforded benzophenones **22a−d**. Benzophenone **22e** was obtained by reacting *p*-chlorophenylmagnesium bromide with aldehyde **26,** in turn obtained from the corresponding ester **25** through standard procedures. Benzophenones **22a−e** were functionalized through a radical-promoted bromination followed by alkylation of pyrrolidine, morpholine, or *N*-Boc-piperazine furnishing intermediates **23a−g**. Ketones **23a,b,d−g**, containing pyrrolidine or morpholine basic moieties, were converted into the corresponding amines **24a,b,d−g** through conversion to oximes followed by reduction with zinc dust. *N*-Boc-piperazine derivative **23c** was converted to **24c** by treatment with ammonia/Ti(IV) isopropoxide followed by $NaBH_4$-mediated reduction.

Reaction of benzhydrylamines **24a,b,d−g** with the appropriate 4-chloroquinolines in ethoxyethanol in the presence of pyridinium chloride resulted in the formation of final compounds **9a−m**. Under the same reaction conditions, **24c** gave rise to

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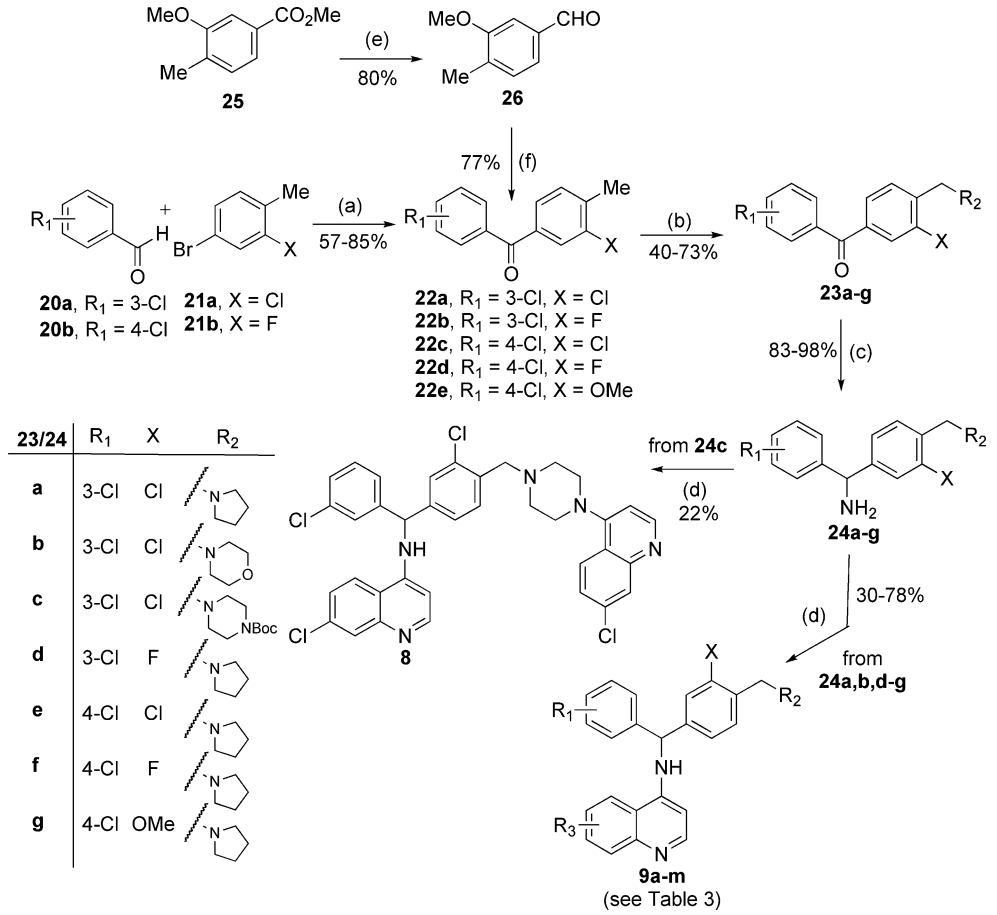
Scheme 4. Synthesis of Compounds 8 and 9a−m[a]

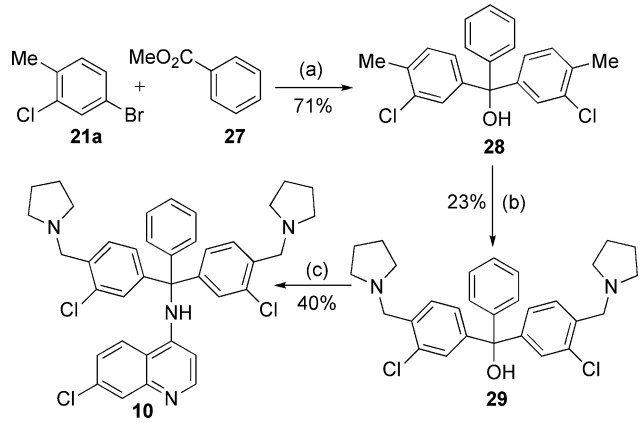


[a]Reagents and conditions: (a) i. **21a,b**, Mg turnings, THF, 80 °C, 1 h, then **20a,b**, 80 °C, 4 h; (b) i. NBS, AIBN, CCl_4, reflux, 4 h; ii. amine, Et_3N, MeCN, 0 °C, 1 h; (c) from **23a,b,d−g**: i. NH_2OH·HCl, $BaCO_3$, MeOH, reflux, 18 h; ii. Zn, AcOH, 50 °C, 18 h; from **23c**: 7 M NH_3 in MeOH, Ti(O-*i*-Pr)$_4$, EtOH, 25 °C, 6 h; then $NaBH_4$, 25 °C, 3 h; (d) 4-chloroquinolines, pyridinium chloride, 2-ethoxyethanol, 135 °C, 18 h; (e) i. $LiAlH_4$, THF, 25 °C, 1 h; ii. MnO_2, THF, 85 °C, 2 h; (f) *p*-chlorophenylmagnesium bromide, THF, 85 °C, 6 h.

compound **8** as the sole reaction product, probably resulting from the in situ deprotection of the Boc-piperazine moiety.

Finally, trityl derivative **10** was prepared as described in Scheme 5. Reaction of the Grignard reagent prepared in situ from aryl bromide **21a** was reacted with benzoic acid methyl ester **27** to afford carbinol **28**. The carbinol was submitted to a bromination reaction in the presence of *N*-bromosuccinimide (NBS) and a catalytic amount of AIBN to afford the corresponding bis-benzyl bromide, which was in turn reacted with pyrrolidine. The resulting alcohol **29** was converted into the corresponding chloride by exposure to thionyl chloride and reacted with 7-chloro-4-aminoquinoline to afford compound **10**.

Scheme 5. Synthesis of Compound 10[a]



[a]Reagents and conditions: (a) **21a**, Mg, THF, 25 °C, 2 h, then **27**, THF, 25 °C, 18 h; (b) i. NBS, AIBN, CCl_4, 85 °C, 2 h; ii. pyrrolidine, Et_3N, MeCN, 0 to 25 °C, 3 h; (c) i. $SOCl_2$, CH_2Cl_2, 0 °C, 20 min, then 45 °C, 4 h; ii. 7-chloro-4-aminoquinoline, Et_3N, MeCN, reflux, 6 h.

RESULTS AND DISCUSSION

1. In Vitro Antiplasmodial Activity and Structure−Activity Relationships (SARs). All of the synthesized compounds were tested in vitro as racemates against the *P. falciparum* CQS strains D10 and NF54, and the CQR strains W2 and K1. Antimalarial activity (IC_{50}, nM) was quantified as inhibition of parasite growth, measured by the production of parasite lactate dehydrogenase (pLDH; D10 and W2 strains) or the incorporation of [^{3}H]hypoxanthine into parasite DNA (NF54 and K1 strains). The results are presented in Tables 1−3.

In our previous studies[26] we hypothesized that within the acidic environment of the DV, the quinoline endocyclic nitrogen of compounds such as **3a,b** coordinates the iron of free heme, eventually leading to an electron-transfer reaction to form putative radical intermediates. Consistent with this view, we previously observed that the electron density, the quinoline

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endocyclic nitrogen, and the dipole moment of the quinoline ring system all appeared to affect the heme-quinoline interaction/reactivity.[26] We also found that the protonatability of the molecule contributes significantly to its ability to kill the parasite.[25−28] CQ and other weak bases diffuse into the DV in their neutral form, whereupon they become protonated in the acidic environment, and these charged species, unable to cross the membrane unassisted, accumulate to high levels within the DV. With this putative mode of action in mind, and in light of our previous findings, the SARs of the analogues described here focused on the effect of structural modifications on the protonatability of the molecule, and on the electron-distribution of the benzhydryl and quinoline systems.

a. Modification of the Protonatable Side Chain at the Benzhydryl System. The antiplasmodial activities of a series of compounds (**4a**−**g**) with different protonatable heterocycles at the benzhydryl system are presented in Table 1. We used heterocycles such as imidazole and piperazines because these moieties are protonatable at pH 5.5 and are able to coordinate metals such as iron (a property that was previously observed to increase the activity of our molecules against CQR strains[25,30]). When the protonatable heterocycle at the benzhydryl system was coupled to the 7-chloro-4-aminoquinoline moiety (**4a**,**b**,**d**−**f**), the resulting compounds were predicted to share similar protonation states at both DV and blood pH values (Table 1). However, subtle differences in their antiplasmodial activities were observed. The imidazole derivative **4a** and its pyrrolidine analogue **3a** both appeared to exhibit low-level cross-resistance with CQ, particularly when the D10 and W2 strains were compared; **4a** and **3a** have resistance index (RI) values of ∼1.9 and 1.6, respectively. The RI is the IC_{50} value in the CQR strain divided by the IC_{50} value in the CQS strain. Hence, a RI close to one indicates that the antiplasmodial activity of the compound is comparable between the CQS and CQR strains. The piperazine analogue **4b** was slightly less active than **3a** against the D10 and W2 strains but had an RI of ∼1, indicating that the CQ-resistance mechanism has little effect on the antiplasmodial activity of this compound. Compound **4c** also contains an unsubstituted piperazine moiety but consistently showed cross-resistance with CQ (RIs of 2.4 and 2.8). This indicates that both the quinoline moiety and the heterocyclic side chain play a role in determining whether a compound displays cross-resistance with CQ. Furthermore, compounds **4f** (in which the piperazine moiety presents a N4-benzyl substituent), **4g** (which contains the ferrocenylmethyl-substituted piperazine), and **5** (a dimeric analogue of **4b**) are less active than the other analogues. It is worth noting that compounds **4g** and **5** have RI values lower than one. This suggests that certain modifications to the benzhydryl side chain can yield compounds that are considerably more active against the CQR strains W2 and K1 than against the CQS strains D10 and NF54. Given this apparent trade-off between cross-resistance and absolute level of activity, **4b** and **4d** appear to have the best balance of these traits within this piperazine series.

b. Piperazine-Tethered Derivatives. We briefly investigated the antiplasmodial activity of compounds **6** and **7** (Table 2), hybrid derivatives that combine the 7-chloro-4-piperazinyl moiety of piperaquine with the polyarylmethyl system of the previously described CLT-based antimalarials. Compounds **6** and **7** showed poor activity against all of the strains examined. This finding is consistent with the idea that for our class of compounds, the conjugation between the polyarylmethyl system and the iron-coordinating and electron transfer moiety (4-aminoquinoline) is necessary for optimal activity.[26,27]

c. Effect of a Third Electron-Withdrawing or -Releasing Substituent at the Benzhydryl System. In the previously reported pharmacokinetic characterization of **3b**, the formation of at least three metabolites was observed.[26] This led us to undertake an in silico approach to identify potential cytochrome P450 (CYP)-mediated sites of metabolism in the lead compounds **3a** and **3b**. From these analyses, which are described in the next section, the pyrrolidinylmethylene moiety and the aromatic ring bearing the protonatable side chain emerged as potential soft spots for the CYP3A4 isoform.

We therefore tested whether these potential sites of metabolism could be masked by the introduction of additional electron-withdrawing or -releasing groups into the benzhydryl side chain. The biological activity of the resulting compounds (**9a**−**m**) was evaluated in D10, W2, NF54, and K1 parasites (Table 3). Introduction of halogens (Cl and F; substituent X in Table 3) into the parent compounds **3a** and **3b** (to obtain compounds **9a**,**b**,**h**,**i**) had little effect on antiplasmodial activity, and in the case of compounds **9b** and **9h**, resulted in improved potency against D10 parasites—albeit with a concomitant increase in cross-resistance with CQ.

The introduction of F, Cl, or a OMe group is not predicted to influence the protonatability of the neighboring basic chain (e.g., **9a**,**b** vs **3b**; **9h**,**i**,**m** vs **3a**). Compounds possessing a pyrrolidine proved to be more potent than compounds carrying a less basic morpholine in the protonatable side chain (**9c** vs **9a** or **9f** vs **9d**). Moreover, the 7-chloro-4-aminoquinoline moiety (**9a**,**b**,**h**,**i**,**m**) was found to impart antiplasmodial activity greater than that of the 6-methoxy- or the 7-trifluoromethyl-4-aminoquinoline moieties (**9d**,**e**,**j**,**k**, and **9g**,**l**, respectively).

Some differences in antiplasmodial potency were observed between the *o*-Cl and *o*-F analogues (substituent X of Table 3). For example, *o*-Cl derivative **9a** was less potent than the corresponding *o*-F analogue **9b**, but the reverse was true for compounds **9h** and **9i**. Yet these compounds have similar protonation states (at both pH 7.4 and 5.5) and similar iron-interacting groups. We previously found that both the electron density at the endocyclic quinoline nitrogen and the polarization of the quinoline ring are also important determinants of potency against malaria parasites.[26] We therefore performed ab initio calculations to predict the electron distribution of compounds **9a**,**b**,**h**,**i** (Figure 1 of the main text and Figure S1 of the Supporting Information).

While all four of the compounds possessed comparable electron densities at the quinoline endocyclic nitrogen, the most potent compounds (**9b** and **9h**, Figure 1) were more polarized along the 4-aminoquinoline moiety (from the exocyclic nitrogen to the endocyclic nitrogen) relative to **9a**. This effect was most evident for **9i** (Figure 1), for which the polarization of the 4-aminoquinoline ring is reversed compared with **9b** and **9h** (Figure 1).

