Design and Synthesis of Potent Antimalarial Agents Based on Clotrimazole Scaffold: Exploring an Innovative Pharmacophore

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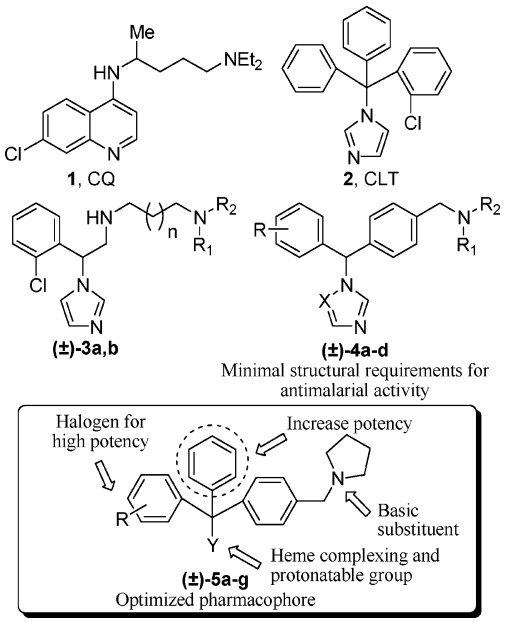
Abstract: Identification of new molecular scaffolds structurally unrelated to known antimalarials may represent a valid strategy to overcome resistance of *P. falciparum* (*Pf*) to currently available drugs. We describe herein the investigation of a new polycyclic pharmacophore, related to clotrimazole, to develop innovative antimalarial agents. This study allowed us to discover compounds characterized by a high in vitro potency, particularly against *Pf* CQ-resistant strains selectively targeting free heme, which are easy to synthesize by low-cost synthetic strategies.

The increasing resistance of the malaria parasite *Plasmodium falciparum* (*Pf*) to currently available drugs and especially to chloroquine (CQ, **1**, Chart 1) demands a continuous effort to develop new effective therapeutic options.[1] Identification of new molecular scaffolds structurally unrelated to existing antimalarial agents represents a valuable strategy to bypass resistance phenomena.

During the intraerythrocytic life cycle of *Pf*, toxic free heme is released into the parasite acidic food vacuole (FV) following host hemoglobin degradation. As a result of its high toxicity, disposal of free heme represents a crucial step for *Pf* survival, and even small perturbations of its detoxification mechanisms could lead to *Pf* death due to the generation of reactive oxygen species. The main pathway of free heme detoxification is the crystallization into inert hemozoin, which accounts for most of the heme clearance in the FV.[2] The remaining heme diffuses through the FV membrane into the cytoplasm where it is inactivated by reduced glutathione.[3]

Interaction with free heme is thought to be responsible for the activity of two of the most potent classes of antimalarial agents known to date, namely, 4-aminoquinolines and endoperoxides. Indeed, the production of reactive radical species as a direct consequence of endoperoxide interaction with Fe(II)-heme, and the formation of complexes between CQ and free heme, prevents its detoxification into hemozoin, causing oxidative stress in the parasite.[4] Consequently, the induction of oxidative stress may represent a promising rationale for antimalarial chemotherapy. Accordingly, free heme represents a valuable target in the design of new antimalarial agents as the lack of interaction with a specific protein target can decrease the potential of inducing resistance under drug pressure. Furthermore, the absence of free heme in the human host could guarantee *Pf* specificity.

Chart 1. Title and Reference Compounds[a]



[a] R, R_1, R_2, X, Y, and *n* are as defined in Table 1.

Clotrimazole (CLT, **2**), a well-known antimycotic drug, is endowed with low in vitro antimalarial activity (W2, IC_{50} = 0.55 μM).[5,6] Several studies, aimed at clarifying its mechanism of action, demonstrated that CLT is able to inhibit the crystallization of free Fe(III)-protoporphyrine (FP; ϵ_{400}) into β-hematin and that, in the same conditions, it forms in vitro complexes with heme in which the imidazole ring results to be a Fe(III) axial ligand.[7] Interestingly, it has also been reported that CLT, interacting with free heme, induces a 10-fold potentiation of heme-dependent hemolysis with respect to CQ.[8] Finally, a recent report suggests that in the presence of H_2O_2, CLT inhibits *Pf* hemoperoxidase, which contains heme as a prosthetic group, by a mechanism based on CLT-one electron oxidation product.[9]

Based on the above-mentioned CLT properties, we used its scaffold to develop novel antimalarial agents, characterized by the lack of interaction with a definite *Pf* or host target protein and with improved pharmacological profile over CLT.

