Design, Synthesis, and Structure–Activity Relationship Studies of 4-Quinolinyl- and 9-Acrydinylhydrazones as Potent Antimalarial Agents

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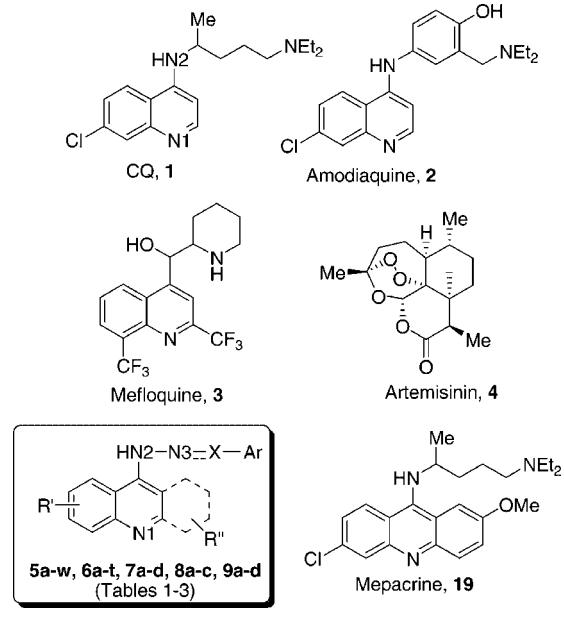
Malaria is a major health problem in poverty-stricken regions where new antiparasitic drugs are urgently required at an affordable price. We report herein the design, synthesis, and biological investigation of novel antimalarial agents with low potential to develop resistance and structurally based on a highly conjugated scaffold. Starting from a new hit, the designed modifications were performed hypothesizing a specific interaction with free heme and generation of radical intermediates. This approach provided antimalarials with improved potency against chloroquine-resistant plasmodia over known drugs. A number of structure−activity relationship (SAR) trends were identified and among the analogues synthesized, the pyrrolidinylmethylarylidene and the imidazole derivatives **5r**, **5t**, and **8b** were found as the most potent antimalarial agents of the new series. The mechanism of action of the novel compounds was investigated and their in vivo activity was assessed.

Introduction

Malaria is endemic to the poorest countries in the world, mainly in tropical and subtropical regions of Africa, Asia, and the Americas. Over 300 million cases are reported annually and 1.5–2.5 million people die from this devastating disease.[1] Although four species of the genus *Plasmodium* cause human malaria, most fatal malaria cases are due to infection by *P. falciparum* (*Pf*). Before the emergence of chloroquine-resistant (CQ-R) parasites, CQ (**1**, Chart 1) was one of the most important and effective antimalarial agents and has been used for decades as drug of choice in several programs aimed toward the global control of malaria. The spread of *Pf* strains resistant to CQ as well as to halofantrine, antifolates, and 4-substituted quinolines, such as amodiaquine (AQ, **2**) and mefloquine (**3**), dramatically reduced the therapeutic options to treat uncomplicated malaria.[2] The natural endoperoxide artemisinin (**4**) and its semisynthetic derivatives are increasingly being used because of their efficacy on CQ-R strains, rapid mode of action, broad range of activity, and low tendency to induce parasite resistance.[3] However, the thermal instability and short half-life of artemisinins (natural and semisynthetic) and the high cost of therapy are the major disadvantages for their widespread use.[4,5] At the moment, two promising drug combinations made of piperaquine or amodiaquine and artemisinin derivatives are under clinical development,[6] while isoquine and derivatives,[7] a class of compounds structurally related to AQ, have shown to be promising drug candidates. However, despite the current efforts to develop new antimalarials, the novel therapeutic options are far from being ideal, so there is an urgent need for newer low-cost safer drugs for the treatment of malaria, specifically designed to be active against *Plasmodium* strains that have acquired resistance to the most commonly prescribed antimalarials. The spread of resistant parasites created a tremendous therapeutic void, and the rate at

Chart 1. Reference and Title Compounds



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which resistance is growing outpaces the development of new effective antimalarials.

After infection, *Pf* establishes an initial asymptomatic liver stage followed by an erythrocytic stage during which the catabolism of hemoglobin (Hb) is a key source of food for the parasite and probably helps to maintain the osmotic integrity of the infected cell.[8] Degradation of host Hb within the acidic (pH $\approx$ 5.5) food vacuole (FV) is a central event for the generation of oxidative stress, which must be controlled by the parasite. Although *Plasmodia* are equipped with a range of antioxidants to maintain redox equilibrium, they must detoxify free heme. Indeed, Hb proteolysis in the acidic FV results in the production of toxic free heme and promotes spontaneous oxidation of oxyferro-protoporphyrin IX (FPIX-Fe(II)) to hydroxyferri-protoporphyrin IX (FPIX-Fe(III), hematin), with the concomitant formation of superoxide anion, hydrogen peroxide, and hydroxyl radicals (reactive oxygen species (ROS)). Parasite heme detoxification represents a well specialized and efficient process characterized by two main pathways: (i) crystallization of hematin dimers (β-hematin) into an inert and insoluble material referred to as the malaria pigment or hemozoin (biomineralization)[9] or (ii) diffusion through the vacuolar membrane to the parasite cytoplasm with the subsequent glutathione-dependent degradation.[10] A number of drugs currently under clinical use exert their activity, exclusively or at least in part, by increasing the oxidative stress in the parasitized erythrocyte. Quinolines and artemisinins react with heme moieties, preventing FP detoxification or forming cytotoxic radicals, respectively. In particular, CQ and related 4-aminoquinoline antimalarials are reported to interact with free heme, to interfere with its detoxification to hemozoin and thus to impair the plasmodium mechanisms of defense against oxidative stress.[11,12] This mechanism of action, characterized by the lack of a specific parasite target protein, accounts for the delayed onset and spreading of CQ-R *Pf* strains, which only occurred after 40 years of heavy drug pressure.[13] Although the molecular bases of CQ resistance are still unclear, in CQ-R strains mutations in *pfcrt* gene (*Pf* CQ-resistance transporter) always occur.[14,15] In particular, altered expression or mutations of *Pf*CRT is involved in ion-dependent FV transport processes that could affect the CQ transport or the microenvironment of the FV (e.g., pH) of resistant parasites.[16] Variations of FV chemical parameters may account for differences in efficacy against CQ-S and CQ-R strains observed for drugs targeting free heme and interfering with heme detoxification. On these bases, free heme represents a privileged target for the design of antimalarials active against CQ-R *Pf* strains. Thus, the aim of the present work was the development of potent compounds, targeting free heme and characterized by low potential of selecting resistant parasites.