2. In Silico Prediction of Potential Sites of Metabolism. Oral administration of **3b** in rats resulted in at least three putative metabolite peaks in the plasma chromatograms,[26] which indicates that this compound undergoes extensive first-pass biotransformation. We therefore performed an in silico study of the potential sites of metabolism (soft spots) for our lead compounds, with the aim of synthesizing new derivatives with functionalities that mask the identified soft spots. We used the P450 Site of Metabolism prediction workflow[33] (implemented in Maestro Suite 2011[32]) to predict metabolism mediated by isoforms CYP3A4, CYP2C9, and CYP2D6. The prediction for isoforms CYP2C9 and CYP2D6 combined induced-fit docking[34] (which determines accessibility

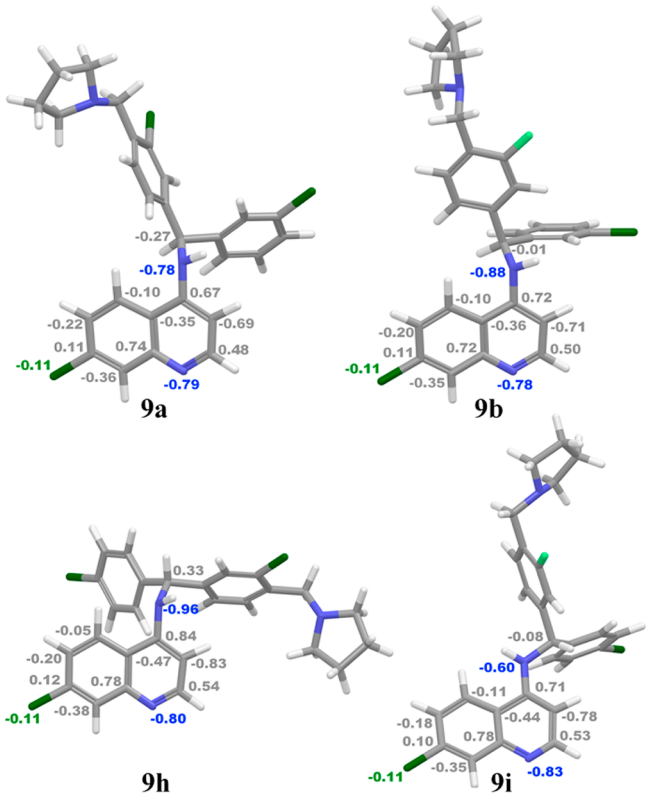

Figure 1. ESP charges from HF/6-31G++* calculations performed in vacuum by Jaguar[31] for compounds **9a**,**b**,**h**,**i**. The compounds are represented by tubes (pictures were generated by Maestro[32]).

of the compounds to the CYP reactive center) with a rule-based approach to intrinsic reactivity.[35] In the case of CYP3A4, which has a highly flexible binding site, only intrinsic reactivity was used to predict soft spots. In silico predictions were performed for each enantiomer of the most promising compounds identified in this study (see Table S1 of the Supporting Information). We focused on addressing the potential soft spots located at the benzhydryl system and at the protonatable side chain, as even small modifications of the 4-aminoquinoline ring can be detrimental to antimalarial activity. Putative CYP3A4-mediated soft spots in the benzhydryl system were mainly localized at the protonatable chain (A, B) and at the ortho position of the aromatic ring bearing the side chain (C) (e.g., **3b** (Figure 2) and **3a** (Figure S2 of the Supporting Information). Predicted CYP2C9- and CYP2D6-mediated soft spots were also mainly localized to the benzylic (A) and pyrrolydine (B) rings for both **3a** and **3b**. Moreover, **3b** contained a further soft spot (D) on the second aromatic ring.

Introduction of a masking functionality in B led to compounds **9a** and **9h**, respectively. The presence of the Cl atom in **9a** was predicted to reduce CYP3A4-mediated metabolism and lead to more stable analogues (Figure 2). Similar results were obtained for **9h** and the F-substituted analogues **9b**,**i** (Table S1 of the Supporting Information).

The presence of the Cl atom also affected the accessibility of positions A and B of both enantiomers of **9a** and **9h** to the iron center of the CYP 2C9 binding site. Representations of **3b** and **9a** inside CYP2C9 (Figure 3) depict how the Cl atom at position C is expected to reduce the accessibility of positions A and B to the iron center.

3. Inhibition of β-Hematin Formation. Interaction with free heme is predicted to be key to the mode of action of these antimalarials. To confirm this activity, compound **4b**, one of the most potent compounds identified in this study, was tested using the β-hematin inhibitory activity (BHIA) assay. As expected, compound **4b** exhibited dose-dependent inhibition of β-hematin formation (Table 4), with an IC_{50} value of 0.67. **4b** was found to be more potent than **3b** in the BHIA assay, yet these two compounds possess similar levels of antiplasmodial activity. However, previous studies of related compounds have observed that activities measured in the BHIA assay do not always match those measured in parasite growth assays.[24] Aside from subtle differences in the pharmacokinetic properties of **4b** and **3b**, the data presented here, together with previous findings on similar compounds,[28] indicate that an interaction with free heme is necessary for antiplasmodial activity, but the data also suggests that an additional mode of action may contribute to the activity of this class of compound.

4. In Vivo Antimalarial Activity against *Plasmodium berghei*. The in vivo antimalarial activities of compounds **4b** and **4d** were evaluated using a *P. berghei* mouse model. Groups of five mice were infected with *P. berghei* and treated orally with compounds **4b**,**d**, **9h**, and **10**, using a range of dosage regimens (summarized in Table 5). The key measurements were (1) the percentage of parasitemia reduction compared to the untreated mice and (2) the prolongation of survival of the infected mice. All compounds drastically reduced parasitemia (>99%). Of note, a single oral dose of **4b** or **4d** at 30 mg/kg substantially prolonged mouse survival. Partial cure was achieved with several doses (e.g., 3 × 30 mg/kg), and administration of three or four doses of **4d** extended the survival of all of the mice until day 30. However, for reasons that are currently unclear, full cure was not observed.

5. Inhibition of CQ Transport via PfCRT by Benzhydryl Quinolines. To determine whether members of this series of compounds interact with a resistance-conferring form of PfCRT ($PfCRT^{CQR}$), we tested a representative set of derivatives (**2**, **3a**,**b**, **4b**,**d**,**e**,**g**, **6**, **7**, **9b**,**h**,**i**, and **10**), for the ability to inhibit CQ transport via this protein. Expression of PfCRT in *Xenopus* oocytes enables direct measurements of $PfCRT^{CQR}$-mediated CQ transport, and thus its inhibition by potential resistance-reversers or quinoline-based antimalarials. The direction of CQ transport in the PfCRT expression system is from the mildly acidic extracellular medium (pH 6.0) into the oocyte cytosol (pH 7.2), which corresponds to the efflux of CQ from the acidic DV (pH 5.5) into the parasite cytosol (pH 7.3). A key advantage of the oocyte system is that it allows interactions with PfCRT to be studied directly and in isolation, without confounding effects such as the binding of drugs to heme or interactions of the compound with other targets or transporters. In an initial experiment in which the derivatives were tested at an extracellular concentration of 100 μM, all 13 compounds inhibited CQ transport via $PfCRT^{CQR}$ (Figure 4 A; $P < 0.001$, ANOVA). None of the compounds affected the diffusion of CQ into oocytes expressing $PfCRT^{CQS}$ ($P > 0.05$, ANOVA). The fact that compounds **3a**,**b**, and **9b**,**h**,**i** inhibited $PfCRT^{CQR}$-mediated CQ transport to a similar extent (70−80% reduction in CQ uptake) indicates that changes to the benzhydryl aromatic ring or the distal chlorinated benzyl group have little effect on the ability of these compounds to interact with $PfCRT^{CQR}$. For comparison, the CQ resistance-reverser verapamil reduced CQ transport via $PfCRT^{CQR}$ by ~74%. The trityl derivatives **6**, **7**, and **10** decreased CQ uptake by 58%, 42%, and 72%, respectively, which suggests that the substrate binding-site of $PfCRT^{CQR}$ can tolerate large, bulky residues. Compounds **4b** and **4d** abolished CQ uptake via $PfCRT^{CQR}$ when present at 100 μM; under these conditions, CQ

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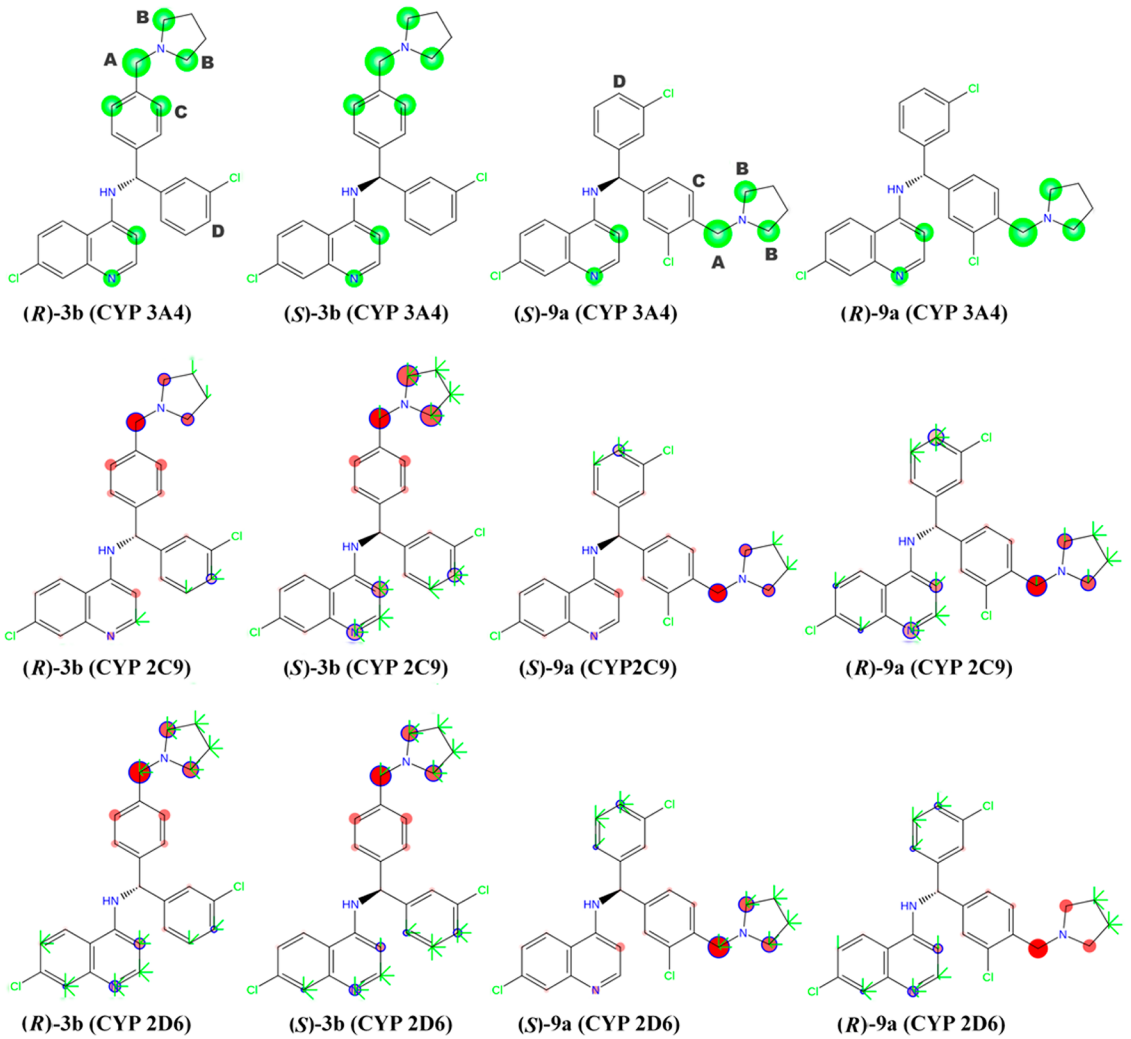

Figure 2. Structures of (*R*)- and (*S*)-enantiomers of compounds **3b** and **9a**, annotated on the basis of predicted CYP-mediated soft spots (A–D). The number of rays indicates the value of the Fe accessibility from IFD. Each full ray represents a unit of accessibility, with the length of the final ray representing the decimal portion. Size of the circles: overall SOM score; the color of the circle indicates the atomic intrinsic reactivity: the redder the circle, the higher the intrinsic reactivity; a blue ring around the circle indicates that the atom passed through the CYP filtering stage. No ring indicates that the atom failed the CYP filtering stage.

accumulation in oocytes-expressing PfCRT^{CQS} was not significantly different from that measured in PfCRT^{CQR}-expressing oocytes ($P > 0.05$, ANOVA). The ability of the piperazine derivatives (**4b,d,e,g**) to inhibit PfCRT^{CQR} appeared to decrease as the bulkiness of the terminal amine substituent was increased. Hence, this position may play an important role in influencing the affinity of interactions with PfCRT^{CQR}. An analysis of the concentration-dependent inhibition of PfCRT^{CQR} by **4b** and **4d** (Figure 4B,C) yielded IC_{50} values of 10.3 ± 0.9 and 10.5 ± 1.0 μM, respectively. These values are significantly lower than that measured previously for verapamil (30 ± 1 μM; $P < 0.005$, unpaired *t*-test) and are similar to the IC_{50} value obtained for the antiretroviral drug saquinavir (13 ± 0.9 μM), the most potent PfCRT^{CQR} inhibitor reported to date.[36] It should be noted that the micromolar concentrations of the compounds used here to inhibit PfCRT^{CQR} are physiologically relevant, given that dibasic compounds are expected to accumulate to high micromolar, and even millimolar, concentrations within the acidic environment of the parasite's DV via weak-base trapping. The ability of **4b** and **4d** to block PfCRT^{CQR}-mediated transport, coupled with their intrinsic antiplasmodial activity, makes these compounds good candidates for use in combination with CQ against CQR malaria. Because halogenation of the benzhydryl group did not affect the extent to which the compounds inhibit PfCRT^{CQR} (cf., **3a** and **3b** with **9b**, **9h**, and **9i**; Figure 4A), it is possible that the same modification would improve the metabolic stability of **4b** and **4d** without diminishing their ability to block PfCRT^{CQR}.

6. In Vitro Toxicity Assays. Cytotoxicity assays were performed on the lead compounds to investigate their effects on cell viability in vitro. IC_{50} values against both human fibroblast (NIH3T3) and hepatic (HepG2) cell lines are presented in Table 6. The data indicate that all of the compounds tested have low cytotoxic potential, although the IC_{50} values obtained with hepatocytes are for the most part lower than those obtained with fibroblasts.

