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Structure–activity relationships of 4-*N*-substituted ferroquine analogues: Time to re-evaluate the mechanism of action of ferroquine

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ABSTRACT

A series of five new alkyl 4-*N*-substituted analogues of ferroquine (FQ, SR97193) were designed, synthesized, and characterized. The antimalarial activity of the compounds was measured against twelve strains of *Plasmodium falciparum*. The compounds were more active than chloroquine (CQ) against all the CQ-resistant clones. For a better understanding of their mechanism of action, their physicochemical properties (lipophilicity and basicity) and their action on the inhibition of β-hematin formation were evaluated. The importance of the intramolecular hydrogen bond in neutral FQ in the antimalarial activity was probed, compared to the methyl analogue **1**.

Results of additional physicochemical measurements suggested new insights into the mechanism of action of FQ in sharp contrast with CQ. We complement here our understanding on the mechanism of action of FQ with the process of catalysis-mediated hemozoin formation at the interface between vacuolar content and membrane lipids.

1. Introduction

Malaria is an enormous health problem placing roughly 40% of the world's population at risk. This parasitic disease causes 600 million clinical cases and up to 3 million people die each year [1] and severe or non fatal cerebral malaria attacks (which represent 40% of pediatric admissions in some sub-Saharan areas) are the cause of neurological and cognitive impairments in African children [2].

Overlap of malaria infection areas with other important infectious diseases can have severe consequences. HIV infection and malaria are synergistic in terms of morbidity in co-infected patients, mainly in pregnant women [3–5].

Among the five *Plasmodium* species transmitting malaria, *P. falciparum* is the most dangerous, particularly due to cerebral and pregnancy malaria severity. Chloroquine (CQ, Chart 1) and other quinoline-based drugs (such as mefloquine, MQ) have been used for the prophylaxis and treatment of malaria. However, resistance to CQ has spread all over the malaria endemic areas and this phenomenon is complicated by the appearance of multiresistant strains [6].

Artemisinin (ART), a sesquiterpene lactone isolated from the shrub *Artemisia annua*, and its semisynthetic derivatives are being used because of their efficacy against CQ-resistant strains. The World Health Organisation (WHO) recommends that Artemisinin Combinations Therapy (ACT) should be given in all countries where the malaria parasite has developed resistance to CQ. These ACTs associate fast-acting ART-derived drugs with other antimalarials with longer half-lives such as MQ. Nevertheless, their thermal instability [7,8] and the high cost of therapy [9] are disadvantages for their global use. The decrease of susceptibility of *P. falciparum* to ART derivatives in some areas is a new preoccupation for the future [10–12].

In the mid-1990s, mainly inspired by the pioneering works of Gérard Jaouen's team [13,14], we launched a drug research program aimed at discovering new antimalarial agents. The bioorganometallic strategy was applied to several antimalarial drugs currently in use [15]. Rapidly, a drug-candidate emerged from a first screening of 50 ferrocenic compounds and ferroquine (FQ, SR97193, Chart 1) was selected. FQ was extremely active against

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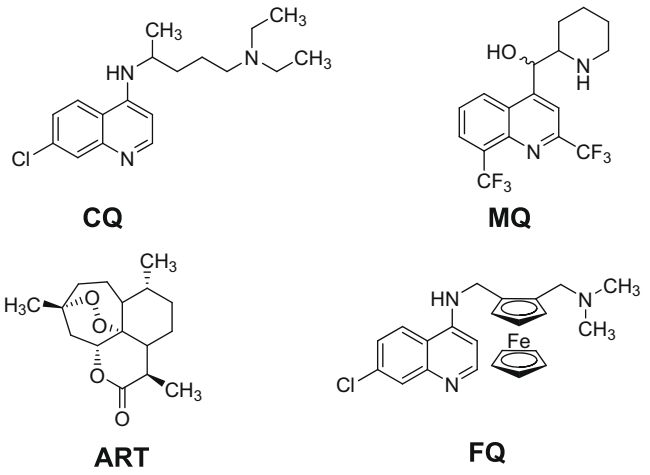


Chart 1. Chemical structures of chloroquine (CQ), mefloquine (MQ), artemisinine (ART) and ferroquine (FQ).