Within a wide program aimed toward developing new antimalarial drugs[17] and taking into account our experience in the synthesis of quinoline-based pharmaceuticals,[18] we recently designed and synthesized a small set of heteroarylhydrazones, which exhibited exceptional potency toward CQ-R *Plasmodia*, inhibiting β-hematin formation in the BHIA assay in vitro.[19] Encouraged by the initial results,[19] we performed a thorough investigation of their structure–activity relationships (SARs). The present paper describes the synthesis and the biological investigation of novel 7-chloroquinolylhydrazones, methoxyquinolylhydrazones, tricyclic derivatives, and finally, hydrazides and sulfonylhydrazides (**5**–**9**, Tables 1−3). Comparing the new compounds with the original set of hydrazones,[19] SAR studies delineated a number of structural features responsible for modulating the in vitro antimalarial activity, thus leading to the development of a number of very potent antimalarials mainly active against CQ-R parasites. The new analogues were specifically designed to accumulate in the FV and interact with free heme, possibly generating toxic radical species capable of interfering with the antioxidant defense mechanisms of the plasmodium. The most potent compounds against the CQ-R strains were selected for further pharmacological investigation aimed toward understanding their mechanism of action and their *in vivo* activity.

Chemistry

Compounds **5r**, **6a**,**c**,**i**,**k**,**q**,**r**, and **8a**−**c** were synthesized as previously described,[19] and their detailed synthetic procedures are reported therein. The general synthesis for the novel hydrazones **5a**−**q**,**s**−**w**, **6b**,**d**−**h**,**j**,**l**−**p**,**s**,**t**, and **7a**−**d** is depicted in Scheme 1. Reaction of appropriate 4-hydrazinoquinolines, 9-hydrazino-1,2,3,4-tetrahydroacridines, 9-hydrazinoacridine, and benzo[*b*][1,5]naphthyridin-10-yl-hydrazine (**10a**−**n**) with the appropriate aromatic and heteroaromatic carboxaldehydes in refluxing ethanol gave the desired hydrazones in good yields (**5**–**8**).

Scheme 2 describes the synthesis of 3-hydroxy-4-(tetrahydro-1*H*-1-pyrrolylmethyl)benzaldehyde **16** and 4-(1*H*-pyrrolidin-1-yl)benzaldehyde **18**, required for the synthesis of compounds **5q**,**t** and **6e**. Esterification of commercially available 3-hydroxy-4-methylbenzoic acid **11** with methanol under acid-catalyzed conditions and subsequent protection of the phenolic hydroxyl group as an acetate afforded compound **12**. α,α′-Azobisisobutyronitrile (AIBN)-catalyzed radical bromination of **12** using *N*-bromosuccinimide (NBS) furnished the corresponding bromo derivative **13** in good yield. This latter was treated with pyrrolidine in alkaline medium to yield the alkylation product **14**. Subsequent reduction with $LiAlH_4$ afforded the corresponding benzyl alcohol **15**, which on oxidation with activated manganese(IV) oxide resulted in the formation of aldehyde **16** in excellent overall yield.

The aldehyde **18** was synthesized in good yields following the Buchwald synthesis, involving the reaction of 4-bromobenzaldehyde **17** with pyrrolidine in the presence of BINAP and NaO-*t*-Bu with a catalytic amount of $Pd_2(dba)_3$.

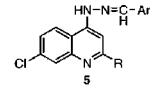
The synthetic pathway for derivatives **9a**−**d** is reported in Scheme 3. EDCI-catalyzed coupling of hydrazine **10a** with the appropriate carboxylic acid in the presence of HOBt and *N*-methylmorpholine provided the corresponding hydrazides **9a**,**b**. Finally, the synthesis of compounds **9c**,**d** was realized by treatment of **10a** with the suitable aromatic sulfonyl chloride in dry pyridine. The experimental details for synthesis, molecular modeling, and biology are given as Supporting Information.

Results and Discussion

1. In Vitro Antimalarial Activity and Structure–Activity Relationships (SARs). The aim of this study was to discover novel chemical entities characterized by high activity against CQ-R *Pf* strains and by a low potential to induce resistance. To reach this goal, we developed compounds selectively targeting free heme and possibly interfering with the redox equilibrium of the malaria parasite forming toxic radicals and increasing oxidative stress in the parasitized erythrocytes.

It is known that heteroaroyl hydrazones and Schiff base hydrazones are able to form complexes with different metals,[20,21] and their metal coordination geometry has been elucidated by X-ray analyses (Conquest 1.9, Cambridge Structural Database System (CSDS)). Moreover, interaction with metal ions and

Table 1. Prevalent Ionic Forms and Antiplasmodial Activity of Compounds **5a**−**w**



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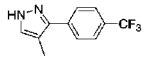
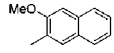
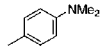
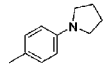
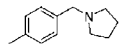
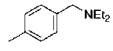
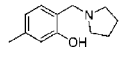
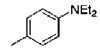
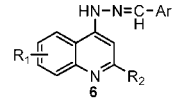
Cmp	Ar	R	Prevalent ionic form (%)[a]						IC_{50} (nM)[b]			
			Tautomer A		Tautomer B		Tautomer C					
			pH 7.2	pH 5.5	pH 7.2	pH 5.5	pH 7.2	pH 5.5	D10[c]	W2[d]	3D7[c]	K1[d]
5a		H	N (98)	P (50) N (50)	P (96)	P (100)	N (100)	N (100)	>3000	760	NT[e]	NT[e]
5b		H	N (97)	P (60)	P (99)	P (100)	-	-	607	814	NT	NT
5c		H	N (97)	P (58)	P (99)	P (100)	N (100)	N (98)	570	627	NT	NT
5d		H	N (97)	P (57)	P (99)	P (100)	-	-	949	1386	169	824
5e		H	N (98)	P (54)	P (99)	P (100)	-	-	504	855	167	577
5f		H	N (94)	P (76)	P (100)	P (100)	-	-	210	256	25.8	53.1
5g		H	N (96)	P/ZW (62)	P (82)	P (100)	N (80)	P (93)	65.2	23.7	5.9	31.6
5h		H	N (97)	P/ZW (54)	P (99)	P (100)	P (53) N (47)	P (98)	197	86.9	61.5	270
5i		H	N (95)	P/ZW (66)	P (98)	P (100)	-	-	453	140	152	626
5j		H	N (94)	P/ZW (58)	P (100)	P (100)	-	-	174	249	48.9	385
5k		H	N (97)	P (59)	P (98)	P (100)	-	-	347	509	125	2128
5l		H	N (96)	P (68)	P (76)	P (99)	-	-	186	258	5.0	39.6
5m		H	N (96)	P (67)	P (100)	P (100)	-	-	276	236	224	3528
5n		H	N (97)	P (61)	P (100)	P (100)	-	-	184	203	400	30.7
5o		H	N (96)	P (66)	P (99)	P (100)	-	-	145	169	31.8	63.7
5p		H	N (95)	P (70)	P (100)	P (100)	-	-	325	383	308	1139
5q		H	N (95)	P (68)	P (100)	P (100)	-	-	189	190	855	427
5r[f]		H	P (95)	DP (68)	DP (98)	DP (100)	-	-	28.9	58.3	19.1	16.4
5s		H	P (96)	DP (63)	DP (98)	DP (100)	-	-	48.3	57.7	2.70	27.2
5t		H	P (92)	DP (66)	DP (77)	DP (99)	-	-	21.4	18.9	0.9	39.6
5u		Me	N (98)	P (50) N (50)	P (96)	P (100)	P (100)	P (100)	1164	1311	NT	NT
5v		Me	N (82)	P (92)	P (100)	P (100)	-	-	122	281	NT	NT
5w		Me	N (72)	DP (53)	P (100)	P (99)	-	-	175	250	300	NT
CQ	-	-	P (88)	DP (87)	DP (100)	DP (100)	-	-	39	280	10	260