7. Mutagenicity Assay: Ames Test. As summarized in Table 7, compounds **4a,b,d** and **9d,i,l** were tested in vitro for mutagenic potential. None of the compounds were mutagenic in the *S. typhimurium* strains TA98 and TA100 at any of the concentrations tested. The number of *S. typhimurium* revertants that arose at each concentration of the test compound was

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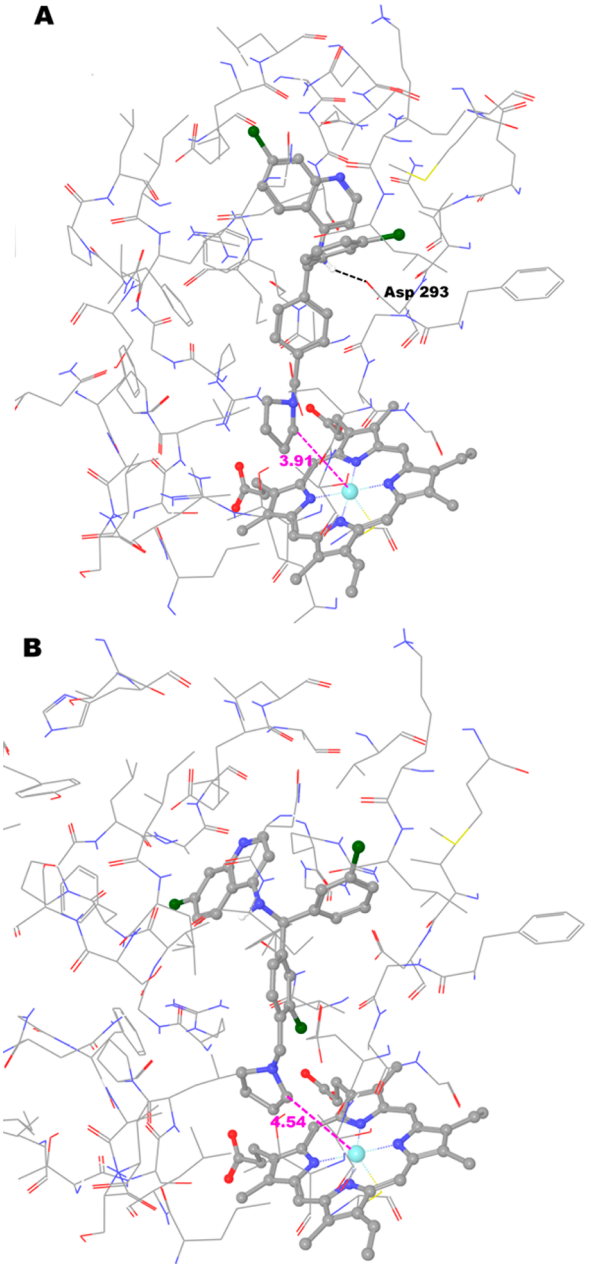

Figure 3. Representative binding poses of compounds **3b** (panel A) and **9a** (panel B) in the 2C9 binding site. The distance of pyrrolidine carbons from the iron center is shown in magenta. The references compounds and heme group were represented by ball and stick (pictures were generated by Maestro[32]).

expressed as a percentage of the number of revertants that arose in the positive control.

8. Preliminary Pharmacokinetic Studies on Compound 4b. Following a 50 mg/kg oral dose, compound **4b** was rapidly taken up into the systemic circulation, with a peak concentration of 0.42 at 2 h. The product was then cleared slowly and was detectable in plasma for up to 48 h. The elimination half-life ($t_{1/2}$), estimated from the sixth hour onward (when the plasma concentration−time curve entered an apparent log−linear phase), was approximately 22 h. This is in contrast with the behavior of hybrid antimalarials previously developed, typified by compound **3b**, which had an elimination $t_{1/2}$ of about 3 h.[27] This relatively longer $t_{1/2}$ was the result of a large volume of

Table 4. β-Hematin Inhibitory Activity Assay of Compounds 3b, 4b, and CQ (1)

compd	IC_{50}[a]
4b	0.67
3b	1.33
1, CQ	1.69

[a]The IC_{50} represents the molar equivalents of compound, relative to hemin, that inhibit β-hematin formation by 50%. Data are the mean of three different experiments in triplicate. Standard errors were all within 10% of the mean.

Table 5. In Vivo Efficacy in a *P. berghei* Rodent Malaria Model

compd	dosage (mg/kg)[a]	% activity[b]	average mice survival days[c]	cured/treated mice[d]
4b	1 × 100	99.5	28.8	2/5
	1 × 30	99.7	15.8	0/5
	3 × 50	99.8	29.6	3/5
	3 × 30	99.8	29.0	1/5
	4 × 30	99.8	30.0	0/5
4d	1 × 100	99.6	29.6	3/5
	1 × 30	99.7	24.0	1/5
	3 × 50	99.8	30.0	2/5
	3 × 30	99.8	30.0	3/5
	4 × 30	99.9	30.0	3/5
9h[e]	1 × 100	99.5	14.0	0/3
	1 × 30	99.4	7.7	0/3
10[e]	1 × 100	99.3	21.3	0/3
	1 × 30	99.4	7.0	0/3

[a]Compounds were formulated in ethanol/Tween 80/water 3/7/90. Data are shown as means with n = 5 mice. [b]% Activity = average parasitemia reduction. [c]Survival of control animals: 6−7 days. [d]Cure = no parasites present at day 30. [e]n = 3 mice.

distribution (V_d/F = 153 L/kg, assuming complete absorption of **4b** from the gastrointestinal tract) in conjunction with an intermediate to high rate of clearance (Cl = dose × F/AUC) of 82 mL/min·kg. CLT pharmacokinetics also shows a large volume of distribution and a high rate of clearance with extensive first-pass biotransformation, resulting in low plasma concentrations of the unchanged parent compound after oral dosing in animals and humans.[37,38] By contrast, CQ is cleared slowly, partly unchanged and partly after cytochrome P450-mediated oxidation to its active monodesethyl- and bisdesethyl-metabolites. The plasma $t_{1/2}$ for CQ is relatively long in all species; in humans it typically averages between 20 to 60 days after a single oral dose. The volume of CQ distribution ranges from 200 to 800 L/kg when calculated from plasma concentrations and about 200 L/kg when estimated from whole blood data because of its marked concentration in erythrocytes (concentrations being 5 to 10 times higher).[39] Compound **4b** distributes into erythrocytes, albeit to a lesser extent than CQ, and regardless of the dosage studied, **4b** had an in vitro blood-to-plasma ratio of approximately 1.9 in mice. As is the case for CQ, the binding of **4b** to erythrocytes may contribute to its potent antimalarial activity in animals. Preliminary tissue distribution studies of compound **4b** were limited to the liver because of the preferential tropism previously demonstrated by **3b** for this tissue.[27] This derivative reached very high levels in the liver; these values were consistently above the blood concentrations from the first sampling point. The mean peak concentration in the liver (128−130 μg/g) occurred between 2 and 6 h, which suggests that this organ may act as a slow equilibrating compartment. However,

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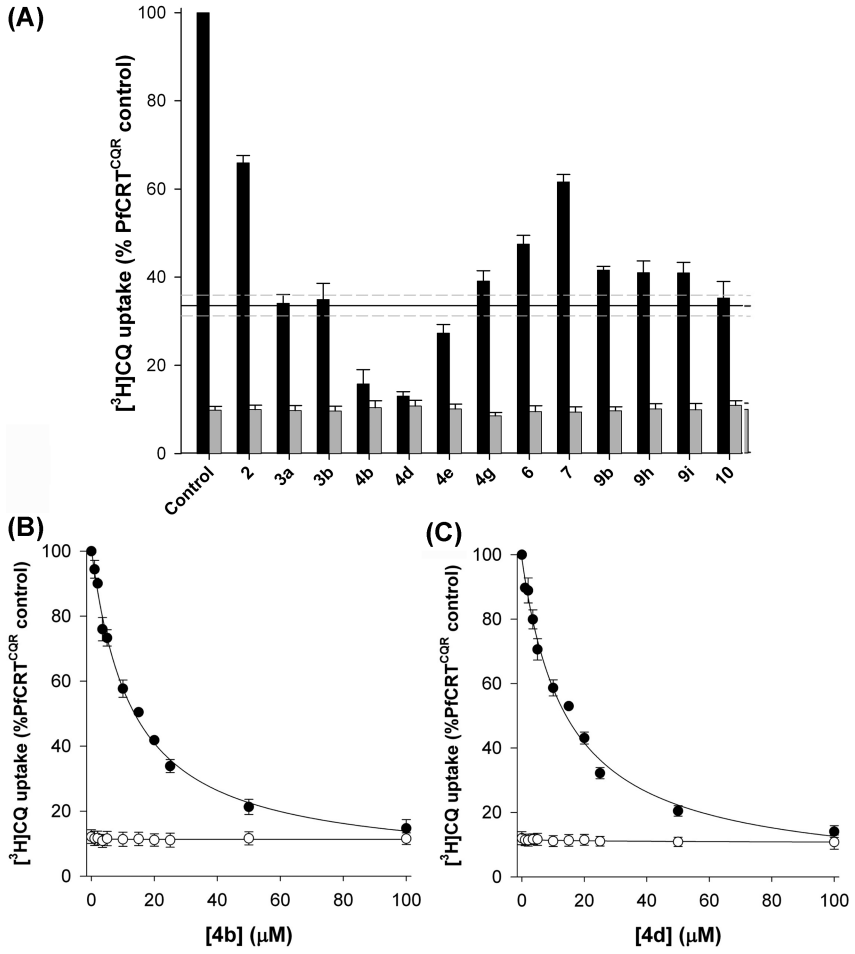

Figure 4. Inhibition of PfCRT^{CQR}-mediated CQ transport by benzhydryl quinolines. (A) [^{3}H]CQ uptake into oocytes expressing Dd2 PfCRT^{CQR} (black bars) or D10 PfCRT^{CQS} (gray bars) in the presence of 100 μM each of the 13 antimalarials tested. All of the compounds caused a significant reduction in CQ uptake via PfCRT^{CQR} ($P < 0.001$; ANOVA). The level of inhibition by the CQ resistance-reverser verapamil is represented as a solid line with SEM from $n = 4$ represented by gray dashed lines. Concentration-dependent effects of **4b** (B) and **4d** (C) on the uptake of [^{3}H]CQ into oocytes expressing Dd2 PfCRT^{CQR} (closed circles) or D10 PfCRT^{CQS} (open circles). The IC_{50} values derived from these data for **4b** and **4d** (10.3 ± 0.9 μM and 10.5 ± 1.0 μM (mean ± SEM; $n = 5$), respectively) were obtained by least-squares fit of the equation $Y = Y_{min} + [(Y_{max} - Y_{min})/(1 + ([inhibitor]/IC_{50})^C]$, where Y is PfCRT^{CQR}-mediated CQ transport, Y_{min} and Y_{max} are the minimum and maximum values of Y, and C is a constant. PfCRT^{CQR}-mediated CQ transport was calculated by subtracting the uptake measured in oocytes expressing D10 PfCRT^{CQS} from that in oocytes expressing Dd2 PfCRT^{CQR}. In all panels, uptake is shown as the mean ± SEM from four to five separate experiments, within which measurements were made from 10 oocytes per treatment. Note that noninjected oocytes and oocytes expressing PfCRT^{CQS} take up [^{3}H]CQ to similar (low) levels via simple diffusion of the neutral species; this represents the "background" level of CQ accumulation in oocytes (refer to ref 17 for full data and a detailed discussion).

between 6 and 48 h after dosing, the concentration of **4b** in the liver decreased almost in parallel with the plasma concentration, with comparable elimination $t_{1/2}$ values of around 29 h, although this could not be properly calculated because of the short sampling interval. Accumulation in rodent liver and other tissues is a well-known characteristic of the highly lipophilic CLT and CQ. Unlike CLT, however, CQ is selectively distributed in melanin-containing tissues where retention is prolonged.[37,39−41]

9. Functional in Vitro Assays for Cardiovascular Effects. A major hurdle in the development of new drugs is reducing the risk of cardiac arrhythmias, such as a long QT syndrome. To evaluate the cardiotoxic potential of compound **4b**, we investigated its effect on Langendorff-perfused rat heart. Under control conditions, left ventricular pressure (LVP) was 59.68 ± 7.86 mmHg ($n = 6$). **4b** reduced LVP in a concentration-dependent manner ($IC_{50} = 9.50 \pm 1.25 \times 10^{-6}$ M, $n = 6$; Figure 5) and also significantly decreased heart rate (HR) and increased RR, PQ, and QRS intervals at the maximum concentration tested (30 μM; Table 8). It is important to note that **4b** increased the QT interval ($P < 0.05$, repeated measures ANOVA), although the values obtained for each concentration were not significantly different from the control value ($P > 0.05$, Dunnett's post test; Table 8). As the QT interval is affected by heart rate, Bazett's formula was used for any heart rate changes to calculate QTc; the resulting values did not vary over the drug range tested (Table 8). By contrast, terfenadine, a well-known arrhythmogenic antihistamine, significantly increased the QT interval from 68.51 ± 2.73 to 99.83 ± 5.24 ms at 10 μM (** $P < 0.01$, Dunnett's post test) and to 125 ± 6.62 ms at 30 μM (** $P < 0.01$). Premature ventricular contractions (i.e., extrasystoles) were recorded in three out of six experiments when **4b** was present. Additionally, a Wenckebach-type second degree atrioventricular block occurred in the presence of 30 μM **4b** in three out of six hearts. In conclusion, **4b** is a negative inotropic and chronotropic agent capable of prolonging PQ interval to such an extent that an atrioventricular block occurred.

■ CONCLUSIONS

This study describes the discovery of antimalarial agents that are potent against CQS and CQR strains in vitro as well as in a

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rodent malaria model. Compound **4b** emerged as one of the most potent agents and, along with compound **4d**, exhibited strong antiplasmodial activity in vivo against *P. berghei* after oral administration. The compounds interfere with the process of heme detoxification, as demonstrated by a BHIA assay. Using the *Xenopus* oocyte expression system, we also demonstrated that hybrids **4b** and **4d** are among the most potent of known inhibitors of the resistance-conferring form of PfCRT. The intrinsic pharmacological properties of a selection of analogues were also evaluated by undertaking preliminary assessments of cytotoxicity, genotoxic potential, and effects on cardiac function. Compound **4b** had an optimal half-life in mice but caused a negative inotropic effect in rat hearts when tested at high concentrations. This issue will be the focus of future work that will aim to optimize this class of otherwise potent and promising antimalarials.