both CQ-susceptible and CQ-resistant *P. falciparum* [16–18]. To date, attempts at trying to induce *in vitro* or *in vivo* resistance in *Plasmodium* have failed [17]. Moreover, no relationship was found between PfCRT protein (responsible for CQ resistance) or other transport proteins already involved in antimalarial resistance in *Plasmodium*, and susceptibility to FQ [17,19,20]. FQ is currently the most advanced antimalarial project of Sanofi-Aventis R&D [21]. As for CQ and ART, the mechanism of action of FQ is partially unknown. FQ forms complexes with hematin in solution and is also able to inhibit the formation of β-hematin [22]. Recently, we demonstrated that the ferrocene core present in FQ was able to generate reactive oxygen species (ROS) under oxidizing conditions, such as those estimated in the food vacuole of the parasite [23]. The cause of the potent activity of FQ on CQ-resistant strains remains biologically unexplained. Nevertheless its activity is strictly linked to the presence of the ferrocene moiety in the lateral side chain of the aminoquinoline ring [24,25].

In our continuing effort to study the mechanism of action of FQ, we have now designed novel derivatives of FQ modified on the nitrogen atom located at the 4-position of the quinoline ring. Indeed such modifications permitted us to study the influence of the basic and lipophilic characteristics of these new derivatives on their antiplasmodial activity (including transport and localization) and on the inhibition of β-hematin formation.

To investigate this hypothesis, the present paper describes the synthesis, and the physicochemical properties, and biological activities of these novel FQ analogues. Comparing the new compounds with the original parents, CQ and FQ, structure–activity relationships studies were conducted around the basicity and the lipophilicity, thus leading to new considerations concerning the localization of FQ in the digestive vacuole (DV) of the parasite.

2. Results

2.1. Synthesis

The modified ferroquines were obtained starting from the commercially available *N*,*N*-dimethylferrocenylmethylamine in five steps as depicted in Scheme 1. The synthesis of aldehyde **6** has been previously described and exhaustively studied [26]. The formation of the C–C bond results from a two-step sequence involving metallation and formylation. According to the adapted Mignonac reaction [27], the ferrocenic aldehyde **6** is then converted to the corresponding amines **7–11** by reductive amination in 42–70% yields. Finally, the SN_{Ar} reaction between the secondary amines **7–11** and 4,7-dichloroquinoline gave compounds **1**–**5** in 36–58% yields.

2.2. Lipophilicity and basicity

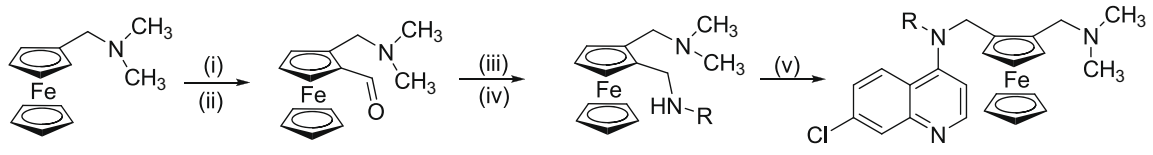
For diprotonic molecules, it is necessary to take into account the ionic state of the potential drugs. The distribution coefficient (log *D*), a pH dependent version of the partition coefficient (log *P*), reflects the true behavior of ionisable compounds in solution at a given pH [28]. The log *D* of CQ, FQ and 4-*N*-substituted FQ analogues **1–5** have been determined by HPLC in *n*-octanol-buffer mixture [29] at pH 5.2 and 7.4. These values of pH are supposed to reflect respectively the vacuolar and the cytosolic pHs in the parasite [30].

Measured log *D* values are reported in Table 1. All the newly synthesized FQ-like compounds **1–5** are 4-*N*-tertiary amine FQ-analogues. Only the reference CQ does not include the ferrocene core in its structure. Derivatives **1–5** differ only by the length of the alkyl chains substituting the amine at the 4-position of the quinoline ring.

At pH 7.4, log *D* values of compounds **1–5** were all found to be between 3 and 4, indicating that the molecules are highly lipid soluble. In contrast to purely organic molecules [31], their log *D* values evolve as a sigmoid function of the length of their alkyl side chains, and not as a linear function. The same trend is observed at vacuolar pH showing a hydrophilic behavior for all the compounds in these acidic conditions.