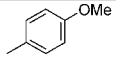
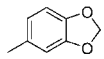
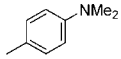
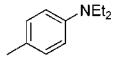
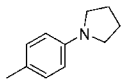
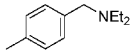
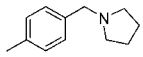
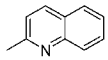
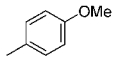
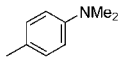
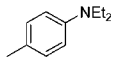
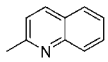
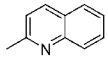
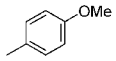
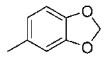
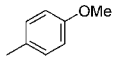
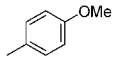
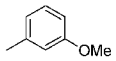
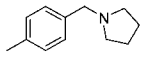
[a] ACD/pKa DB version 10.00 software (Advanced Chemistry Development Inc., Toronto, Canada). DP = diprotonated form; P = protonated form; ZW = zwitterionic form; *N* = neutral form. [b] IC_{50} values are the mean of at least three determinations; standard errors were all within 10% of the mean. [c] CQ-sensitive clone. [d] CQ-resistant clone. [e] NT = not tested. [f] Reference 19.

generation of radical intermediates has been proposed as the mechanism of action of hydrazone antitumorals.[22] Accordingly, since the quinoline system is known to possess iron complexation properties,[21] we envisioned to combine quinolines with

Table 2. Prevalent Ionic Forms and Antiplasmodial Activity of Compounds **6a−t**

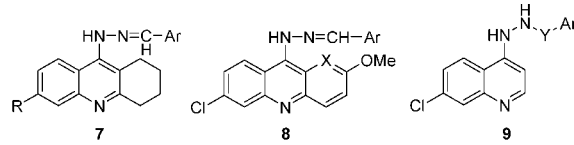


6

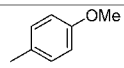
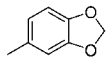
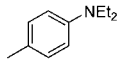
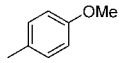
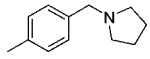
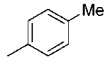
Cmp	Ar	R_1	R_2	Prevalent ionic form (%)[a]						IC_{50} (nM)[b]			
				Tautomer A		Tautomer B		Tautomer C					
				pH 7.2	pH 5.5	pH 7.2	pH 5.5	pH 7.2	pH 5.5	D10[c]	W2[d]	3D7[c]	K1[d]
6a[f]		6-OMe	H	P (61)	P (99)	P (100)	P (100)	-	-	99.4	128	65.1	NT[e]
6b		6-OMe	H	P (55)	P (98)	P (100)	P (100)	-	-	132	197	NT[e]	NT[e]
6c[f]		6-OMe	H	P (67)	P (96)	P (100)	P (100)	-	-	82.5	103	NT[e]	NT[e]
6d		6-OMe	H	P (67)	DP (56)	P (100)	P (99)	-	-	142	166	22.9	172
6e		6-OMe	H	P (68)	P (89)	P (100)	P (100)	-	-	115	185	404	462
6f[f]		6-OMe	H	DP (59)	DP (99)	DP (98)	DP (100)	-	-	39.2	79.3	11.0	55.1
6g		6-OMe	H	DP (60)	DP (99)	DP (98)	DP (100)	-	-	24.8	99.4	16.7	86.1
6h		6-OMe	H	N (60)	P (97)	P (100)	P (100)	N (100)	N (100)	73	96	125	159
6i[f]		8-OMe	H	N (96)	P (65)	P (100)	P (100)	-	-	519	254	5703	-
6j		8-OMe	H	N (97)	P (60)	P (100)	P (100)	-	-	715	903	326	769
6k[f]		8-OMe	H	N (96)	P (65)	P (100)	P (100)	-	-	338	356	NT[e]	NT[e]
6l		8-OMe	H	N (94)	P/ZW (48)	P (100)	P (99)	-	-	442	791	206	125
6m		8-OMe	Me	N (92)	P (81)	P (100)	P (100)	N (100)	N (100)	404	324	789	117
6n		7-OMe	H	P (54)	P (98)	P (100)	P (100)	N (100)	N (100)	108	140	NT[e]	213
6o		7-OEt	Me	P (92)	P (100)	P (100)	P (100)	-	-	212	232	805	537
6p		6-OMe	Me	P (87)	P (100)	P (100)	P (100)	-	-	132	184	507	149
6q[f]		6-OMe	Me	P (89)	P (100)	P (100)	P (100)	-	-	103	150	31.1	62.3
6r[f]		6,7-OCH_2O	H	P (76)	P (99)	P (99)	P (100)	-	-	210	163	156	467
6s		6,7-OCH_2O	Me	P (92)	P (100)	P (100)	P (100)	-	-	197	229	4984	NT[e]
6t		6,7-OCH_2O	H	DP (73)	DP (99)	DP (92)	DP (100)	-	-	35.6	46.5	26.7	58
CQ	-	-	-	P (88)	DP (87)	DP (100)	DP (100)	-	-	39	280	10	260

[a] ACD/pKa DB version 10.00 software (Advanced Chemistry Development Inc., Toronto, Canada); DP = diprotonated form; P = protonated form; ZW = zwitterionic form; N = neutral form; A = anionic form. [b] IC_{50} values are the mean of at least three determinations; standard errors were all within 10% of the mean. [c] CQ-sensitive clone. [d] CQ-resistant clone. [e] NT = not tested. [f] Reference 19.