Table 6. Cytotoxic Activity against 3T3 and HepG2 Cell Lines of 4a,b,d, 9d,i,l,h, 10, and reference compounds 3b, CQ, and CLT

	TC_{50} (μM)	
compd	3T3	HepG2
4a	35	22
4b	1.8	1.8
4d	16	<4
9d	24	12
9i	25	12
9l	34	19
9h	16	4.0
10	15	6.1
3b	9.0	4.3
CQ	>80	31
CLT	>48	>48

EXPERIMENTAL SECTION

Chemistry. Starting materials and solvents 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 or Bruker 400 MHz spectrometer 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 value of chemical shifts (δ) are given in ppm and coupling constants (J) in hertz (Hz). Mass spectra were recorded utilizing electron spray ionization (ESI). Yields refer to purified products and are not optimized. All moisture-sensitive reactions were performed under argon atmosphere using oven-dried glassware and anhydrous solvents. Elemental analyses were performed in a Perkin-Elmer 240C elemental analyzer, and the results were within (0.4% of the theoretical values). All compounds tested in the biological assays were analyzed by combustion analysis (CHN) to confirm the purity ≥95%.

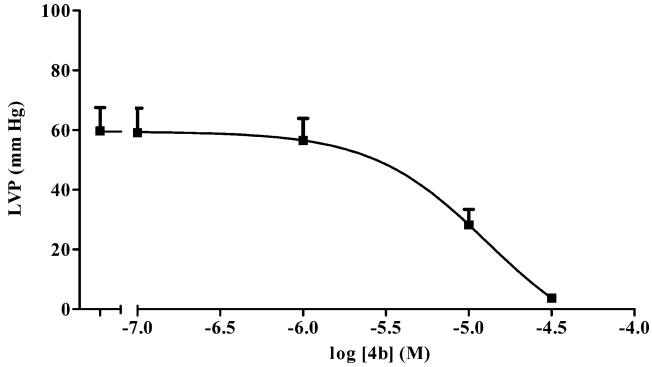

Figure 5. Concentration−effect relationship of **4b** on left ventricular pressure in Langendorff perfused rat hearts. On the ordinate scale, response is reported as mmHg. Each value represents mean ± SEM (n = 6).

Table 7. Ames Test on Compounds 4a,b,d, 9d,i,l,h, 10, and reference compounds 3b and CQ

4a			**4b**			**4d**			**9d**			**9i**		
	% R ± SD[a]			% R ± SD[a]			% R ± SD[a]			% R ± SD[a]			% R ± SD[a]	
C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100
0	5 ± 5	5 ± 4	0	3 ± 4	20 ± 20	0	7 ± 6	3 ± 4	0	8 ± 5	4 ± 1	0	6 ± 4	7 ± 13
2	0	6 ± 3	1.5	2 ± 3	19 ± 24	1	9 ± 8	6 ± 3	1.5	3 ± 3	3 ± 0	1.5	4 ± 0	12 ± 1
4	7 ± 3	6 ± 3	3	4 ± 4	22 ± 18	2	7 ± 7	5 ± 2	3	0	4 ± 1	3	5 ± 5	10 ± 3
9	0	7 ± 10	6	6 ± 3	13 ± 10	4	4 ± 4	3 ± 3	6	0	4 ± 1	6	3 ± 4	10 ± 3
18	3 ± 6	5 ± 2	12	4 ± 8	17 ± 26	8	16 ± 10	5 ± 1	12	3 ± 5	4 ± 1	13	3 ± 2	11 ± 3
35	3 ± 3	2 ± 2	24	4 ± 4	27 ± 26	17	11 ± 8	7 ± 8	24	3 ± 3	5 ± 0	25	11 ± 5	11 ± 1
70	2 ± 3	2 ± 2	48	4 ± 0	28 ± 9	34	4 ± 8	2 ± 2	48	3 ± 3	10 ± 4	50	8 ± 4	11 ± 4
+[c]	100 ± 9	100 ± 44	+[c]	100 ± 31	100 ± 55	+[c]	100 ± 13	100 ± 11	+[c]	100 ± 14	100 ± 10	+[c]	100 ± 11	100 ± 25
9l			**9h**			**10**			**3b**			CQ (**1**)		
	% R ± SD[a]			% R ± SD[a]			% R ± SD[a]			% R ± SD[a]			% R ± SD[a]	
C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100	C[b]	TA98	TA100
0	6 ± 4	6 ± 6	0	10 ± 0	6 ± 6	0	3 ± 4	4 ± 4	0	5 ± 4	5 ± 2	0	10 ± 11	30 ± 31
2	1 ± 2	4 ± 2	2	17 ± 6	4 ± 2	7	1 ± 2	4 ± 3	1	2 ± 4	5 ± 9	2.5	24 ± 8	36 ± 21
4	1 ± 2	3 ± 1	4	0	3 ± 1	15	1 ± 2	5 ± 1	2	2 ± 2	2 ± 1	5	14 ± 22	21 ± 14
8	1 ± 2	1 ± 1	16	3 ± 6	1 ± 1	24	5 ± 6	6 ± 4	4	0	0	10	61 ± 83	26 ± 14
17	4 ± 4	4 ± 2	24	10 ± 10	4 ± 2	40	3 ± 4	4 ± 2	8	6 ± 2	5 ± 2	20	29 ± 14	7 ± 12
33	4 ± 4	8 ± 3	40	10 ± 10	8 ± 3	61	5 ± 2	4 ± 6	16	14 ± 2	10 ± 8	40	19 ± 22	38 ± 36
66	8 ± 7	1 ± 1	80	0	1 ± 1	80	0	0	32	2 ± 4	2 ± 3	80	50 ± 8	23 ± 11
+[c]	100 ± 11	100 ± 1	+[c]	100 ± 26	100 ± 1	+[c]	100 ± 16	100 ± 6	+[c]	100 ± 10	100 ± 25	+[c]	100 ± 0	100 ± 60

[a]% R ± SD = % of revertants ± standard deviation. [b]C = concentration (μM). [c]+ = positive control.

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Table 8. Effects of 4b on HR, RR, PQ, QRS, QT, and QTc in Langendorff Perfused Rat Hearts[a]

4b (μM)	HR (BPM)	RR (ms)	PQ (ms)	QRS (ms)	QT (ms)	QTc (ms)
none	236.68 ± 7.83	258.62 ± 8.87	42.67 ± 2.29	13.92 ± 0.42	73.01 ± 4.52	145.78 ± 10.53
0.1	235.08 ± 8.44	256.97 ± 9.38	42.53 ± 2.05	14.00 ± 0.45	74.00 ± 4.52	145.15 ± 10.28
1	233.54 ± 9.17	260.13 ± 9.49	42.38 ± 2.04	14.18 ± 0.48	76.50 ± 4.46	151.17 ± 10.20
10	216.05 ± 12.09	294.00 ± 21.22	50.56 ± 3.07	17.00 ± 2.03	94.50 ± 6.83	177.33 ± 9.91
30	116.31 ± 27.00**	317.00 ± 20.39**	75.67 ± 5.54**	22.67 ± 2.40**	94.33 ± 8.61	168.83 ± 13.41

[a]Each value represents mean ± SEM (n = 6). **P < 0.01 vs control (repeated measures ANOVA, Dunnet's post test). HR: frequency, RR: cycle length, PQ: atrioventricular conduction time, QRS: intraventricular conduction time, QT: duration of ventricular depolarization and repolarization, i.e., the action potential duration.

(4-Chlorophenyl)(4-(imidazol-1-ylmethyl)phenyl)-methanone (12). To a suspension of NaH (60% in mineral oil, 25.6 mg, 0.64 mmol) in dry *N*,*N*-DMF (6.0 mL) was slowly added a solution of imidazole (43.6 mg, 0.64 mmol) in *N*,*N*-DMF (3.0 mL), and the resulting mixture was stirred at 25 °C. After 1 h, the reaction mixture was cooled to 0 °C, and a solution of **11a**[28] (99.0 mg, 0.32 mmol) in *N*,*N*-DMF (2.0 mL) was added. The reaction mixture was stirred at 25 °C for 1 h; afterward the solvent was removed and the residue was taken up in water. The aqueous phase was extracted with ethyl acetate, and the organic phase was dried over Na_2SO_4. The solvent was removed, and the residue was purified by column chromatography (6% methanol in ethyl acetate) to afford the title compound as a white low-melting solid (94.4 mg, 82%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75−7.68 (m, 4H), 7.57 (s, 1H), 7.43 (d, J = 8.5 Hz, 2H), 7.23 (d, J = 8.2 Hz, 2H), 7.11 (s, 1H), 6.92 (s, 1H), 5.20 (s, 2H).

(4-Chlorophenyl)(4-(imidazol-1-ylmethyl)phenyl)methanol (13). To a stirred solution of **12** (0.9 g, 3.0 mmol) in ethanol (10 mL) was added sodium borohydride (0.16 g, 4.5 mmol) at 0 °C, and the resulting mixture was stirred at 0 °C for 1 h. Thereafter, the reaction mixture was quenched with dropwise addition of water (5 mL), and the solvent was evaporated under reduced pressure. The residue was treated with water and extracted with chloroform (3 × 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated. The residue was chromatographed (6% methanol in chloroform) to afford **13** as a brown oil (0.73 g, 82%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34−7.23 (m, 7H), 7.04 (d, J = 8.2 Hz, 2H), 6.88 (s, 1H), 6.81 (s, 1H), 5.76 (s, 1H), 5.00 (s, 2H); MS (ESI) m/z [M + H]$^+$ 299.

(4-Chlorophenyl)(4-(*N*-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methanone (14a). To a stirred solution of **11a**[28] (2.89 g, 9.3 mmol) in dry acetonitrile (75 mL) were added 1-*tert*-butoxycarbonylpiperazine (2.08 g, 11.2 mmol) and triethylamine (2.5 mL, 18.6 mmol) at 0 °C, and the resulting mixture was stirred for 1 h at 0 °C. Thereafter, the reaction mixture was quenched with water (2 mL), and the solvent was evaporated under reduced pressure. The residue was treated with water and extracted with chloroform (2 × 40 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated. The residue was chromatographed (2% methanol in dichloromethane) to afford **14a** as a white low-melting solid (1.96 g, 51%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73−7.69 (m, 4H), 7.43−7.40 (m, 4H), 3.55 (s, 2H), 3.43−3.40 (m, 4H), 2.40−2.37 (m, 4H), 1.42 (s, 9H); MS (ESI) m/z 415 [M + H]$^+$.

(3-Chlorophenyl)(4-(*N*-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methanone (14b). Starting from **11b**[28] (10 g, 32.6 mmol), the title compound was prepared following the above-described procedure and was obtained as a white low-melting solid (9.7 g, 72%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76−7.73 (m, 3H), 7.67−7.64 (m, 2H), 7.56−7.38 (m, 3H), 3.58 (s, 2H), 3.46−3.44 (m, 4H), 2.41 (m, 4H), 1.45 (s, 9H); MS (ESI) m/z 437 [M + Na]$^+$.

(4-Chlorophenyl)(4-(*N*-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methanol (15a). Starting from **14a** (2.1 g, 5.1 mmol), the title compound was prepared following the procedure described for the synthesis of **13** and was obtained as a white low-melting solid (1.9 g, 88%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29−7.20 (m, 8H), 5.69 (s, 1H), 3.98 (br, 1H), 3.41 (s, 2H), 3.34 (m, 4H), 2.30 (m, 4H), 1.41 (s, 9H); MS (ESI) m/z 417 [M + H]$^+$.

(3-Chlorophenyl)(4-(*N*-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methanol (15b). Starting from **14b** (9.7 g, 23.5 mmol), the title compound was prepared following the procedure described for the synthesis of **13** and was obtained as a white low-melting solid (7.3 g, 75%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (s, 1H), 7.30−7.25 (m, 7H), 5.80 (s, 1H), 3.47 (s, 2H), 3.39 (br, 4H), 2.36 (br, 4H), 1.44 (s, 9H); MS (ESI) m/z 439 [M + Na]$^+$.

***N*-((4-Chlorophenyl)(4-(imidazol-1-ylmethyl)phenyl)-methyl)-7-chloro-4-aminoquinoline (4a).** To a solution of **13** (0.30 g, 1.0 mmol) in dry dichloromethane (8.0 mL) was added a solution of thionyl chloride (450 μL, 6.2 mmol) in dry dichloromethane (4.0 mL). The mixture was stirred at 0 °C for 20 min and thereafter was heated to 45 °C for 4 h. The volatiles were removed, and the residue was treated with dry toluene (3 × 4 mL) and concentrated under reduced pressure to remove residual thionyl chloride. The resulting hydrochloride salt was suspended in dry acetonitrile (12 mL), and to this solution, cooled at 0 °C, was added a solution containing triethylamine (430 μL, 3.0 mmol) and 7-chloro-4-aminoquinoline[42] (213 mg, 1.2 mmol). Thereafter, the reaction mixture was heated to 80 °C for 6 h. The solvent was evaporated under reduced pressure, and the residue was treated with water and extracted with ethyl acetate (4 × 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by flash column chromatography (2% methanol in chloroform) to afford the title compound as a white solid (0.17 g, 38%); mp (ethanol) 229−231 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (d, J = 5.3 Hz, 1H), 7.99 (d, J = 2.1 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.52 (s, 1H), 7.42−7.23 (m, 7H), 7.17 (s, 1H), 7.14 (s, 1H), 7.08 (s, 1H), 6.91 (s, 1H), 6.21 (d, J = 5.4 Hz, 1H), 5.71 (d, J = 4.3 Hz, 1H), 5.39 (d, J = 4.3 Hz, 1H), 5.12 (s, 2H).

***N*-((4-Chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)-methyl)-7-chloro-4-aminoquinoline (4b).** Starting from **15a** (0.84 g, 2.0 mmol) and 7-chloro-4-aminoquinoline[42] (0.43 g, 2.4 mmol), *N*-((4-chlorophenyl)(4-(1-*tert*-butoxycarbonylpiperazin-4-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline was obtained following the above-described procedure as brown oil (0.51 g, 44%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (d, J = 5.2 Hz, 1H), 7.91 (s, 1H), 7.80 (d, J = 8.7 Hz, 1H), 7.31−7.24 (m, 9H), 6.21 (d, J = 5.2 Hz, 1H), 5.75 (d, J = 4.3 Hz, 1H), 5.69 (d, J = 3.5 Hz, 1H), 3.46 (s, 2H), 3.39 (m, 4H), 2.35 (m, 4H), 1.43 (s, 9H); MS (ESI) m/z 577 [M + H]$^+$. To a solution of the above compound (0.16 g, 0.28 mmol) in methanol (4 mL) was added a mixture of acetyl chloride (400 μL) in methanol (4 mL) at 0 °C, and the mixture was stirred at 25 °C for 2 h. Thereafter, the solvent was removed, and the residue was treated with water and triethylamine (1.0 mL) and extracted with dichloromethane (3 × 30 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by column chromatography (5% methanol in dichloromethane) to afford the title compound as white solid (90.0 mg, 68%); mp (ethanol) 190−192 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (d, J = 5.3 Hz, 1H), 7.95 (d, J = 2.1 Hz, 1H), 7.73 (d, J = 9.0 Hz, 1H), 7.39−7.22 (m, 9H), 6.22 (d, J = 5.3 Hz, 1H), 5.69 (d, J = 4.4 Hz, 1H), 5.47 (d, J = 4.4 Hz, 1H), 3.47 (s, 2H), 2.90−2.83 (m, 4H), 2.40 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2, 149.3, 148.5, 139.6, 139.5, 138.7, 135.2, 134.0, 130.2, 129.4, 129.2, 128.8, 127.6, 125.9, 121.2, 117.4, 101.5, 63.4, 61.7, 54.7, 46.3; MS (ESI) m/z 477 [M + H]$^+$.