It is known that CLT elicits its antimycotic activity through the inhibition of fungi cytochrome P450 14α-lanosterol-demethylase (14-LD). On the other hand, interaction with human P450 cytochromes, and the subsequent interference with its own metabolism and that of a number of endogenous and exogenous chemicals, represents a critical issue for CLT systemic administration. Inhibition of cytochrome P450 is linked to the coordination of the nucleophilic nitrogen of the azole heterocyclic ring to the P450 heme iron in the ferric state. Accordingly, the main goal of this work was the design of CLT-related free heme selective ligands, with improved antimalarial potency and

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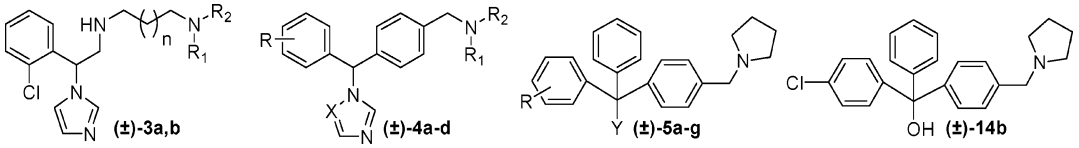
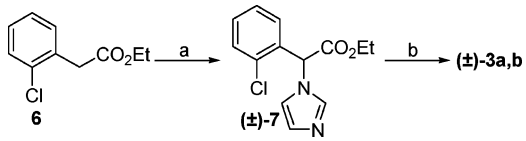
Table 1. Antiplasmodial Activity of Compounds **3a**,**b**, **4a**−**d**, **5a**−**g**, and **14b**



							prevalent ionic form[a,b]		IC$_{50}$[c] (μM)			
cmpd	*n*	R	R$_1$	R$_2$	X	Y	% at pH 7.4	% at pH 5.5	D10[d]	W2[e]	3D7[d]	K1[e]
3a	1		Et	Et			DP (50.5)	TP (86.6)	6.41	4.46	1.90	3.08
3b	0		−(CH$_2$)$_4$−				P (80.5)	TP (79.9)	9.43	3.05	2.61	0.92
4a		2-Cl	−(CH$_2$)$_4$−		CH		P (91.6)	DP (87.1)	2.18	1.70	0.011	0.11
4b		3-Cl	−(CH$_2$)$_4$−		CH		P (89.4)	DP (89.9)	1.42	1.03	0.11	0.20
4c		4-Cl	−(CH$_2$)$_4$−		CH		P (88.8)	DP (90.5)	1.66	1.13	0.31	0.17
4d		2-Cl	Et	Et	N		P (99.4)	P (99.9)	>10	>10	>10	0.79
5a		3-Cl				1*H*-imidazole	P (93.1)	DP (84.0)	0.23	0.10	0.047	0.023
5b		4-Cl				1*H*-imidazole	P (92.7)	DP (84.9)	0.13	0.059	0.0070	0.0050
5c		4-Cl				1*H*-1,2,4-triazole	P (99.1)	P (99.9)	6.92	3.83	8.91	2.15
5d		4-Cl				pyrrolidine	DP (62.3)	DP (99.2)	4.81	2.57	0.94	1.99
5e		4-Cl				CN	P (99.1)	P (100)	nt[f]	nt	5.07	2.64
5f		4-OMe				1*H*-imidazole	P (90.7)	DP (88.4)	2.04	1.84	8.13	5.93
5g		4-Cl				−CH$_2$-1*H*-imidazole	P (83.3)	DP (93.9)	nt	nt	0.75	0.52
14b		4-Cl				OH	P (99.5)	P (100)	8.3	nt	>10	2.17
CLT, **2**							N (95.0)	P (80.6)	0.55	0.49	0.060	0.25
CQ, **1**							P (91.9)	DP (87.4)	0.022	0.28	0.010	0.26

[a] TP = triprotonated form; DP = diprotonated form; P = protonated form; and N = neutral form (ACD/pKa DB version 9.00 software (Advanced Chemistry Development, Inc., Toronto, Canada)). [b] Percentage of prevalent ionic form in brackets. [c] IC$_{50}$ values are the mean of at least three determinations. Standard errors were all within 10% of the mean. [d] CQ-S clone. [e] CQ-R clone. [f] nt = not tested.