We can notice a similar difference between log $D_{7.4}$ and log $D_{5.2}$ around 3.5 for compounds **FQ**, **1** and **2** corresponding to a small substituent like a hydrogen, a methyl or an ethyl on the amine at the 4-position of the quinoline ring. On the other hand, this difference is considerably modified for the other 4-*N*-substituted FQ-analogues **3–5** with values around 2.7. These data reflect a different behavior for these molecules compared to the parent drug FQ when the side chain is longer than two carbon atoms. It is thus clear that this type of substitution increases particularly the lipophilicity of compounds **3–5** at vacuolar pH.

CQ, FQ and 4-*N*-substituted FQ analogues **1**–**5** pK_a determinations were carried out by potentiometric titration. Compared to



Scheme 1. (i) *t*-BuLi, N_2 atm., Et_2O; (ii) DMF; (iii) RNH_2, anh.THF; (iv) $NaBH_4$, anh.MeOH; (v) 4,7-dichloroquinoline, K_2CO_3, TEA, NMP, N_2 atm., 135 °C.

Table 1
Experimental pK_a and log *D* values at two different pH reflecting the vacuolar and cytosolic environment of the FQ derivatives, and calculated vacuolar accumulation ratios

Compound	log $D_{5.2}$	log $D_{7.4}$	pK_{a1}	pK_{a2}	VAR
CQ	−1.20	0.85	7.94 ± 0.02[a]	10.03 ± 0.02[a]	112
FQ	−0.77	2.95	7.00 ± 0.01	8.45 ± 0.02	5248
1	−0.56	3.04	7.05 ± 0.02	8.80 ± 0.03	3981
2	0.09	3.42	7.10 ± 0.04	9.10 ± 0.02	2137
3	0.83	3.68	6.70 ± 0.02	9.20 ± 0.02	708
4	1.2	3.97	6.85 ± 0.01	9.05 ± 0.02	589
5	1.4	3.99	6.70 ± 0.02	8.90 ± 0.02	389

[a] Values taken from Ref. [22].

FQ, the protonatable aliphatic tertiary amine groups (N24) of the side chain in compounds **1**–**5** display higher pK_{a2} values, with differences between 0.5 and 0.7 units. In the structure of FQ, the presence of the hydrogen on the 4-amino atom (N11) allows the establishment of an intramolecular H-bond with the basic side chain. The nitrogen lone pair involved in this H-bonding is no more available and the molecule is (quite) folded. To the contrary, compounds **1**–**5** should adopt a more flexible open conformation due to the absence of the H-bonding, protonation can occur more easily due to the larger accessibility to water. (see Section 2.6).

In FQ, a second protonation can occur at the endocyclic amino group (N1) of the quinoline ring to form a planar resonance-stabilized structure between the quinolinium and the ammonium. In compounds **1**–**5**, the substitution on the 4-amino atom (N11) of the quinoline ring induces a steric hindrance with the hydrogen atom at position 5. The conformation is no more planar and the tertiary amino group is twisted out the plane of the quinoline ring. This deformation diminishes the resonance stabilization energy and leads to a slight decrease of the pK_{a1} values.

2.3. Vacuolar accumulation ratio (VAR)

Previous investigations with FQ have indicated a decreased accumulation of FQ compared to CQ [22]. The vacuolar accumulation ratios (VAR) were predicted from a derivation of the Hendelson–Hasselbach equation using the calculated log *D* values. For a diacidic base, the log *D* is a function of the acidity constants (pK_a) and the partition coefficient (log *P*):

$$\log D_{\text{calculated}} = \log P - \log[1 + 10^{\wedge}(pK_{a1} - pH) + 10^{\wedge}(pK_{a1} + pK_{a2} - pH). \quad (1)$$

In view of the high degree of variability of the methods (the pK_a were determinated in dioxane–water mixture and the log *P* in octanol–water mixture),we used here for analysis the log *D* values as measured in the current study. This issue has been also previously discussed [15]. For CQ, FQ and compounds **1**–**5**, the VAR were calculated as follows:

$$\text{VAR} = 10^{\wedge}(\log D_{7.4} - \log D_{\text{pH}}), \quad (2)$$

and reported in Table 1. Clearly, FQ is expected to accumulate 50 times more than CQ in the parasitic DV. In this series, a decrease of the VAR is observed as a function of the length of the alkyl side chain. Thus, the effect of alkyl substitution on the nitrogen at the position 4 of the quinoline ring is accompanied with a decreased accumulation. This effect is due to variations of basicity and lipophilicity of compounds **1**–**5**.