***N*-((4-Chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)-methyl)-6-methoxy-4-aminoquinoline (4c).** Starting from **15a** (0.88 g, 2.1 mmol) and 6-methoxy-4-aminoquinoline[42] (0.44 g, 2.5 mmol), *N*-((4-chlorophenyl)(4-(1-*tert*-butoxycarbonylpiperazin-4-ylmethyl) phenyl)methyl)-6-methoxy-4-aminoquinoline was obtained

as a brown low-melting solid (0.37 g, 31%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (d, J = 4.9 Hz, 1H), 7.99 (d, J = 9.3 Hz, 1H), 7.32−7.23 (m, 9H), 7.02 (s, 1H), 6.25 (d, J = 5.2 Hz, 1H), 5.74 (d, J = 4.1 Hz, 1H), 5.54 (s, 1H), 3.90 (s, 3H), 3.50 (s, 2H), 3.42 (br, 4H), 2.38 (br, 4H), 1.45 (s, 9H); MS (ESI) m/z 573 $[M + H]^+$. The above compound was converted into the title compound (pale yellow oil, 50 mg, 75%) following the procedure described for the synthesis of **4b**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (d, J = 4.9 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.32−7.25 (m, 9H), 7.03 (s, 1H), 6.21 (d, J = 4.9 Hz, 1H), 5.69 (d, J = 4.1 Hz, 1H), 5.27 (s, 1H), 3.87 (s, 3H), 3.47 (s, 2H), 2.87 (m, 4H), 2.40 (m, 4H), 1.90 (s, 1H); MS (ESI) m/z 473 $[M + H]^+$.

***N*-((3-Chlorophenyl)(4-(piperazin-1-ylmethyl)phenyl)-methyl)-7-chloro-4-aminoquinoline (4d).** Starting from **15b** (9.7 g, 23.5 mmol) and 7-chloro-4-aminoquinoline,[42] *N*-((3-chlorophenyl)(4-(1-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methyl)-7-chloro-4-aminoquinoline was obtained following the above-described procedure as white low-melting solid (7.3 g, 54%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, J = 5.3 Hz, 1H), 7.97 (d, J = 2.1 Hz, 1H), 7.73 (d, J = 9.0 Hz, 1H), 7.50−7.13 (m, 9H), 6.23 (d, J = 5.4 Hz, 1H), 5.69 (d, J = 4.3 Hz, 1H), 5.45 (d, J = 4.4 Hz, 1H), 3.48 (d, J = 8.9 Hz, 2H), 3.46−3.31 (m, 4H), 2.55−2.25 (m, 4H), 1.44 (s, 9H); MS (ESI) m/z 577 $[M + H]^+$. The above compound was converted into the title compound (white solid, 76%) following the procedure described for the synthesis of **4b**; mp (ethyl acetate/hexane) 209−211 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.41 (d, J = 5.3 Hz, 1H), 7.96 (d, J = 2.1 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.43−7.19 (m, 9H), 6.22 (d, J = 5.4 Hz, 1H), 5.67 (d, J = 4.4 Hz, 1H), 5.45 (d, J = 4.4 Hz, 1H), 3.47 (s, 2H), 2.91−2.80 (m, 4H), 2.40 (br, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2, 149.3, 148.4, 143.2, 139.4, 138.8, 135.2, 130.6, 130.2, 129.3, 128.4, 127.6, 125.9, 125.6, 121.2, 117.4, 101.5, 63.4, 61.9, 54.7, 46.3; MS (ESI) m/z 477 $[M + H]^+$.

***N*-((4-Chlorophenyl)(4-(1-methylpiperazin-4-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (4e).** To a solution of **4b** (50 mg, 0.11 mmol) in a mixture of 1% acetic acid in ethanol (3 mL) were added formaldehyde (37% in water, 3 μL, 0.11 mmol) and $NaCNBH_3$ (13.0 mg, 0.22 mmol). After 6 h, a saturated solution of $NaHCO_3$ was added, the solvent was removed and the aqueous phase extracted with dichloromethane. The organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The residue was dissolved in 1:1 dichloromethane−*n*-hexane mixture and crystallized affording **4e** (49 mg, 95%) as white solid; mp (1:1 dichloromethane/*n*-hexane) 181−183 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, J = 5.3 Hz, 1H), 7.97 (d, J = 2.1 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.47−7.18 (m, 9H), 6.22 (d, J = 5.4 Hz, 1H), 5.69 (d, J = 4.3 Hz, 1H), 5.41 (d, J = 4.3 Hz, 1H), 3.51 (s, 2H), 2.46 (s, 8H), 2.28 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2, 149.3, 148.4, 139.7, 139.5, 138.6, 135.2, 134.0, 130.2, 129.5, 129.3, 128.8, 127.6, 125.9, 121.0, 117.3, 101.5, 62.6, 61.7, 55.2, 53.0, 46.1; MS (ESI) m/z 491 $[M + H]^+$.

***N*-((4-Chlorophenyl)(4-((4-benzylpiperazin-1-yl)methyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (4f).** Starting from **4b** (50 mg, 0.11 mmol) and benzaldehyde (11 μL, 0.11 mmol), the title compound was prepared following the procedure described for **4e**. The reaction crude was purified by column chromatography (5% methanol in chloroform) to afford **4f** as white solid (48.0 mg, 80%); mp (ethyl acetate/*n*-hexane) 98−100 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, J = 5.3 Hz, 1H), 7.99 (d, J = 2.1 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 7.41−7.24 (m, 14H), 6.23 (d, J = 5.3 Hz, 1H), 5.69 (d, J = 4.3 Hz, 1H), 5.33 (d, J = 4.3 Hz, 1H), 3.52 (s, 4H), 2.48 (s, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.1, 149.2, 148.5, 139.6, 139.5, 138.7, 138.2, 135.2, 134.0, 130.2, 129.5, 129.4, 129.2, 128.9, 128.4, 127.6, 127.3, 125.9, 121.2, 117.4, 101.5, 63.3, 62.7, 61.7, 53.3, 53.2; MS (ESI) m/z 567 $[M + H]^+$.

***N*-((4-Chlorophenyl)(4-(1-(ferrocen-1-ylmethyl)piperazin-4-ylmethyl)phenyl)methyl)-7-chloro-4-aminoquinoline (4g).** Starting from **4b** (50 mg, 0.11 mmol) and ferrocenyl aldehyde (23 mg, 0.11 mmol), the title compound was prepared following the procedure described for **4e**. The reaction crude was purified by column chromatography (5% methanol in chloroform) to afford **4g** as orange solid (43.0 mg, 78%); mp (ethyl acetate/*n*-hexane) 125−128 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (d, J = 5.3 Hz, 1H), 7.98 (d, J = 2.1 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 7.43−7.18 (m, 9H), 6.22 (d, J = 5.3 Hz, 1H), 5.68 (d, J = 4.3 Hz, 1H), 5.36 (d, J = 4.3 Hz, 1H), 4.16 (s, 2H), 4.11 (s, 7H), 3.48 (s, 2H), 3.39 (s, 2H), 2.45 (s, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.1, 149.3, 148.5, 139.6, 139.5, 138.6, 135.2, 134.0, 130.2, 129.4, 129.2, 128.8, 127.5, 125.9, 121.2, 117.4, 101.5, 82.3, 70.6, 68.7, 68.3, 62.6, 61.7, 58.5, 52.9, 52.6; MS (ESI) m/z 675 $[M + H]^+$.

***N*,*N*′-Bis(4-(4-chlorobenzoyl)phenylmethyl)piperazine (16).** Piperazine (0.14 g, 1.61 mmol) was added to a solution of **11a**[27] (0.25 g, 0.81 mmol) and triethylamine (0.22 mL, 1.61 mmol) in dry acetonitrile (5 mL), cooled to 0 °C. After 1 h, water was added, and the volatiles were removed. The aqueous phase was extracted with chloroform. The organic extracts were dried over Na_2SO_4, and the solvent was removed. The residue was purified by column chromatography (2% methanol in dichloromethane) to afford the title compound (66% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.80−7.67 (m, 8H), 7.51−7.38 (m, 8H), 3.59 (s, 4H), 2.52 (s, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.3, 143.9, 138.9, 136.2, 131.6, 130.2, 129.2, 128.8, 62.8, 53.4.

***N*,*N*′-Bis(4-(4-chlorochlorobenzyl)phenylmethyl)piperazine (17).** Starting from ketone **16**, the corresponding alcohol *N*,*N*′-bis(4-(4-chlorohydroxybenzyl)phenylmethyl)piperazine was obtained following the procedure used for the synthesis of **13**. ^{1}H NMR (300 MHz, DMSO) δ 7.40−7.29 (m, 8H), 7.27 (d, J = 7.8 Hz, 4H), 7.18 (d, J = 7.6 Hz, 4H), 5.93 (d, J = 3.9 Hz, 2H), 5.67 (d, J = 3.6 Hz, 2H), 3.37 (s, 4H), 2.31 (s, 8H); ^{13}C NMR (75 MHz, DMSO) δ 145.4, 144.5, 137.5, 131.8, 129.3, 128.7, 126.7, 74.0, 62.5, 53.3. The above alcohol was dissolved in dry dichloromethane cooled to 0 °C and treated with thionyl chloride (6 equiv). The resulting mixture was heated to 45 °C for 4 h. Subsequently, the volatiles were removed, and the crude chloride (90% yield after two steps) was used in the next step without further purification.

***N*,*N*′-Bis(4-(4-chloro(7-chloroquinolin-4-ylamino)benzyl)-phenylmethyl)piperazine (5).** A mixture of **17** (0.39 g, 0.67 mmol), 7-chloro-4-aminoquinoline[42] (0.24 g, 1.35 mmol), and triethylamine (0.37 mL, 2.6 mmoL) in acetonitrile (5 mL) was heated under reflux for 5 h. Afterward the solvent was removed and the residue was purified by column chromatography (2% methanol in chloroform) to afford the title compound (0.42 g, 72%) as a pale yellow low-melting solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, J = 5.1 Hz, 2H), 7.99 (s, 2H), 7.73 (d, J = 8.9 Hz, 2H), 7.41−7.19 (m, 18H), 6.23 (d, J = 5.2 Hz, 2H), 5.70 (d, J = 3.6 Hz, 2H), 5.47 (s, 2H), 3.54 (s, 4H), 2.53 (s, 8H).

***N*-((4-Chlorophenyl)((4-pyrrolidin-1-ylmethyl)phenyl)-methyl)-*N*′-(7-chloroquinolin-4-yl)piperazine (6).** To a solution of **18a**[26] (0.31 g, 1.0 mmol) in dry dichloromethane (8 mL) was added a solution of thionyl chloride (450 μL, 6.2 mmol) in dry dichloromethane (4 mL) at 0 °C, and the mixture was stirred at 0 °C for 20 min and thereafter heated to 45 °C for 4 h. The volatiles were removed, and the residue was treated with dry acetonitrile (3 × 4 mL) and concentrated under reduced pressure to remove residual thionyl chloride. The resulting hydrochloride salt was suspended in dry acetonitrile (12 mL), and to this solution were added triethylamine (430 μL, 3.0 mmol) and 7-chloro-4-(piperazin-1-yl)quinoline[29] (0.31 g, 1.2 mmol) at 0 °C. Thereafter, the reaction mixture was heated to 80 °C for 6 h. The solvent was evaporated under reduced pressure, and the residue was treated with water and extracted with ethyl acetate (4 × 30 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by column chromatography (2% methanol in chloroform) to afford **6** (0.12 g, 22%) as a brown oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.69 (d, J = 5.0 Hz, 1H), 8.01 (d, J = 2.1 Hz, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.45−7.31 (m, 5H), 7.31−7.18 (m, 4H), 6.79 (d, J = 5.1 Hz, 1H), 4.33 (s, 1H), 3.56 (s, 2H), 3.26−3.15 (m, 4H), 2.65 (s, 4H), 2.53−2.43 (m, 4H), 1.82−1.72 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.1, 152.1, 150.3, 141.2, 140.5, 138.7, 135.0, 132.9, 129.5, 129.4, 129.1, 129.0, 127.9, 126.2, 125.4, 122.1, 109.0, 75.4, 60.5, 54.5, 52.5, 52.0, 23.6; MS (ESI) m/z 531 $[M + H]^+$.

***N*-(Bis(4-chlorophenyl)((4-pyrrolidin-1-ylmethyl)phenyl)-methyl)-*N*′-(7-chloroquinolin-4-yl)piperazine (7).** Starting from **18b** (0.37 g, 0.89 mmol) and 7-chloro-4-(piperazin-1-yl)quinoline[29] (0.26 g, 1.08 mmol), the title compound was prepared following the above-described procedure and was obtained as a yellow oil (0.34 mg, 59%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.70 (d, J = 5.0 Hz, 1H), 8.00 (d, J = 2.1 Hz, 1H), 7.74 (d, J = 9.0 Hz, 1H), 7.57−7.35 (m, 6H), 7.35−7.20 (m, 7H), 6.84 (d, J = 5.1 Hz, 1H), 3.57 (s, 2H), 3.35 (s, 4H), 3.01 (s, 2H), 2.52 (s, 4H), 2.03 (s, 2H), 1.86−1.68 (m, 4H); MS (ESI) m/z 641 $[M + H]^+$.