Scheme 1[a]



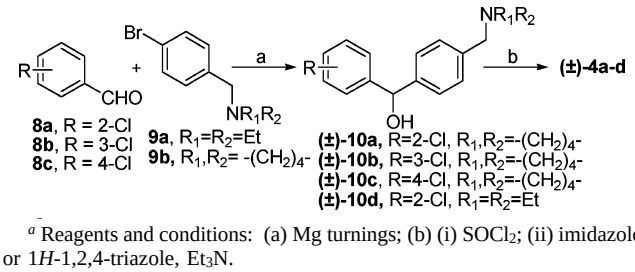
[a] Reagents and conditions: (a) (i) NBS, AIBN; (ii) imidazole sodium salt; (b) (i) DIBAL; (ii) 1-(2-aminoethyl)pyrrolidine (for **3a**) or 3-diethylamino-propylamine (for **3b**), 1% AcOH in MeOH, NaBH$_3$CN.

minimized side effects. To achieve activity against both CQ-sensitive (CQ-S) and CQ-resistant (CQ-R) *Pf* strains, we reasoned that an effective antimalarial should rapidly accumulate and be activated inside the microenvironment of the parasite FV (i.e., pH 5.5; redox milieu −250 mV; presence of free heme), generating toxic radical intermediates. On these bases, we designed novel antimalarials characterized by improved penetration into the FV where the acidic environment promotes the spontaneous oxidation of haemoglobin-derived Fe(II) heme to Fe(III) heme with the formation of superoxide ions generating H_2O_2 and hydroxyl radicals. Accordingly, our design strategy focused on compounds with appropriate electronic features that would generate radical intermediates in the *Pf* FV environment.

With these goals in mind, we dismantled the structure of CLT to identify the minimal structural requirements responsible for antimalarial activity, introducing a series of substituents able to modulate (i) the propensity to form active radicals, (ii) the ability to selectively complex free heme, and (iii) the FV penetration (Chart 1 and Table 1). The designed compounds **3**−**5** are characterized by the presence of a phenyl- (**3a**,**b**), diphenyl- (**4a**−**d**), or triphenylmethyl (**5a**−**g**) moiety in which we introduced a protonatable group to increase tropism for the FV. Specific heme-complexing groups were also exploited (Chart 1).

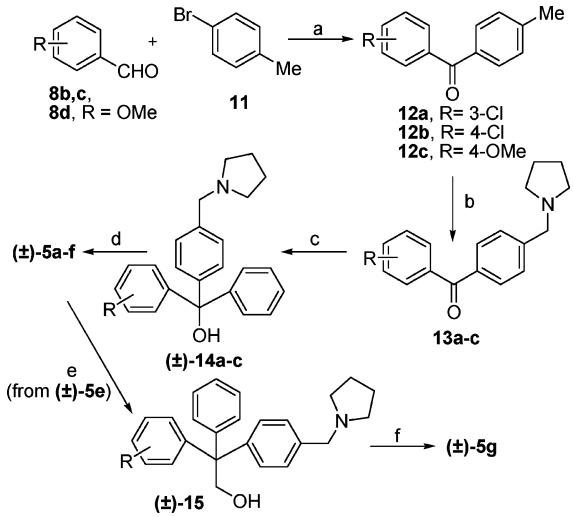
Synthesis of the novel compounds is shown in Schemes 1−3. All synthesized compounds were tested in vitro as racemates against a series of *Pf* strains, namely, the CQ-S D10 and 3D7 and the CQ-R W2 and K1 strains. Experimental details are provided as Supporting Information.

Scheme 2[a]



[a] Reagents and conditions: (a) Mg turnings; (b) (i) SOCl$_2$; (ii) imidazole or 1*H*-1,2,4-triazole, Et$_3$N.

Scheme 3[a]



[a] Reagents and conditions: (a) Mg turnings; (b) (i) NBS, AIBN; (ii) pyrrolidine, Et$_3$N; (c) PhMgBr; (d) (i) SOCl$_2$; (ii) imidazole (for **5a**,**b**, **5f**), 1*H*-1,2,4-triazole (for **5c**), pyrrolidine (for **5d**), Et$_3$N or TMSCN, TiCl$_4$ (for **5e**); (e) (i) DIBAL; (ii) NaBH$_4$; (f) (i) SOCl$_2$; (ii) imidazole, K$_2$CO$_3$, NaI.