Table 3. Prevalent Ionic Forms and Antiplasmodial Activity of Compounds **7a−d**, **8a−c**, and **9a−d**



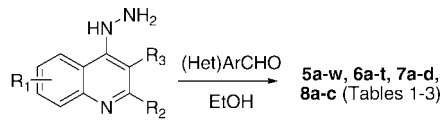
7 8 9

Cmp	Ar	R	X	Y	Prevalent ionic form (%)[a] Tautomer A pH 7.2	Tautomer A pH 5.5	Tautomer B pH 7.2	Tautomer B pH 5.5	Tautomer C pH 7.2	Tautomer C pH 5.5	IC_{50} (nM)[b] D10[c]	W2[d]	3D7[c]	K1[d]
7a		H	-	-	P (92)	P (100)	P (100)	P (100)	-	-	942	2365	NT[e]	NT[e]
7b		H	-	-	P (96)	P (100)	P (100)	P (100)	-	-	618	923	NT[e]	NT[e]
7c		H	-	-	P (92)	DP (61)	P (100)	P (99)	-	-	986	1467	1075	NT[e]
7d		Cl	-	-	N (69)	P (96)	P (99)	P (100)	-	-	760	1333	930	2645
8a[g]		-	CH	-	P (83)	DP (91)	DP (98)	DP (100)	-	-	96.8	137	112	NT[e]
8b[g]		-	CH	-	N (83)	P/ZW (81)	P (94)	P (100)	N (87)	P (88)	62	31	1.0	4.5
8c[g]		-	N	-	ZW (71)	P/ZW (95)	N (100)	N (97)	N (99)	N (57)	71.7	26.9	283	198
9a		-	-	CO	N (98)	N (53) P (47)	N (99)	N (80)	-	-	6474	NA[f]	5460	NA[f]
9b		-	-	CO	N (99)	N (57)	N (100)	N (91)	-	-	NA[f]	NA[f]	8834	NA[f]
9c		-	-	SO_2	N (92)	N (50) P (50)	N (58)	N (94)	-	-	5842	5056	3021	8460
9d		-	-	SO_2	N (93)	N (64)	A (55) N (45)	N (96)	-	-	4271	2526	2162	599
CQ	-	-	-	-	P (88)	DP (87)	DP (100)	DP (100)	-	-	39.0	280	10.0	260

[a] ACD/pKa DB version 10.00 software (Advanced Chemistry Development Inc., Toronto, Canada); DP = diprotonated form; P = protonated form; ZW = zwitterionic form; N = neutral form; A = anionic form. [c] CQ-sensitive clone. [d] CQ-resistant clone. [e] IC_{50} values are the mean of at least three determinations; standard errors were all within 10% of the mean. [f] NT = not tested. [g] NA = not active (IC_{50} > 10000). [h] Reference 19.

arylhydrazone moieties to obtain potent antimalarials.[19] We performed specific structural modifications of the original diarylhydrazone class of antimalarials to reach a fine-tuning of the antimalarial potency. Our design efforts were mainly dictated by the hypothesis that antiplasmodial activity might be directly related to the ability both to bind intracellular iron and to form redox-active iron complexes that generate cytotoxic ligand-centered radical species. To validate our hypothesis, we exploited the effect on antimalarial potency of quinolyl, acridinyl, and tetrahydroacridinyl hydrazone systems. Although the exact mode of action of CQ has not been fully clarified, it is generally accepted that CQ readily enters the parasite FV along the pH gradient and complexes FPIX-Fe(III).[10,23,24] The exact structure of the complex intermediate formed between 4-aminoquinoline antimalarials and heme is still unclear. Most of the modeling studies and interpretation of NMR data performed at neutral or alkaline pH support heme/4-aminoquinolines interaction as a face-to-face $\pi-\pi$ alignment, although the X-ray structure of the complex has never been obtained. A solid-state NMR study of acid-precipitated CQ−FPIX-Fe(III) aggregates provided the first direct evidence of a strong interaction, probably a covalent complex, between the endocyclic quinoline nitrogen (N1, Chart 1) and heme iron(III),[25] similar to the iron−nitrogen axial coordination bond of the quinoline ring of metaquine to heme, later proposed.[26] Accordingly, by specific substituents, we modulated the electron density at N1 of our polycyclic hydrazones, optimizing the interaction of the hydrazone-N1 with the heme iron center. As a consequence of this binding, in the specific microenvironment of the plasmodium FV, an electron transfer reaction could be facilitated leading to the formation of radical intermediates, stabilized by delocalization within the highly conjugated hydrazone scaffold. The resulting radical could evolve either directly reacting with other chemical entities in the *Pf* environment or undergoing the reductive cleavage at the hydrazone linker, thus generating other toxic species.[21,27,28] Following this hypothesis, compounds

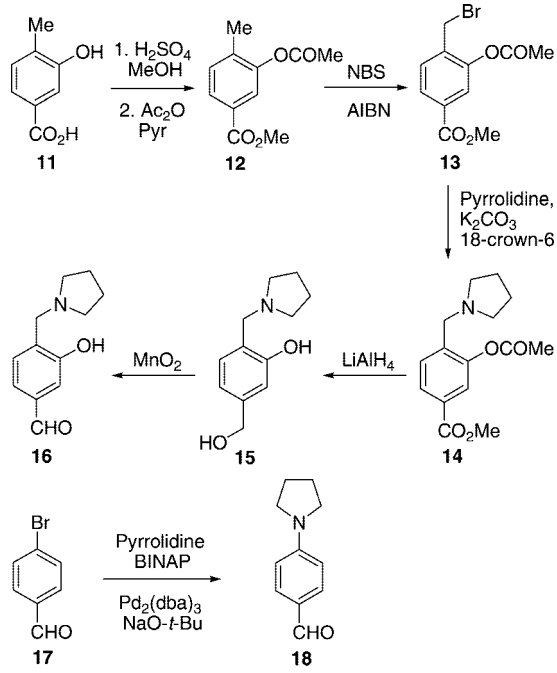
Scheme 1. Preparation of Final Compounds **5a**−**w**, **6a**−**t**, **7a**−**d**, and **8a**−**c**