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(3-Chlorophenyl)(4-methyl-3-chlorophenyl)methanone (22a). A mixture of **21a** (15.0 g, 73 mmol), magnesium turnings (1.8 g, 73 mmol), and a catalytic amount of iodine in anhydrous THF (150 mL) was heated under reflux until the formation of the Grignard reagent. A solution of **20a** (15.6 mL, 146 mmol) in dry THF (100 mL) was added dropwise, and the resulting mixture was heated to 83 °C for 4 h. The reaction mixture was quenched with 20 mL of saturated solution of ammonium chloride. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude residue was purified by flash chromatography (2% ethyl acetate in *n*-hexane) to give **22a** as a white solid (18.0 g, 78%); mp (ethyl acetate/hexane) 133−134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78−7.75 (m, 2H), 7.64−7.55 (m, 3H), 7.45−7.44 (m, 2H), 2.46 (s, 3H); MS (ESI) *m*/*z* 287 $[M + Na]^+$.

(3-Chlorophenyl)(4-methyl-3-fluorophenyl)methanone (22b). Starting from **21b** (1.0 g, 5.3 mmol) and **20a** (1.2 mL, 10.6 mmol), the title compound was prepared following the procedure described for **22a** and was obtained in 57% yield as a white solid; mp 93−95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (s, 1H), 7.64 (d, *J* = 7.6 Hz, 1H), 7.56 (d, *J* = 7.6 Hz, 1H), 7.53−7.42 (m, 3H) 733−7.25 (m, 1H), 2.37 (s, 3H).

(4-Chlorophenyl)(4-methyl-3-chlorophenyl)methanone (22c). Starting from **21a** (3.0 g, 14.6 mmol) and **20b** (3.3 mL, 29.3 mmol), the title compound was obtained as white solid (2.3 g, 60%); mp (ethyl acetate/hexane) 131−133 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (d, *J* = 1.7 Hz, 1H), 7.73 (d, *J* = 6.7 Hz, 2H), 7.55 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.47 (d, *J* = 6.7 Hz, 2H), 7.34 (d, *J* = 7.9 Hz, 1H), 2.46 (s, 3H); MS (ESI) *m*/*z* 265 $[M + H]^+$.

(4-Chlorophenyl)(4-methyl-3-fluorophenyl)methanone (22d). Starting from **21b** (10.0 g, 53 mmol) and **20b**, the title compound was obtained as white solid (11.2 g, 85%); mp (ethyl acetate/hexane) 115−116 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (d, *J* = 8.5 Hz, 2H), 7.47−7.44 (m, 4H), 7.32−7.26 (m, 1H), 2.36 (s, 3H).

3-Methoxy-4-methylbenzaldehyde (26). To a stirred suspension of $LiAlH_4$ (4.2 g, 111 mmol) in dry THF was added a solution of **25** (10.0 g, 55.5 mmol) in dry THF dropwise at 0 °C. The reaction was stirred at 25 °C for 1 h. Afterward the excess of $LiALH_4$ was quenched by careful addition of water, and the aqueous layer was extracted with dichloromethane. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford 3-methoxy-4-methylbenzyl alcohol (7.0 g, 84%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.12 (d, *J* = 7.8 Hz, 1H), 6.92−6.85 (m, 2H), 4.65 (s, 2H), 3.84 (s, 3H), 2.21 (s, 3H). To a solution of the above compound (6.0 g, 39.5 mmol) in dry THF was added MnO_2 (17.0 g, 394.7 mmol), and the mixture was stirred under reflux for 2 h. Thereafter, the solid was filtered off, the solvent was evaporated in vacuo, and the crude residue was purified by column chromatography (20% ethyl acetate in *n*-hexane) affording **26** (5.6 g, 95%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.40−7.23 (m, 3H), 3.89 (s, 3H), 2.29 (s, 3H).

(4-Chlorophenyl)(3-methoxy-4-methylphenyl)methanone (22e). To a stirred solution of **26** (3.5 g, 23.3 mmol) in dry THF (10 mL) was added dropwise 4-chlorophenylmagnesium bromide (1 N solution in THF, 11.6 mL, 11.6 mmol), and the mixture was heated under reflux for 6 h. After cooling to room temperature, the reaction mixture was quenched by dropwise addition of a saturated solution of ammonium chloride. The aqueous phase was extracted with ethyl acetate and washed with brine. The combined organic layers were dried over Na_2SO_4 and evaporated. The crude residue was purified by flash chromatography (2% ethyl acetate in *n*-hexane) to afford the title compound as a white solid (2.3 g, 77%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (d, *J* = 6.4 Hz, 2H), 7.45 (d, *J* = 6.4 Hz, 2H) 7.32−7.20 (m, 3H), 3.88 (s, 3H), 2.30 (s, 3H); MS (ESI) *m*/*z* 261 $[M + H]^+$.

(3-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanone (23a). To a solution of **22a** (14.0 g, 52.8 mmol) in CCl_4 (40 mL) were added NBS (11.1 g, 62.0 mmol) and AIBN (catalytic amount), and the resulting suspension was heated under reflux for 4 h. After cooling to room temperature, the formed succinimide was filtered off and the solvent was evaporated. A mixture of the above bromide (11.3 g, 32.7 mmol), pyrrolidine (4.1 mL, 50.0 mmol), and triethylamine (9.1 mL, 66.4 mmol) in dry acetonitrile (150 mL) was stirred at 0 °C for 1 h; thereafter, the reaction mixture was quenched with water (50 mL) and the volatiles were removed. The aqueous phase was extracted with chloroform, the combined organic layers were dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by column chromatography (2% methanol in chloroform) to afford **23a** as yellow amorphous solid (10.9 g, 98%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (m, 2H), 7.65−7.47 (m, 4H), 7.44−7.42 (m, 1H), 3.82 (s, 2H), 2.63 (br, 4H), 1.84 (br, 4H); MS (ESI) *m*/*z* 334 $[M + H]^+$.

(3-Chlorophenyl)(3-chloro-4-(morpholin-4-ylmethyl)-phenyl)methanone (23b). Starting from **22a** and morpholine, the title compound was obtained following the above-described procedure in 62% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79−7.58 (m, 6H), 7.47−7.45 (m, 1H), 3.75 (m, 4H), 3.69 (s, 2H), 2.56 (m, 4H); MS (ESI) *m*/*z* 350 $[M + H]^+$.

(3-Chlorophenyl)(3-chloro-4-(1-*tert*-butoxycarbonylpipera-zin-4-ylmethyl)phenyl)methanone (23c). Starting from **22a** and *N*-Boc-piperazine, the title compound was obtained following the above-described procedure in 55% yield as a pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 10.2 Hz, 2H), 7.67−7.61 (m, 3H), 7.59−7.53 (m, 1H), 7.45−7.39 (m, 1H), 3.67 (s, 2H), 3.45 (br, 4H), 2.48 (br, 4H), 1.45 (s, 9H); MS (ESI) *m*/*z* 449 $[M + H]^+$.

(3-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanone (23d). Starting from **22b** and pyrrolidine, the title compound was prepared following the above-described procedure and was obtained in 57% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.70−7.62 (m, 1H), 7.62−7.35 (m, 5H), 3.77 (s, 2H), 2.60 (s, 4H), 1.82 (s, 4H); MS (ESI) *m*/*z* 318 $[M + H]^+$.

(4-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanone (23e). Starting from **22c** and pyrrolidine, the title compound was obtained following the above-described procedure in 73% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79−7.69 (m, 3H), 7.68−7.58 (m, 2H), 7.50−7.40 (m, 2H), 3.81 (s, 2H), 2.62 (s, 4H), 1.82 (s, 4H); MS (ESI) *m*/*z* 334 $[M + H]^+$.

(4-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanone (23f). Starting from **22d** and pyrrolidine, the title compound was obtained following the above-described procedure in 57% yield as white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79−7.62 (m, 2H), 7.59−7.45 (m, 2H), 7.44−7.37 (m, 3H), 3.72 (s, 2H), 2.55 (m, 4H), 1.77 (m, 4H); MS (ESI) *m*/*z* 318 $[M + H]^+$.

(4-Chlorophenyl)(3-methoxy-4-(pyrrolidin-1-ylmethyl)-phenyl)methanone (23g). Starting from **22e** and pyrrolidine, the title compound was prepared following the procedure described for **23a** and was obtained in 40% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.7 Hz, 2H), 7.50 (d, *J* = 7.7 Hz, 1H), 7.46 (d, *J* = 8.7 Hz, 2H), 7.35 (d, *J* = 1.4 Hz, 1H), 7.30−7.24 (m, 1H), 3.88 (s, 3H), 3.77 (s, 2H), 2.65 (s, 4H), 1.83 (s, 4H); MS (ESI) *m*/*z* 330 $[M + H]^+$.

(3-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanamine (24a). To a solution of **23a** (3.3 g, 10.1 mmol) in methanol (150 mL) were added hydroxylamine hydrochloride (1.6 g, 23.3 mmol) and $BaCO_3$ (4.6 g, 23.3 mmol), and the mixture was heated under reflux for 18 h. The resulting white precipitate was filtered off, and the solvent was evaporated under reduced pressure to furnish 3.4 g (97%) of crude oxime (MS (ESI) *m*/*z* 318 $[M + H]^+$) which was used in the next step without further purification. The above oxime (0.70 g, 2.0 mmol) was dissolved in acetic acid (5 mL), Zn (5.2 g, 79.4 mmol) was added, and the resulting mixture was heated to 50 °C and stirred at the same temperature for 18 h. Subsequently, the solid was filtered off, the volatiles were removed, and the residue was taken up in a saturated solution of $NaHCO_3$. The aqueous phase was extracted with chloroform, and the combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by column chromatography (5% methanol in chloroform) to afford the title compound (0.45 g, 68%) as colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44−7.24 (m, 3H), 7.23−7.19 (m, 4H), 5.13 (s, 1H), 3.72 (s, 2H), 2.57 (br, 4H), 1.79 (br, 4H); MS (ESI) *m*/*z* 335 $[M + H]^+$.

(3-Chlorophenyl)(3-chloro-4-(morpholin-4-ylmethyl)-phenyl)methanamine (24b). Starting from **23b** (2.9 g, 8.0 mmol), the title compound was prepared following the above-described procedure and was obtained as a yellow oil (1.7 g, 62%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43−7.38 (m, 3H), 7.26−7.20 (m, 4H), 5.12 (s, 1H), 3.71−3.68 (m, 4H), 3.57 (s, 2H), 2.51−2.48 (m, 4H), 1.92 (br, 2H); MS (ESI) *m*/*z* 351 $[M + H]^+$.

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(3-Chlorophenyl)(3-chloro-4-(1-*tert*-butoxycarbonylpiperazin-4-ylmethyl)phenyl)methanamine (24c). A mixture of **23c** (0.50 g, 1.1 mmol), $Ti(O\text{-}i\text{-}Pr)_4$ (0.66 mL, 2.2 mmol), and NH_3 (7 M in MeOH, 0.80 mL, 5.5 mmol) was stirred at 25 °C for 6 h; afterward $NaBH_4$ (63 mg, 1.7 mmol) was added, and the resulting mixture was stirred at 25 °C for further 3 h. The reaction was then poured into aqueous NH_3, and the resulting inorganic precipitate was filtered off and washed with ethyl acetate. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4, and the solvent was removed. The residue was purified by column chromatography (5% methanol in chloroform) to furnish the title compound (0.31 g, 63%) as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44−7.31 (m, 3H), 7.28−7.14 (m, 4H), 5.68 (br, 2H), 5.11 (s, 1H), 3.56 (s, 2H), 3.45−3.36 (m, 4H), 2.47−2.38 (m, 4H), 1.43 (s, 9H); MS (ESI) m/z 450 $[M + H]^+$.

(3-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanamine (24d). Starting from **23d**, the title compound was prepared following the procedure described for the synthesis of **24a** and was obtained in 74% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38−7.31 (m, 2H), 7.29−7.14 (m, 3H), 7.11−7.04 (m, 2H), 5.15 (s, 1H), 3.66 (s, 2H), 2.55 (s, 4H), 1.78 (s, 4H); MS (ESI) m/z 319 $[M + H]^+$.

(4-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanamine (24e). Starting from **23e**, the title compound was prepared following the procedure described for the synthesis of **24a** and was obtained in 68% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58−7.05 (m, 7H), 5.14 (s, 1H), 3.71 (s, 2H), 2.57 (s, 4H), 1.78 (s, 4H); MS (ESI) m/z 335 $[M + H]^+$.

(4-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanamine (24f). Starting from **23f**, the title compound was prepared following the procedure described for the synthesis of **24a** and was obtained in 85% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44−7.24 (m, 3H), 7.23−7.08 (m, 4H), 5.11 (s, 1H), 3.69 (s, 2H), 2.94 (br, 4H), 2.69 (br, 4H); MS (ESI) m/z 319 $[M + H]^+$.