In general, the antimalarial potency trend for the three series of compounds is triphenylmethyl **5** > diphenylmethyl **4** > monophenylmethyl **3**. In particular, compounds **3a** and **3b** were found to be significantly less potent than CLT against both CQ-S and CQ-R *Pf* strains, while an increase of activity was noted

Table 2. β-Hematin Inhibitory Activity Assay of Compounds **5b**, CLT (**2**), and CQ (**1**)

cmpd	IC_{50}[a]
5b	2.53
CLT, **2**	2.50
CQ, **1**	1.69

[a] Molar equivalents of the compound relative to hemin able to inhibit β-hematin formation by 50%. IC_{50} values are the mean of at least three determinations. Standard errors were all within 10% of the mean.

when a further aromatic ring was introduced. The IC_{50} values of compounds **4a**−**c** are in the range of 0.010 and 0.30 μM against 3D7 and K1 *Pf* strains, suggesting that the presence of a diphenylmethyl moiety is a minimal structural requirement for antimalarial activity. The potency strongly increased when an additional aromatic ring was introduced (**5a** vs **4b** and **5b** vs **4c**). The electronic properties of the aromatic systems, modulated by the introduction of specific substituents, affected the antimalarial potency. Indeed, while the position of the chlorine substituent had a weak effect on the activity of compounds **4a**−**c**, in the triarylmethyl series **5**, the Cl position was responsible for a fine-tuning of the antimalarial activity against CQ-S and CQ-R strains (**5a** vs **5b)**, according to the hypothesized generation of a radical (i.e., planar) intermediate. On the other hand, a dramatic loss of activity could be observed when an electron-withdrawing chlorine atom was replaced by an electron-donating methoxy group (**5b** vs **5f**). Following our design hypothesis, the imidazole ring was considered as the heme interacting group of choice. Indeed, we hypothesized a dual role for the imidazole moiety, heme-complexing group, since it is known to give a strong axial interaction with Fe(III) and substrate for a one-electron oxidation reaction, generating a trityl radical. Accordingly, interference with heme interacting properties by substituting imidazole with pyrrolidine (**5d**), hydroxy (**14b**), or cyano (**5e**) groups caused a drastic loss of activity. Moreover, a drop in activity versus CQ-S and CQ-R strains was also registered linking the imidazole system to the triarylmethyl moiety through a bridged methylene (**5b** vs **5g**), interfering with the hypothesized generation of the trityl radical. Interestingly, replacement of the imidazole by a triazole (**4d** and **5c**), another heme coordinating group present in known antifungal agents, strongly decreased antimalarial potency. This could be explained by the fact that at the FV pH (5.5) the imidazole group is protonated while the triazole group is neutral (Table 1). A critical structural feature of the new antimalarials is the presence of a protonatable nitrogen in the side chain that confers higher potency with respect to CLT against both CQ-S and CQ-R *Pf* strains (**5b** vs CLT). It is worthy of note that the protonatable nitrogen on the side chain of our compounds was rationally introduced for several reasons. In fact, being protonated at the blood pH of 7.4 (Table 1), it could be the key factor to provide high penetration in the infected erythrocytes, while its combination with a group protonated only at the FV pH of 5.5 (such as the imidazole group) could guarantee compound accumulation, thus increasing potency and selectivity. The combination of a protonatable group at pH 7.4 (pyrrolidinylmethyl) to a chlorine atom at position 4 provided the most potent compound of the series against CQ-R *Pf* strains (**5b**, 3D7, IC_{50} = 7.0 nM and K1, IC_{50} = 5.0 nM), being more potent than CQ and CLT. Compound **5b** showed a dose-dependent inhibition of β-hematin formation using the β-hematin inhibitory activity (BHIA) assay (Table 2).[10] According to our design hypothesis, although compound **5b** was found much more potent than CLT on different *Pf* strains, its in vitro BHIA was found very similar to that of CLT, suggesting that **5b** may better accumulate in the FV. On the other hand, although similar protonation states are found for CQ and **5b** (Table 1), the much higher in vitro antimalarial potency of **5b** vs CQ, compared to their inhibitory potency in the BHIA assay, suggests that a further mechanism of action, besides inhibition of hemozoin formation through the interaction with hemin (Fe(III)-FP chloride), may be responsible for killing the parasite.

On the basis of our docking studies and bioinformatic analysis, the basic side chain substituent, besides to increase the pharmacokinetics properties of the new molecules with respect to CLT, was also designed to improve selectivity for free heme with respect to heme as cytochromes P450 prosthetic group. To hit this mark, CLT was subjected to flexible docking studies into the 14-LD homology model[11] of *C. albicans*. At this purpose, it has to be underlined that the electronic and steric interactions with the residues present in the active site of 14-LD (Figure 1C) may not be the only determinant of substrate selectivity, as the external surface and the access channel properties may play a key role.[12] Thus, we also considered the presence of positively charged residues on the external surface of the active site (Figure 1A) and in the putative substrate access channel (Figure 1B) of 14-LD. Most of the positively charged residues identified in *C. albicans* 14-LD, including the K108 at the active site, are conserved in the other fungi P450 orthologs as well as in the active site of CYP3A4, the most important enzyme for drug metabolism in humans, where two arginine residues are placed in the same region occupied by K108 (Figure 1C). Our studies led to the hypothesis that the introduction of