10a-m

	R_1	R_2	R_3
a	7-Cl	H	H
b	7-Cl	Me	H
c	6-OMe	H	H
d	6-OMe	Me	H
e	8-OMe	H	H
f	7-OMe	H	H
g	7-OEt	Me	H
h	8-OMe	Me	H
i	6,7-OCH_2O-	H	H
j	6,7-OCH_2O-	Me	H
k	H	-$(CH_2)_4$-	
l	6-Cl	-$(CH_2)_4$-	
m	6-Cl		
n	6-Cl		

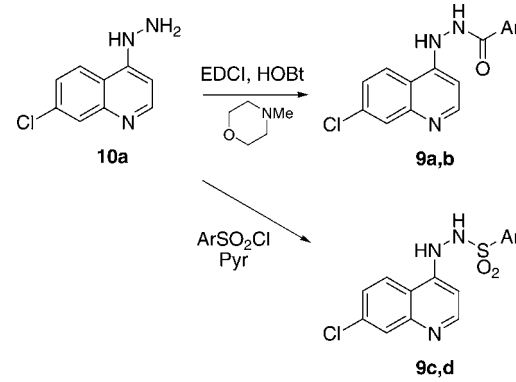
Scheme 2. Preparation of Intermediates **16** and **18**



reported in Tables 1−3 were designed with the aim to define the structural and electronic properties necessary to modulate the antimalarial activity. Accordingly, specific 4-aminoquinoline substitutents, coupled to different aromatic systems (i.e., arylidenes or heterocycles), were explored and the key role of the hydrazone linker between the two aromatic moieties was assessed.

The new hydrazones and their derivatives were tested in vitro against a series of *Pf* strains, namely, the CQ-S D10 and 3D7 and the CQ-R W2 and K1 strains. The antimalarial activity (IC_{50}, nanomolar) was quantified as inhibition of parasite growth, measured with the production of parasite lactate dehydrogenase (D10 and W2; asynchronous culture) or the incorporation of [^{3}H]-hypoxanthines (3D7 and K1, synchronous culture).[17a] Results are reported in Tables 1–3.

Scheme 3. Synthesis of Final Compounds **9a**−**d**



(i). Effect of the Structural and Electronic Properties of 4-Hydrazinoquinoline Ring Substituents. To fully investigate the effect of different substituents at the 4-hydrazinoquinoline system on antimalarial activity, we performed a comprehensive computational analysis (see Experimental Section), on all possible tautomers of compounds **5m**, **6a**, **6j**, **6o**, **6q**, **6r**, and **7a**. In fact, all these compounds share the same *p*-OMe-benzylidene moiety, while presenting diversely substituted 4-hydrazinoquinoline systems. Assuming an iron−nitrogen axial coordination bond with heme, the steric hindrance and the electron density at N1 were likely to affect the strength of this interaction.

The results of our pK_a calculations indicated that the introduction of the 6-OMe (**6a**) and 6,7-methylendioxy (**6r**) groups increased the protonatability of the 4-aminoquinoline moiety with respect to the 7-Cl analogue (**5m**), affecting the percentage of protonated forms at cytoplasmic pH (Tables 1 and 2) and, consequently, the antimalarial activity. Nevertheless, our quantum-mechanical calculations revealed that all these substituents show a comparable effect on the overall 4-aminoquinoline ring electronic distribution, related to a similar molecular dipole moment (Figure 1SIA,B, Supporting Information). On the contrary, an 8-OMe group as in **6j** decreased the electron density/protonatability at N1 (Table 2), which affected the dipole direction of the 4-aminoquinoline scaffold (Figure 1SI panels A,B vs C,D). This effect, together with the steric hindrance at C8 disfavored the interaction between free heme iron and N1 and consequently decreased the antimalarial activity (**6a** vs **6j**, Table 2). According to our hypothesized mechanism of action, the introduction of a methyl group at C2 (**6q**) increased the electron density and protonatability at N1 (AM1 full optimized partial charge = −0.15) and, consequently, the antimalarial activity (**6q** vs **6j,** Table 2). Replacement of the 6-OMe group of **6q** by a more hindered ethoxy group at C7 (**6o**), although maintaining a similar electronic effect at N1, significantly lowered the activity against CQ-S and CQ-R strains (**6o** vs **6q**), due to unfavorable steric effects. Analogously, introduction of a further saturated fused cycle (tetrahydroacridine) as in **7a**,**d**, with or without a chlorine substituent, dropped down the potency against the strains tested (Table 3). The resulting antiplasmodial activity of these compounds confirmed the significant role played by the substituent on the 4-aminoquinoline ring. As hypothesized, the antimalarial activity against all tested strains varied according to the polarization of the 4-aminoquinoline moiety. In particular, potency is related to the electron density at N1, which is crucial for the accumulation inside the FV and for the interaction with iron. Moreover, the presence of a strong molecular dipole moment, from the exocyclic N2 to N1 (Chart 1), in the protonated form of the most

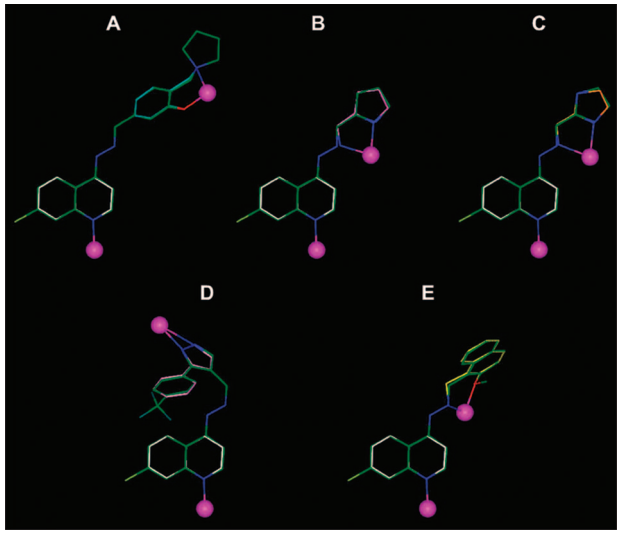
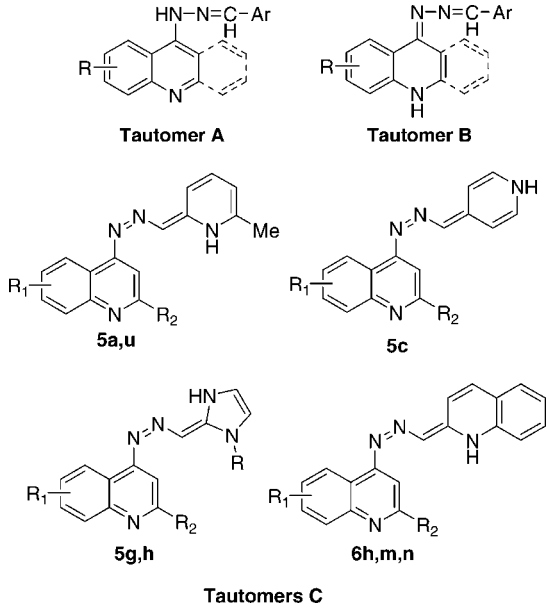