(4-Chlorophenyl)(3-methoxy-4-(pyrrolidin-1-ylmethyl)-phenyl)methanamine (24g). Starting from **23g**, the title compound was prepared following the procedure described for the synthesis of **24a** and was obtained in 70% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33−7.24 (m, 5H), 6.89−6.86 (m, 2H), 5.11 (s, 1H), 3.78 (s, 3H), 3.62 (s, 2H), 2.54 (br, 4H), 1.75 (br, 4H); MS (ESI) m/z 331 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(1-(7-chloroquinolin-4-yl)-piperazin-4-ylmethyl)phenyl)methyl)-7-chloro-4-aminoquinoline (8).** A mixture of **24c** (0.21 g, 0.47 mmol), 4,7-dichloroquinoline (0.14 g, 0.70 mmol), and pyridinium chloride (81.0 mg, 0.70 mmol) in ethoxyethanol (10.0 mL) was heated to 135 °C and kept at the same temperature for 5 h. The reaction mixture was allowed to cool to 25 °C, the volatiles were removed, the residue was taken up in water, and the aqueous phase was extracted with chloroform (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, and the solvent was removed. The residue was purified by column chromatography (5% methanol in chloroform) to afford the title compound in 22% yield, as a pale yellow solid; mp (ethyl acetate/*n*-hexane) 139−141 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (d, J = 5.0 Hz, 1H), 8.47 (d, J = 5.3 Hz, 1H), 8.08−7.99 (m, 2H), 7.95 (d, J = 9.0 Hz, 1H), 7.73 (d, J = 9.0 Hz, 1H), 7.56 (d, J = 7.9 Hz, 1H), 7.47−7.31 (m, 5H), 7.30−7.20 (m, 3H), 6.82 (d, J = 5.1 Hz, 1H), 6.24 (d, J = 5.3 Hz, 1H), 5.68 (d, J = 4.2 Hz, 1H), 5.37 (s, 1H), 3.75 (s, 2H), 3.26 (br, 4H), 2.82 (br, 4H); MS (ESI) m/z 672 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9a).** 4,7-Dichloroquinoline (102 mg, 0.51 mmol) and pyridinium chloride (59 mg, 0.51 mmol) were added to a solution of **24a** (156 mg, 0.47 mmol) in 2-ethoxyethanol (10 mL), and the resulting solution was heated to 135 °C for 5 h. The solvent was evaporated and the residue was suspended in water (10 mL), the aqueous phase was extracted with chloroform, and the combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography (5% methanol in chloroform) to afford **9a** as a white solid (120 mg, 52%); mp (ethyl acetate/*n*-hexane) 134−136 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, J = 5.3 Hz, 1H), 7.99 (s, 1H), 7.73 (d, J = 8.9 Hz, 1H), 7.52 (d, J = 7.8 Hz, 1H), 7.40 (d, J = 9.0 Hz, 1H), 7.37−7.15 (m, 6H), 6.23 (d, J = 5.3 Hz, 1H), 5.66 (d, J = 4.0 Hz, 1H), 5.38 (d, J = 3.7 Hz, 1H), 3.74 (s, 2H), 2.60 (s, 4H), 1.81 (s, 4H); MS (ESI) m/z 496 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9b).** Starting from **24d** and 4,7-dichloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 56% yield as white solid; mp (ethyl acetate/*n*-hexane) 190−192 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (d, J = 5.3 Hz, 1H), 8.00 (s, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.52−7.18 (m, 6H), 7.11 (d, J = 7.8 Hz, 1H), 7.00 (d, J = 10.4 Hz, 1H), 6.23 (d, J = 5.3 Hz, 1H), 5.67 (d, J = 4.1 Hz, 1H), 5.35 (d, J = 4.1 Hz, 1H), 3.68 (s, 2H), 2.57 (s, 4H), 1.80 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.2, 159.9, 152.2, 149.3, 148.2, 142.7, 141.6, 135.4, 132.5, 130.7, 129.4, 128.8, 127.7, 126.1, 125.6, 123.1, 121.0, 117.3, 114.6, 114.3, 101.5, 61.5, 54.4, 52.7, 23.7; MS (ESI) m/z 480 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(morpholin-4-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9c).** Starting from **24b** and 4,7-dichloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 58% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (d, J = 5.2 Hz, 1H), 7.88 (s, 1H), 7.85 (d, J = 9.1 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 7.36−7.27 (m, 3H), 7.27−7.18 (m, 4H), 6.20 (d, J = 5.3 Hz, 1H), 5.85 (d, J = 4.0 Hz, 1H), 5.66 (d, J = 4.0 Hz, 1H), 3.66 (s, 4H), 3.55 (s, 2H), 2.47 (s, 4H); MS (ESI) m/z 512 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-6-methoxy-4-aminoquinoline (9d).** Starting from **24a** and 6-methoxy-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 30% yield as a pale yellow solid; mp (ethyl acetate/*n*-hexane) 103−105 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37 (d, J = 5.27 Hz, 1H), 7.95 (d, J = 9.4 Hz, 1H), 7.51 (d, J = 7.9 Hz, 1H), 7.36−7.22 (m, 7H), 6.98 (d, J = 2.0 Hz, 1H), 6.23 (d, J = 5.3 Hz, 1H), 5.66 (d, J = 3.8 Hz, 1H), 5.09 (d, J = 3.8 Hz, 1H), 3.93 (s, 3H), 3.74 (s, 2H), 2.61 (m, 4H), 1.81 (m, 4H); MS (ESI) m/z 492 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-6-methoxy-4-aminoquinoline (9e).** Starting from **24d** and 6-methoxy-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 45% yield as reddish solid; mp (ethyl acetate/hexane) 95−97 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (d, J = 5.1 Hz, 1H), 7.95 (d, J = 9.2 Hz, 1H), 7.48−7.22 (m, 7H), 7.12 (d, J = 7.6 Hz, 1H), 7.07−6.96 (m, 2H), 6.23 (d, J = 5.1 Hz, 1H), 5.67 (d, J = 3.7 Hz, 1H), 5.11 (s, 1H), 3.93 (s, 3H), 3.67 (s, 2H), 2.57 (s, 4H), 1.79 (s, 4H); MS (ESI) m/z 476 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(morpholin-4-ylmethyl)-phenyl)methyl)-6-methoxy-4-aminoquinoline (9f).** Starting from **24b** and 6-methoxy-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 60% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (d, J = 5.27 Hz, 1H,), 7.88 (d, J = 9.1 Hz, 1H), 7.46 (d, J = 7.9 Hz, 1H), 7.32−7.20 (m, 7H), 7.13 (d, J = 2.6 Hz, 1H), 6.20 (d, J = 5.3 Hz, 1H), 5.66 (d, J = 4.1 Hz, 1H), 5.57 (d, J = 4.1 Hz, 1H), 3.81 (s, 3H), 3.71−3.68 (m, 4H), 3.57 (s, 2H), 2.51−2.48 (m, 4H); MS (ESI) m/z 508 $[M + H]^+$.

***N*-((3-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-trifluoromethyl-4-aminoquinoline (9g).** Starting from **24a** and 7-trifluoromethyl-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 30% yield as a pale yellow solid; mp (ethyl acetate/hexane) 84−86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, J = 4.8 Hz, 1H), 8.28 (s, 1H), 7.97 (d, J = 8.2 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.36−7.17 (m, 6H), 6.31 (d, J = 5.2 Hz, 1H), 5.68 (d, J = 3.9 Hz, 1H), 5.62 (s, 1H), 3.74 (s, 2H), 2.61 (s, 4H), 1.80 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.3, 148.1, 147.8, 142.3, 140.4, 137.5, 135.4, 134.8, 131.5, 130.7, 128.7, 128.4, 128.0, 127.6, 125.9, 125.7, 121.1, 120.8, 120.6, 102.5, 61.5, 56.9, 54.5, 23.8; MS (ESI) m/z 530 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9h).** Starting from **24e** and 4,7-dichloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was in 46% yield as white solid; mp (ethyl acetate/hexane) 215−217 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (d, J = 5.2 Hz, 1H), 8.00 (s, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.51 (d, J = 7.9 Hz, 1H), 7.45−7.09 (m, 7H), 6.22 (d, J = 5.3 Hz, 1H), 5.67 (d, J = 4.0 Hz, 1H), 5.33 (s, 1H), 3.74 (s, 2H), 2.60 (s, 4H), 1.81 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2, 149.3, 148.3, 140.8, 139.0, 137.7, 135.3, 134.8, 134.3, 131.4, 129.6, 129.3, 128.9, 128.4, 126.0, 125.9, 121.1, 117.4, 101.6, 61.3, 57.0, 54.6, 23.8; MS (ESI) m/z 496 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9i).** Starting from **24f** and 4,7-dichloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 78% yield as white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.41 (d, J = 4.7 Hz, 1H), 7.95 (s, 1H), 7.43 (m, 5H) 7.09 (d, J = 7.6 Hz, 1H), 6.98 (d, J = 10.3 Hz, 1H), 6.21 (d, J = 4.7 Hz, 1H), 5.67 (s, 1H), 5.51 (s, 1H), 3.65 (s, 2H), 2.54 (m, 4H), 1.77 (m, 4H); MS (ESI) m/z 480 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-chloro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-6-methoxy-4-aminoquinoline (9j).** Starting from **24e** and 6-methoxy-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 40% yield as a pale yellow solid; mp (ethyl acetate/hexane) 93−95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (d, J = 5.1 Hz, 1H), 7.91 (d, J = 9.2 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 7.36−7.17 (m, 7H), 7.07 (s, 1H), 6.20 (d, J = 5.1 Hz, 1H), 5.67 (d, J = 3.4 Hz, 1H), 5.37 (d, J = 3.4 Hz, 1H), 3.85 (s, 3H), 3.72 (s, 2H), 2.59 (s, 4H), 1.79 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.3, 148.6, 147.6, 144.2, 141.2, 139.4, 137.3, 134.6, 134.1, 131.6, 131.3, 129.4, 129.1, 128.5, 126.1, 120.7, 119.6, 101.6, 99.6, 61.3, 57.0, 55.9, 54.6, 23.8; MS (ESI) m/z 492 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-6-methoxy-4-aminoquinoline (9k).** Starting from **24f** and 6-methoxy-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 50% yield as white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (d, J = 5.0 Hz, 1H), 7.94 (d, J = 9.2 Hz, 1H), 7.49−7.20 (m, 6H), 7.17−6.93 (m, 3H), 6.22 (d, J = 5.0 Hz, 1H), 5.69 (d, J = 3.8 Hz, 1H), 5.18 (s, 1H), 3.91 (s, 3H), 3.68 (s, 2H), 2.58 (s, 4H), 1.80 (s, 4H); MS (ESI) m/z 476 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-fluoro-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-trifluoromethyl-4-aminoquinoline (9l).** Starting from **24f** and 7-trifluoromethyl-4-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 35% yield as white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, 1H, J = 5.0 Hz), 8.30 (s, 1H), 7.91 (d, 1H, J = 7.4 Hz), 7.62 (d, 1H, J = 8.8 Hz) 7.43−7.26 (m, 5H), 7.11 (d, 1H, J = 7.9 Hz), 7.0 (d, 1H, J = 10.3 Hz), 6.32 (d, 1H, J = 5.0 Hz), 5.71 (d, 1H, J = 4.1), 5.46 (d, 1H, J = 3.8 Hz), 3.67 (s, 2H), 2.56 (m, 4H), 1.79 (m, 4H); MS (ESI) m/z 514 $[M + H]^+$.

***N*-((4-Chlorophenyl)(3-methoxy-4-(pyrrolidin-1-ylmethyl)-phenyl)methyl)-7-chloro-4-aminoquinoline (9m).** Starting from **24g** and 4,7-dichloroquinoline, the title compound was prepared following the procedure described for the synthesis of **9a** and was obtained in 53% as white solid; mp (ethyl acetate/hexane) 188−190 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (d, J = 5.3 Hz, 1H), 7.99 (d, J = 2.1 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.44−7.20 (m, 6H), 6.86 (d, J = 7.7 Hz, 1H), 6.79 (d, J = 1.5 Hz, 1H), 6.24 (d, J = 5.3 Hz, 1H), 5.68 (d, J = 4.3 Hz, 1H), 5.36 (d, J = 4.1 Hz, 1H), 3.76 (s, 3H), 3.67 (s, 2H), 2.60 (s, 4H), 1.80 (s, 4H); MS (ESI) m/z 492 $[M + H]^+$.

(Phenyl)bis(3-chloro-4-methylphenyl)methanol (28). A solution of **21a** (2.4 mL, 18.38 mmol) in THF (5 mL) was added dropwise to a suspension of magnesium turnings (446 mg, 18.38 mmol) and iodine (catalytic amount) in freshly distilled THF (6 mL), and an exothermic reaction was observed. The mixture was stirred at room temperature until the complete formation of the Grignard reagent; thereafter, a solution of **27** (1.0 g, 7.35 mmol) in dry THF was added dropwise, and the mixture was stirred at 25 °C for 18 h. The reaction mixture was quenched with 20% ammonium chloride solution. The aqueous layer was extracted with diethyl ether, and the combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The crude residue was purified by flash chromatography (5% ethyl acetate in *n*-hexane) to give **28** in 71% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41−7.24 (m, 7H), 7.23−7.01 (m, 4H), 2.78 (br, 1H), 2.40 (s, 6H).

(Phenyl)bis(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methanol (29). NBS (997 mg, 5.60 mmol) and AIBN (92 mg, 0.56 mmol) were added to a solution of **28** (1.0 g, 2.8 mmol) in CCl_4 (15 mL), and the resulting suspension was heated at 85 °C for 2 h. After cooling to room temperature, the formed succinimide was filtered off and the solvent was evaporated. Triethylamine (1.4 mL, 9.90 mmol) and pyrrolidine (545 μL, 6.61 mmol) were added to a solution of the above bromide (1.1 g, 2.1 mmol) in dry acetonitrile, at 0 °C. Thereafter, the reaction mixture was heated to 80 °C for 4 h. The solvent was evaporated under reduced pressure, and the residue was treated with water and extracted with ethyl acetate (4 × 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by flash column chromatography (2% ethyl acetate in dichloromethane) to afford the title compound in 30% yield as a pale yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45−7.30 (m, 4H), 7.31−7.19 (m, 5H), 7.17−7.04 (m, 2H), 3.73 (s, 4H), 2.59 (s, 8H), 1.77 (s, 8H); MS (ESI) m/z 495 $[M + H]^+$.

***N*-((Phenyl)bis(3-chloro-4-(pyrrolidin-1-ylmethyl)phenyl)-methyl)-7-chloro-4-aminoquinoline (10).** Starting from **29** and 4-amino-7-chloroquinoline, the title compound was prepared following the procedure described for the synthesis of **4a** and was obtained in 40% yield as a pale yellow solid; mp (ethyl acetate/*n*-hexane) 109−111 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (d, J = 5.4 Hz, 1H), 7.97 (d, J = 2.0 Hz, 1H), 7.74 (d, J = 9.0 Hz, 1H), 7.43 (d, J = 7.9 Hz, 3H), 7.31 (s, 7H), 7.20−2.16 (m, 2H), 6.17 (s, 1H), 5.88 (d, J = 5.4 Hz, 1H), 3.72 (s, 4H), 2.59 (s, 8H), 1.80 (s, 8H); MS (ESI) m/z 655 $[M + H]^+$.

■ COMPUTATIONAL DETAILS

Molecule Preparation. Three-dimensional structure building was carried out on Cooler Master Centurion 5 (Intel Core2 Quad CPU Q6600 @ 2.40 GHz) with Ubuntu 10.04 LTS (long-term support) operating system running Maestro 9.2 (Schrödinger, LLC, New York, NY, 2011). Molecular energy minimizations were performed by MacroModel[43] using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA) force field 2005.[44,45] The solvent effects were simulated using the analytical Generalized-Born/Surface-Area (GB/SA) model,[46] and no cutoff for nonbonded interactions was selected. Polak−Ribiere conjugate gradient (PRCG)[47] method with 5000 maximum iterations and 0.001 gradient convergence threshold was employed.

Ab Initio QM Calculations. Structure optimization at higher theory levels was performed by Jaguar (version 7.8, Schrödinger, LLC, New York, NY, 2011). All QM calculations were at the Hartree−Fock (HF) level using the 6-31++G* basis for all atoms and performed on Cooler Master Centurion 5 (Intel Core i5−2500 CPU @ 3.30 GHz Quad) with Ubuntu 10.04 LTS (long-term support) OS. The parameters on SCF window were changed in accurate for accuracy level, the maximum iterations were set to 100 and the energy change of 5×10^{-7} hartree was employed. Concerning the optimization window, 500 as maximum steps and default convergence criteria were used. The calculations were performed, taking into account the solvation effects and in vacuum. Solvation was included by solving the Poisson−Boltzmann equations with a realistic molecular surface (van der Waals radius plus solvent radius about each atom) using the Jaguar solvation model (PBF).[48] We used a dielectric constant for water of 80.37 and a probe radius of 1.4 Å for water. The properties established were ESP charges and surfaces. The output files were saved as .mae and the charges analyzed in Maestro suite 2011.