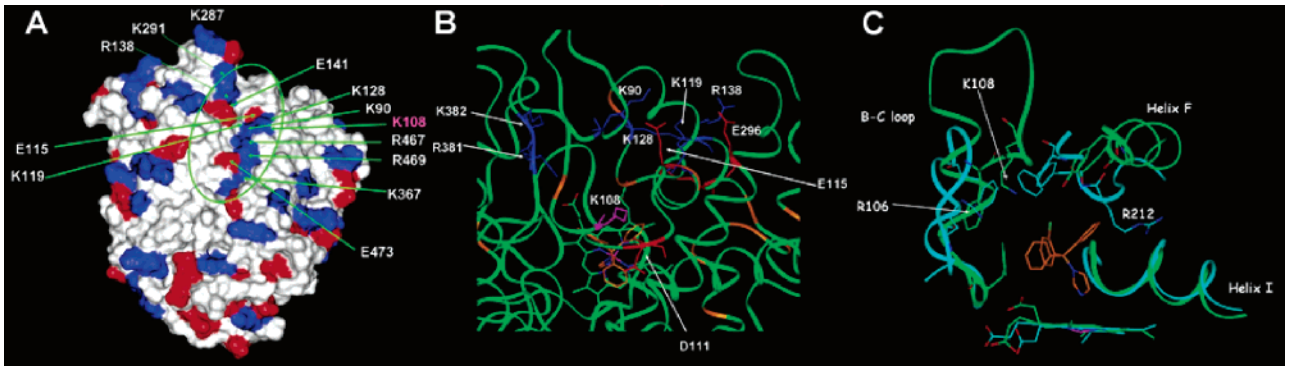


Figure 1. (A) Connolly surface of 14-LD homology model: positive, negative, and neutral amino acids are colored in blue, red, and white, respectively. (B) Zoom of the putative substrate access channel. Mutated (orange), positive (blue), and negative (red) amino acids are evidenced. K108 is colored in magenta. (C) Superimposition of CLT (orange)/14-LD (green) complex and X-ray structure of human cytochrome P450 3A4 (cyan). Positively charged residues are evidenced. Heteroatoms are colored: O = red, N = blue, Fe = magenta, and Cl = light green. Hydrogen atoms are omitted for clarity.

Table 3. Antimycotic Activity of Compounds **4a**−**c**, **5b**, and CLT (**2**)

	4a MIC[a]	**4b** MIC	**4c** MIC	**5b** MIC	**CLT**, **2** MIC
C. albicans	>32	>32	>32	32	0.03
C. krusei[b]	>32	>32	>32	16	2
C. parapsilosis[c]	>32	>32	>32	32	2
C. neoformans	>32	>32	>32	>32	2
A. flavus[d]	>32	>32	>32	>32	4
A. fumigatus	>32	>32	>32	>32	4
A. fumigatus R	>32	>32	>32	>32	4

[a] Minimum inhibitory concentrations (μg/mL) were calculated after 24 h of exposure, and the results were confirmed after 48 h. [b] ATCC 6258. [c] ATCC 22019. [d] ATCC 204304.

a protonatable group should decrease compound interaction with heme as a P450s prostetic group. These results were confirmed by the determination of the antifungal activity of selected compounds against a panel of fungal species (*Aspergillus* spp., *Candida* spp., and one isolate of *Cryptococcus neoformans*). In fact, antifungal activity of azoles, including CLT, is mediated by inhibition of cytochromes such as 14-LD. As shown in Table 3, none of the new compounds, including **5b**, bearing the pyrrolidinylmethyl chain, had any activity against these fungi.

In summary, a novel polycyclic pharmacophore was exploited to design potent antimalarials against CQ-R strains. The design strategy mainly focused on development of polycyclic compounds structurally related to CLT with improved selectivity for free heme over P450 cytochromes such as 14-LD. The compounds were specifically designed to easily penetrate and accumulate into the FV of the parasite.

This study allowed us to identify a novel class of antimalarials, typified by compound **5b**, characterized by a potent in vitro activity, with low cytotoxicity (CLT, $IC_{50} = 70$ μM in KB cell line; **5b**, $IC_{50} = 58$ and 70 μM in Daudi human cell lines and human lymphocytes, respectively) and low antimycotic activity against *Candida* spp. and *Aspergillus* species. Further studies are in progress to assess the in vivo antimalarial activity and pharmacokinetic properties of this promising lead compound.

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Supporting Information Available: Experimental details for the new compounds **3a**,**b**, **4a**−**d**, and **5a**−**g**; molecular modeling and pharmacology; and elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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