Figure 1. Resulting conformers at pH 5.5 of **5t** (A), **5f** (B), **5g** (C), **5l** (D), and **5o** (E) were superimposed on the X-ray structure of iron complexes containing the following moieties: (A−E) quinoline (white; CSD code FOFROH); (A) 2-(aminomethyl)phenol (cyan; CSD code DAMQAJ); (B) (1*H*-pyrrol-2-yl)methanimine (magenta; CSD code WEFFUJ); (C) (1*H*-imidazol-2-yl)methanimine (orange; CSD code FEJCAZ); (D) 3-phenyl-1*H*-pyrazole (pink; CSD code IDABIY), and (E) 3-(iminomethyl)naphthalen-2-ol (yellow; CSD code QASSOT). The superimposition was made fitting the putative iron coordinating atoms. Iron atoms' vdW volume (magenta) are scaled (0.3) for clarity. Heteroatoms are colored: C, green; O, red; N, blue; Fe, magenta; Cl, light green, and F, dark green. All hydrogens have been omitted for clarity.

Chart 2. Tautomers A and B for the Title Compounds and Tautomers C for **5a**,**c**,**g**,**h**,**u** and **6h**,**m**,**n**



active compounds supported the hypothesis of a strong relationship between potency (**6a** ≅ **6q** > **6r** > **5m** > **6o** > **6j** > **7a**, Tables 1–3) and ability to promote redox reactions after iron binding. Finally, the presence of substitutions able to hinder the hypothesized interaction with iron (i.e., at C2 (**7a**), C7 (**6o**), and C8 (**6j**)) were detrimental for antimalarial activity.

(ii). Effects of the Arylidene Substituents. The introduction of electron-donating groups, such as ethers, dimethylamino, and diethylamino at the *para*-position of the arylidene moiety did not significantly affect the antimalarial activity of the corresponding compounds with respect to unsubstituted analogues (**6i** vs **6j–l**, Table 2). Even introducing different substituents on the quinoline system, electron-donating groups at *meta* and *para* positions of the arylidene system (**5m**,**n**,**p**,**q**,**v**,**w** or **6a**−**e**, **6p**, **6s**, or **7b**−**d**; Tables 1−3) provided compounds with a similar trend in potency. Indeed, the antimalarial activity of this set of compounds mainly depends upon the nature of the 4-aminoquinoline substituent.

On the contrary, a significant increase of potency against CQ-S and CQ-R strains was observed when we introduced a *p*-diethylaminomethyl or a *p*-pyrrolidinemethyl group on the arylidene ring, protonated at pH 7.2 (**6f**[19] vs **6d** and **6g** vs **6e** in the 6-MeO quinoline series, **5r**[19] vs **5q** and **5s** vs **5p** in the 7-Cl quinoline series, or **6t** vs **6r** in the 6,7-methylendioxy quinoline series and **8a**,[19] Tables 1−3). These extra protonatable functions were introduced to mimic the basic aliphatic nitrogen of CQ and AQ, critical for drug sequestration into the acidic FV of *Pf*.[29] Moreover, in the case of **5t**, the introduction of an extra basic group combined with an arylidene *m*-hydroxyl provided a second iron coordination site in the molecule (Conquest 1.9, CSDS) (Figure 1A), further improving potency especially against 3D7 and W2 *Pf* strains (**5t** vs **5r**, Table 1). In general, analogues protonated at pH 7.2 (**5r**−**t**, **6f**,**g**, and **6t**) are more potent than their unprotonated analogues (**5m**,**n**, **5p**,**q**, **6a**−**e**, and **6r**,**s**) and represent some of the most potent compounds of this series (Tables 1 and 2). Referring to the protonation state of the molecule, it has to be underlined that, when the extra aminomethyl basic chain was introduced in the 2-(benzylidene)-1-(6-methoxyquinolin-4-yl)hydrazine scaffold the resulting compound was mainly diprotonated at cytoplasmic pH (**6f** and **6g**, Tables 1 and 2); consequently the antimalarial potency against all tested CQ-R strains increased to a lesser extent than in the corresponding 7-Cl derivatives (**5r** vs **6g** and **5s** vs **6f**, Table 1), monoprotonated at the same value of pH (probably due to different FV accumulation). This demonstrated the importance of a fine-tuning of the electronic properties in order to optimally balance the tropism for the FV and the propensity to react with free heme.

(iii). Heteroarylidene versus Arylidene Substituents. When arylidene analogues were compared with their heteroarylidene counterparts, a dramatic influence on the antimalarial activity was observed (Tables 1 and 3). In particular, the introduction of a furan ring (**5d**) led to a 2–5-fold drop in potency against D10 and W2 strains when compared with the analogue bearing a *p*-OMe benzylidene ring (**5d** vs **5m**, Table 1) and to those analogues bearing other five-membered heterocycles (**5e**−**l** vs

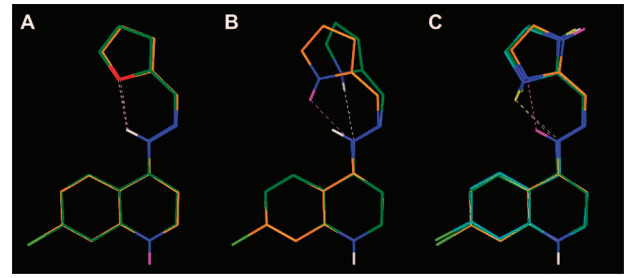


Figure 2. Superimposition of the most stable conformers of all possible tautomers of **5d** (A), **5f** (B), and **5g** (C). The carbon atoms and the hydrogens are colored: tautomer A (C green and H white); tautomer B (C orange and H magenta), and tautomer C (C cyan and H yellow). Heteroatoms are colored: O, red; N, blue; Cl, light green. All hydrogens, except those involved in hydrogen bonds and those of protonable nitrogens, have been omitted for clarity. Hydrogen bonds (dashed lines) are colored according to the hydrogens color.