P450 Site of Metabolism. Sites of metabolism for all molecules reported in this study were predicted by P450 site of metabolism (P450 SOM) workflow[33] implemented in Schrödinger suite 2011 (Schrödinger, LLC, New York, NY, 2011). For each molecule P450 SOM performed a calculation of intrinsic reactivity coupled to induced fit docking (IFD)[46] for the selected isoform of cytochrome. The reactivity

rules have been parametrized in P450 SOM to predict atomic reactivity profiles for promiscuous P450 enzymes that are thought to be mostly independent of structural restrictions on the binding poses. The reactivity is predicted with a linear free energy approach based on the Hammett and Taft scheme, where the reactivity of a given atom is the sum of a baseline reactivity rate and a series of perturbations determined by the connectivity.[35] The induced-fit docking approach is a variation on the normal protocol.[49] The initial sampling is enhanced by generating multiple starting conformations, so that a wider range of poses is found in the initial docking stage. The initial docking includes van der Waals scaling of the receptor and alanine mutation of the most flexible residues. In the Prime[49] refinement stage, any residue with an atom within 5 Å of any ligand pose is selected for side-chain prediction. The subsequent minimization includes the ligand, side chains, and backbones of the flexible residues. The ligand is then redocked into each of the low-energy protein conformations, determined by a 40 kcal/mol cutoff. There is no final scoring stage, because all poses are considered in determining which atoms are sufficiently accessible to the reactive heme iron. Any atom within the cutoff distance of 5 Å from the heme iron is considered as a potential site of metabolism.[35]

Parasite Growth and Drug Susceptibility Assay. *P. falciparum NF54 and K1 Strains.* P. *falciparum* drug-sensitive strain NF54 and drug-resistant strain K1 were cultivated in a variation of the medium previously described, consisting of RPMI 1640 supplemented with 0.5% ALBUMAX II, 25 mM HEPES, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine, and 100 μg mL^{-1} neomycin.[50] Human erythrocytes served as host cells. Cultures were maintained in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers at 37 °C. Compounds were dissolved in DMSO (10 mg mL^{-1}), diluted in hypoxanthine-free culture medium, and titrated in duplicate over a 64-fold range in 96-well plates. Infected erythrocytes (1.25% final hematocrit and 0.3% final parasitemia) were added into the wells. After 48 h incubation, 0.5 μCi of [^{3}H]hypoxanthine was added per well, and the plates were incubated for an additional 24 h. Parasites were harvested onto glass-fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich). The results were recorded and expressed as a percentage of the untreated controls. Fifty percent inhibitory concentrations (IC_{50}) were estimated by linear interpolation.[51] Assays were run in duplicate and repeated at least once. Artesunate and chloroquine were used as positive controls.

P. falciparum D10 and W2 Strains. The CQS (D10) and the CQR (W2) strains of *P. falciparum* were sustained in vitro as described by Trager and Jensen.[50] Parasites were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMax (Invitrogen, Milan, Italy), 0.01% hypoxanthine, 20 mM HEPES, and 2 mM glutamine. All cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O_2, 5% CO_2, and 94% N_2. Compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is nontoxic to the parasite). Drugs were placed in 96 well flat-bottom microplates (COSTAR) and serial dilutions made. Asynchronous cultures with parasitemia of 1−1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method in control and drug-treated cultures. Antiplasmodial activity is expressed as the 50% inhibitory concentrations (IC_{50}). Each IC_{50} value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

Inhibition of Hemin Crystallization (BHIA Assay). β-Hematin formation was assayed by a spectrophotometric microassay BHIA (β-hemeatin inhibitory assay) previously reported.[52] Hemin in DMSO (0.4 μmol/well) was distributed in 96-well microplates. Compounds dissolved in water or DMSO were added in doses ranging from 0.25 to 8 mol equiv to hemin. In control wells, water or DMSO were added, maintaining in each well the same DMSO:water ratio of 1:1 in a final volume of 100 μL. β-Hematin formation was initiated by the addition of 100 μL of 8 M acetate buffer (pH 5). Plates were incubated at 37 °C for 18 h to allow the complete reaction. The drug concentration required to inhibit β-hemeatin formation by 50% (IC_{50}) was determined.

Measurements of CQ Transport in *X. laevis* Oocytes Expressing PfCRT. Expression of wild-type and CQ resistance-conferring forms of PfCRT (from the strains D10 and Dd2, respectively) at the plasma membrane of *X. laevis* oocytes was achieved as described previously.[17] Briefly, oocytes were injected with cRNA-encoding PfCRT (20 ng per oocyte), and the uptake of [^{3}H]CQ (0.3 μM; 20 Ci/mmol; ARC) was measured 3−5 days postinjection as detailed elsewhere.[17] Uptake measurements were made over 1−2 h at 27.5 °C in medium that contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM MES, 10 mM Tris-base (pH 6.0), and 15 μM unlabeled CQ. Statistical comparisons were made with the Student's *t*-test for unpaired samples or with ANOVA in conjunction with Tukey's multiple comparisons test.

In Vivo Antimalarial Evaluation. *Plasmodium berghei in Vivo Model.* From a donor mouse with approximately 30% parasitaemia ($PbANKA\text{-}GFP_{CON}$),[53] heparinized blood (containing 50 μL of 200 units mL^{-1} heparin) was taken and diluted in physiological saline to 10^8 parasitized erythrocytes per mL. Of this suspension, 0.2 mL was injected intravenously (iv) into experimental groups of five mice, and a control group of five mice. Multiple dosing regimens were carried out as previously described. Briefly, 6, 24, 48, and 72 h after infection (6 h time point omitted during 3 times of treatment), the experimental groups were treated with a single oral daily dose. Twenty-four hours after the last drug treatment (96 h after infection), 1 μL of tail blood was taken and the parasitaemia determined with a FACScan. For single-dose treatment, mice were dosed 24 h after infection and the parasitemia was assessed 48 h after dosing (72 h after infection). The difference between the mean value of the control group and those of the experimental groups was calculated and expressed as a percent relative to the control group (= activity). The survival of the animals was monitored up to 30 d. Mice surviving for 30 d were checked for parasitaemia by slide reading. A compound was considered curative if the animal survived to day 30 postinfection with no detectable parasites.

Toxicity Evaluation. *Materials.* Dulbecco's Modified Eagle's Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM), trypsin solution, and all the solvents used for cell culture were purchased from Lonza (Switzerland). Glutaraldehyde (25% solution in water) and sodium cacodylate trihydrate (98%) were supplied from Sigma-Aldrich (Germany). Mouse immortalized fibroblasts NIH3T3 were purchased from American Type Culture Collection (Manassas, VA) and human caucasian hepatocyte carcinoma cells HepG2 from The European Collection of Cell Cultures (UK). The mutagenicity assay was supplied by Biologik srl (Trieste, Italy).

Cell Cultures and Cytotoxicity Assay. NIH3T3 and HepG2 were utilized for cytotoxicity experiments. NIH3T3 were maintained in DMEM and HepG2 in EMEM at 37 °C in a humidified atmosphere containing 5% CO_2. The culture media were supplemented with 10% fetal calf serum (FCS), 1% L-glutamine−penicillin−streptomycin solution, and 1% MEM nonessential amino acid solution. Once at confluence, cells were washed with 0.1 M PBS, taken up with trypsin−EDTA solution, and then centrifuged at 1000 rpm for 5 min. The pellet was resuspended in medium solution (dilution 1:15). Cell viability after 24 h of incubation with the different compounds was evaluated by Neutral Red Uptake (Sigma-Aldrich, Switzerland) by the procedure previously reported.[54] The data processing included the Student's *t* test with $p < 0.05$ taken as significance level.

Mutagenicity Assay: Ames Test. The TA100 and TA98 strains of *Salmonella typhimurium* were utilized for mutagenicity assay. Approximately 107 bacteria were exposed to 6 concentrations of each test compound, as well as a positive and a negative control, for 90 min in medium containing sufficient histidine to support approximately two cell divisions. After 90 min, the exposure cultures were diluted in pH indicator medium lacking histidine and aliquoted into 48 wells of a 384-well plate. Within two days, cells which had undergone the reversion to *His* grew into colonies. Metabolism by the bacterial colonies reduced the pH of the medium, changing the color of that well. This color change can be detected visually or by microplate reader. The number of wells

containing revertant colonies were counted for each dose and compared to a zero-dose control. Each dose was tested in six replicates.

Pharmacokinetic Evaluation. *Drug Administration and Plasma and Liver Sampling.* Male CD1 albino mice, 25−30 g (Charles River, Italy), were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L., n.116, G.U., suppl.40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, 0J L358, 1, Dec 12, 1987; Guide for the Care and Use of Laboratory, U.S. National Research Council, 1996). Mice were given **4b** orally (50 mg/kg, dissolved in ethanol:Tween-80 (30:70 v/v) and then diluted with water) and were killed by decapitation under deep anesthesia 15 and 30 min and 1, 2, 3, 6, 24, and 48 h after dosing. Mixed arteriovenous trunk blood was collected in heparinized tubes and centrifuged at 3000*g* for 10 min, and the plasma was stored at −20 °C. Liver was removed immediately, blotted with paper to remove excess surface blood, and quickly frozen in dry ice. Compound **4b** was extracted from plasma and liver homogenate by a solid−liquid extraction procedure and quantified by RP HPLC with fluorescence detection (lambda excitation = 255 nm; lambda emission = 365 nm). Briefly, to 0.05 mL of plasma or liver homogenate (1 g/50 mL, 1 M CH_3COONa/methanol, 40:60 v/v) were added 0.15 mL of 1 M CH_3COONa and 1 mL of hexane containing 1% of isoamilic alcohol. After agitation and centrifugation, the supernatant was re-extracted with 0.15 mL of mobile phase and then recentrifugated. A 0.1 mL amount of mobile phase was injected into the chromatographic column. Separation was performed on a Brownlee Lab Spheri 5 RP 8 (4.6 × 250 mm; 5 μm). The mobile phase consisted of 0.5 M MeCOONa/MeCN/MeOH/Et_3N (19.9/40/40/0.1, v/v) at pH 7, delivered isocratically at a flow rate of 1 mL/min. Approximate retention times were 8.5 min for compound **4b** and 5.6 min for the internal standard. Standard curves were prepared daily by spiking blank mouse plasma and liver homogenate. The within-day and day-to-day precision varied between −6% and 10%, regardless of the tissue analyzed. The in vitro distribution of compound **4b** between blood cells and plasma was studied by incubating different concentrations (250−1000 ng/mL) with fresh mouse blood and plasma at 37 °C and analyzing plasma concentrations. The ratio of drug in plasma (control) to the concentration of drug in plasma fraction (obtained from whole blood) averaged 1.9 ± 0.3, indicating preferential partitioning of **4b** into red blood cells. Standard compartmental analysis was performed for calculation of the usual pharmacokinetic parameters. Plasma and liver concentrations were expressed as mean ± SD.

Electrophisiological Studies on Isolated Heart. *Materials and Methods.* All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy (Feb 8, 2012). Principles of laboratory animal care (NIH publication No. 85−23, revised 1996) were followed. Male Sprague−Dawley rats (300−400 g) were anesthetized with a mixture of Ketavet (0.3 mg/kg; Gellini, Italy) and Rompum (0.08 mg/kg; Bayer, Germany) containing heparin (5000 U/kg), decapitated, and bled. The hearts, spontaneously beating, were rapidly explanted and mounted on a Langendorff apparatus for retrograde perfusion via the aorta at a constant flow rate of 10 mL/min using a peristaltic pump (Alitea C4Midi, Watson-Marlowe, Sweden), as described elsewhere.[55] The perfusion medium was a Krebs−Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.5, Na pyruvate 2, and EDTA 0.5, bubbled with a 95% O_2−5% CO_2 gas mixture (pH 7.4), and kept at 37 °C. The hearts were allowed to equilibrate for at least 15 min before drug exposure. Heart contractility was measured as left ventricle pressure (LVP) by means of a latex balloon attached to a short polythene tubing, inserted into the left ventricle via the mitral valve and connected to a pressure transducer (BLPR, WPI, Germany). The balloon was inflated with deionized water from a microsyringe until a left ventricular end diastolic pressure of 10 mmHg was obtained. A surface electrocardiogram (ECG) was recorded by means of two steel electrodes, one placed on the apex and the other on the left atrium of the heart. The ECG analysis included the following measurements: RR (cycle length), HR (frequency), PQ (atrioventricular conduction time), QRS (intraventricular conduction time), and QT (duration of ventricular depolarization and repolarization, i.e., the action potential duration). LVP and ECG were recorded with a digital PowerLab data acquisition system (PowerLab 8/30; ADInstruments, Castle Hill, Australia) and analyzed by using Chart Pro for Windows software (Power Lab; ADInstruments). The sampling rate was 1 kHz. LVP was calculated by subtracting the left ventricular diastolic pressure from the left ventricular systolic pressure. As the QT interval is affected by heart rate (shortened with more rapid heart rates), correction factors are routinely used to avoid confounding effects of heart rate changes. Bazett's formula was used for any heart rate changes ($QTc = QT/(RR)^{1/2}$).

Chemicals. Terfenadine was supplied by Sigma Chimica, Italy. **4b** was dissolved in water, and terfenadine in dimethyl sulfoxide (DMSO). The resulting concentrations of DMSO (below 0.03%) did not affect the responses (data not shown).

Statistical analysis. Data are reported as means ± SEM; *n* (indicated in parentheses) represents the number of rat hearts. Analysis of data were accomplished using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Statistical analyses and significance as measured by repeated ANOVA (followed by Dunnet's post test), were obtained using GraphPad InStat version 3.06 (GraphPad Software) as appropriate. In all comparisons, $P < 0.05$ was considered significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1. Tables S1 and S2 (elemental analyses). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ACT, artemisinin-based combination therapy; CQ, chloroquine; DV, digestive vacuole; PfCRT, *Plasmodium falciparum* chloroquine-resistance transporter; CLT, clotrimazole; CQS, CQ-sensitive; CQR, CQ-resistant; AIBN, α,α'-azobis(isobutyronitrile); IFD, induced-fit docking; BHIA, β-hematin inhibitory activity; LVP, left ventricle pressure; *N*,*N*-DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide

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