Table 4. β-Hematin Inhibitory Activity Assay of Compounds **5d**,**g**,**l**,**r** and CQ

compd	IC_{50}[a]
5d	1.13
5g	0.65
5l	0.83
5r	0.98
CQ	1.68

[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.

5d). Our computational studies revealed that, although inducing a polarization of the hydrazone linker similar to **5m**, the furan ring of **5d** drove the formation of an intramolecular hydrogen bond between the heterocyclic oxygen and the exocyclic 4-hydrazinoquinoline N2. Consequently, this hydrogen bonding affected the tautomeric equilibrium, stabilizing one tautomer (namely, tautomer A, Chart 2, Figure 2A) and thus reduced the antimalarial potency (Table 1). It is well-known that 4-aminoquinolines are present in equilibrium between two tautomeric forms accounting for their peculiar properties, such as (i) the increased electron density at N1 (dipole moment), (ii) the consequent higher degree of protonatability with respect to 4-unsubstituted quinolines, and (iii) their chemical reactivity.[30] Consequently, we hypothesized that the poor antimalarial activity of **5d** was related to its lower propensity to give an electron transfer reaction with heme iron necessary for the generation of toxic radical intermediates. On the other hand, our data demonstrated that, although endowed with low antimalarial activity, **5d** was still able to strongly interact with Fe(III) heme, being highly active in the BHIA assay (Table 4). As expected, compounds **5e** and **5k**, characterized by moieties with similar electron properties to **5d** but with heteroatoms less prone to form hydrogen bonds, such as thiophene and 3,5-dimethylisoxazole rings, respectively, resulted slightly more potent than **5d** (Table 1). In particular, our quantum-mechanical calculations showed a very weak propensity of the sulfur bridged atom (i.e., low electron density) of **5e** to establish H-bond interactions. On the contrary, our quantum-mechanical calculations indicated that the isoxazole (**5k**) could generate an intramolecular H-bond; nevertheless, our conformational analysis revealed that it is prevented by the steric hindrance provided by the two methyl substituents.

Following the same rationale, we introduced a pyrrole heterocycle obtaining compound **5f** with improved antimalarial activity especially against the 3D7 and K1 *Pf* strains (**5f** vs **5d**, **5e**, and **5m**; Table 1). The pyrrole ring is a H-bond donating group being able to establish a hydrogen bond either with the exocyclic quinoline N2 (Figure 2B) or with the hydrazone linker N3 (Chart 1). The formation of the hydrogen bond with N3 is possible in both the neutral (24% at pH 5.5) and protonated forms (most abundant at pH 5.5). Indeed, in the most stable conformers, the H-bond donating properties of the pyrrole nitrogen allowed the stabilization of both 4-aminoquinoline tautomers (Figure 2B), without affecting the tautomeric equilibria. In addition, the intramolecular H-bond revealed the presence of a further iron coordination site in the molecule, as revealed by a fragment based search in the Cambridge Structural Database (CSD) (Conquest 1.9, CSDS) and depicted in Figure 1B.

When the five-membered heterocycles are substituted by pyridines or quinolines (**5a–c**,**u** and **6h**,**m**,**n**), we uncovered a third tautomeric form (tautomer C, Tables 1 and 2 and Chart 2), which involves the hydrazone NH and the pyridine or quinoline N with a resulting modification of the electron density at the hydrazone linker. In general, pyridine derivatives **5a−c**,**u** and the quinoline analogue **6h** were slightly less than or equally potent to the corresponding *p*-OMe arylidene derivatives (**5a−c**,**u** vs **5m**, **6h** vs **6a**, Tables 1 and 2). The quantum-mechanical studies evidenced that these heterocycles improved the electron density on the hydrazone linker similarly to the *p*-OMe phenyl substituent of **5m** and **6a**.

On these bases, we decided to introduce a 2-imidazolyl group (Chart 2, **5g**), which is also characterized by the presence of a third tautomer but, compared with the other heterocycles, by a higher degree of protonability (Table 1). Taking into account the chemical−physical properties of the imidazole ring, our conformational analysis was performed on the prevalent ionic forms at pH 7.2 and 5.5 of all the three possible tautomers (A, B, and C, Chart 2). The full optimization of the resulting conformers by the quantum-mechanical method AM1 (see Experimental Section, Supporting Information) revealed some unique features of **5g**, such as (i) a high electron density at N1, (ii) a strong electron flow from N1 to the exocyclic quinoline N2 in the protonated form, (iii) the presence of strongly polarized hydrogen bonds between N2 and the imidazole nitrogens, which stabilized the global minima of the three tautomers in a planar conformation, thus favoring tautomeric exchanges (Figure 2C), and finally, (iv) an electron flow from the 4-aminoquinoline moiety to the imidazole ring, which induced the polarization of the hydrazone linker. In particular, as evidenced by pK_a calculations and confirmed by computational studies, the imidazole ring drives the formation of an intramolecular zwitterion due to the exchange of a proton from the imidazole nitrogens to the N2. Moreover, in the most stable conformers of all **5g** tautomers, we uncovered an additional iron coordination site (Conquest 1.9, CSDS; Figure 1C). These electronic and conformational features allowed **5g** to be one of the most potent compounds of the series endowed with high antimalarial activity against both CQ-R and CQ-S *Pf* strains (Table 1).

Introduction of a methyl group at the imidazole NH (**5h**), which impaired the annular tautomerism, significantly decreased the antimalarial potency (**5h** vs **5g**, Table 1). Moreover, further support to our hypothesis was provided by compounds **5i** and **5j**, characterized by the presence of a 4-imidazolyl or a 3-methyl-4-imidazolyl ring, respectively. In these isomers, the formation of a third tautomer is not possible, switching the electronic effect of the imidazole ring from withdrawing to donating (**5i** and **5j** vs **5g**, Table 1). In agreement with the results of our calculations, **5i** and **5j** displayed a drop in antimalarial potency with respect to **5g** (Table 1). Interestingly, the presence of a methyl-substituted imidazole (**5j**) improved imidazole protonatability at pH 5.5, **5j** accumulation into the FV, and consequently, its antimalarial potency compared to **5i** (Table 1).

On these bases, when the mepacrine-like system (mepacrine, **19**, Chart 1) of derivatives **8a–c**,[19] characterized by the favorable electronic effects of the 7-chloro and the 6-methoxy substituents, was combined to the presence of a 2-imidazolyl group (**8b**), we obtained one of the most potent compounds of the series, especially against 3D7 and K1 strains (Table 3). The introduction of an extra N at the 5 position of the acridine system gave an analogue (**8c**) that retained the activity against D10 and W2 strains while being weakly potent against 3D7 and K1 strains (**8c** vs **8b**, Table 3). This drop of activity could be explained by comparing the conformational and electronic properties of **8b** and **8c**. In particular, the presence of the acridine nitrogen

Table 5. In Vitro Cellular Toxicity of Compounds **5d**−**l**,**r**,**t**, **8b**, and CQ (**1**)

compd	toxicity (KB cells), ED_{50} (μM)
5d	19.9
5e	10.4
5f	4.8
5g	19.5
5h	17.1
5i	29.1
5j	73.8
5k	171
5l	1.2
5r	19.5
5t	152
8b	5.1
CQ(**1**)	260

(N5) in **8c** altered the electronic distribution on the whole system, reducing (i) the electron density at N1, possibly affecting iron interaction properties, (ii) the protonatability of the acridine system, critical for FV accumulation, and (iii) the capability to promote intramolecular hydrogen exchanges between the imidazole nitrogens and the exocyclic acridine N2, related to the propensity to form radical intermediates.

Finally, encouraged by our previous results[17b] and by the potency of multisite iron complexing compounds **5g** and **5t** (Table 1, Figure 1A,C), we introduced specific aromatic moieties potentially able to coordinate iron (ConQuest 1.9, CSDS) such as 3-(4-trifluoromethylphenyl)1*H*-pyrazol-4-yl (**5l**) and 2-methoxynaphthalen-1-yl (**5o**) (Figure 1D,E). This led to the discovery of two compounds endowed with potent antimalarial activity, especially against 3D7 and K1 strains (Table 1). The lower activity of **5o** with respect to **5l** could be due to the formation of an intramolecular hydrogen bond between the methoxy group and the exocyclic quinoline N2, which similarly to what was observed for **5d** (Figure 2A) stabilized tautomer A, thus altering the electronic properties of the compound.

(iv). The Effect of the Hydrazone Linker. The hydrazone tether allows the electronic conjugation between the quinoline/acridine system and the (hetero)arylidene moiety. The critical role played by this molecular fragment in the antimalarial activity of our series is demonstrated by the comparison of the pharmacological profile of compounds **9a** and **9b** (Table 3) with that of their analogues **5g** and **5i** (Table 1), respectively. Indeed, our data confirmed the complete loss of activity displayed by derivatives **9a**−**d**, characterized by the presence of an acylhydrazino (**9a**,**b**) or a sulfonylhydrazino linker (**9c**,**d**).

2. β-Hematin Inhibitory Activity Assay. Selected compounds (**5d**,**g**,**l**,**r**) were screened for inhibition of β-hematin formation by using the β-hematin inhibitory activity (BHIA) assay (hemin (FeIII) in dimethylsulfoxide/acetate buffer at pH 5.0, 37 °C, 18 h).[31] All tested compounds showed a dose-dependent inhibition in the BHIA assay (Table 4) and were found to be more potent than CQ in inhibiting hemozoin formation, suggesting that the quinolyl hydrazones described are able to interact with free hematin possibly with a higher affinity than CQ. Since **5d** inhibited β-hematin formation similarly to **5g** (Table 4) but showed a reduced antimalarial activity (Table 1), these results suggested that, besides the interaction with FPIX-Fe(III), a further mechanism of action is required for the antimalarial activity of this class of CQ analogues.

3. In Vitro Cytotoxicity. Cytotoxicity of compounds **5d**−**l**,**r**,**t**, **8b**, and CQ (**1**) was assessed by using KB cells, a cell line derived from a human carcinoma of the nasopharynx system, and results are reported in Table 5. The tested compounds are endowed with in vitro toxicity ranging from 5 to 170 μM. With the exception of **8b**, the most potent compounds of the series displayed a toxicity vs antimalarial activity ratio higher than 200 thus demonstrating good selectivity indices.

Table 6. In Vivo Antimalarial Activity of Selected Compounds against *Plasmodium berghei* ANKA in BALB/c Mice after i.p. Administration

compd	dose (mg/kg/day) i.p.	% suppression on day 4[a]	mice alive on day 4
5r	30	83	5/5
5t	30	71	5/5
6a	30	38	4/5
8a	30	19	5/5
8b	30	40	5/5
CQ (**1**)	10	98	5/5

[a] Percent suppression = $[(C - T)/C] \times 100$; where C = parasitaemia in control group and T = parasitaemia in treated group.

4. In Vivo Antimalarial Activity. Selected compounds that displayed the best in vitro activities were administered intraperitoneally to infected mice to determine whether the antimalarial activities were retained in vivo. Compounds **5r**,**t** and **8b** were submitted to the full suppressive 4-day Peters' test[32] against *P. berghei* ANKA in BALB/c mice, and the results are reported in Table 6. At the dose of 30 mg/kg, none of the compounds tested was able to completely clear parasitemia in the 4 days of administration. Compound **8b**, which displayed the higher in vitro activity against 3D7 and K1 strains, acheived only 40% reduction in parasitemia with respect to untreated control mice. Compounds **5r** and **5t** gave 83% and 71% reduction of parasitemia, respectively, after i.p. administration at the same dose. Further tests are ongoing to clarify whether the reduced in vivo antimalarial activity of the compounds tested, especially of **8b**, compared with the high in vitro activity, is due to low bioavailability and hence to the pharmacokinetic properties of these compounds or whether activity is different between rodent and human strains of *Plasmodium*.

Conclusions

In summary, a series of novel and highly potent antimalarial agents were designed and synthesized. In particular, compounds **5g**,**r**–**t**, **6f**–**g**, and **8b** showed remarkable antimalarial potency in vitro, especially against CQ-R *Pf* strains. Accumulation inside the FV and interaction with free heme possibly followed by their activation to form toxic radical intermediates able to overcome the antioxidant defense mechanisms of the parasite seem to be the bases of their mechanism of action. In fact, oxidative stress represents one of the most promising rationales for the development of antimalarial chemotherapy. The low antimalarial activity of **5d** together with its potent effect on the BHIA assay seems to confirm our hypothesis. Compounds **5r**,**t** showed a promising antiplasmodial activity in vivo against *P. berghei* ANKA after i.p. administration at a dose of 30 mg/kg. Their further pharmacological and pharmacokinetic development is currently under progress. Notably, starting from commercially available starting materials, the development of these novel antimalarials requires few synthetic steps and is characterized by low cost of goods.

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Supporting Information Available: Experimental section, table of elemental analyses, and Figure 1SI. This material is available free of charge via the Internet at http://pubs.acs.org.

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