2.4. Antimalarial activity

The new compounds were evaluated *in vitro* against twelve different *P. falciparum* strains (Table 2). The three strains 3D7, D6 and IMT8425 were defined as susceptible to CQ with IC_{50} values lower than 100 nM, whereas the other strains were classified as resistant to CQ with IC_{50} values higher than 100 nM. As limitation to the IC_{50} values could lead to erroneous conclusions about the supposed efficacy of the compounds, the IC_{90} also have to be evaluated and discussed.

Against the CQ-susceptible strains, the new compounds **1**–**5** were 1.6–8.9 times less active than CQ. All showed superior IC_{50} and IC_{90} values to CQ (and FQ). To the contrary and even if they remain less active than FQ itself, comparison of their IC_{50} shows that the modified FQs **1**–**5** are 1.3–13.5 times more active than CQ against the nine CQ-resistant strains. As for MQ, their efficacy is quite constant regardless of the level of CQ-resistance of the strains, the IC_{50} values of CQ ranging from 262.4 to 879.0 nM. The same remarks can be made about the IC_{90} values. None of these compounds showed enhanced activity relative to MQ and dihydroartemisinin (DHA), the active metabolite of ART. In general, introduction of an alkyl group on the 4-amino atom of the quinoline ring was not favorable to the antimalarial activity. Nevertheless, these molecules remain efficient on strongly resistant *P. falciparum* strains, such as K14 and FCM29. Among, these FQ-derivatives, the highest antimalarial activity of compound **4** bearing the *n*-butyl group is intriguing. Indeed, only small (non-significant) differences between the inhibition of the parasite development of CQ-susceptible (with $\langle IC_{50}\rangle$ = 45 nM) and CQ-resistant (with $\langle IC_{50}\rangle$ = 53 nM) clones are noticed. The same trend is observed with $\langle IC_{90}\rangle$ values.

As previously reported, FQ was highly active against CQ-susceptible and CQ-resistant strains. Cross-resistance was estimated by pairwise correlation of IC_{50} values of all 12 strains. No correlation was found between responses to FQ and CQ (r^2 = 0.246), suggesting that no cross-resistance exists between FQ and CQ (as previously reported on other laboratory strains and isolates) [19,20].

2.5. Inhibition of β-hematin

The new FQ-derivatives were tested for their ability to inhibit β-hematin formation, the synthetic equivalent of hemozoin [32,33]. A modified version of Egan's assay [34] was used. The minor changes are summarized here: (i) as β-hematin should not be formed before the start of the experiment, hematin was added last, (ii) microtubes were kept at 60 °C before addition of sodium acetate in order to avoid re-precipitation, and to obtain the exact volume which determines the pH value of the reaction mixture. The interpretation of the results is not straightforward since both the IC_{50} value and the drug:hematin ratio must be taken into account. The most potent inhibitors were the compounds which showed the highest percentage of inhibition at the lowest drug:hematin ratio, which corroborated with a low IC_{50} value.

As reported in Table 3, the new FQ-derivatives showed a high inhibition of β-hematin formation. The best inhibitor was compound **4** bearing the *n*-butyl group. Ninety-seven percent of inhibition of β-hematin formation is reached with an IC_{50} value of 0.4. The drug:hematin ratio (3:1) is comparable to the other compounds of the series and to CQ. It is reasonable to assume that the flexible *n*-butyl group can adopt a particular conformation (wrapped around the ferrocene moiety?) leading to a similar mode of interaction as for FQ. Nevertheless, FQ remains the best inhibitor in terms of the drug:hematin ratio and IC_{50} value. It is interesting to mention here the types of inhibition profiles observed in this series (Fig. 2). Indeed, whereas CQ had a sigmoid behavior as for amodiaquine (AQ) [35], FQ had a rapid maximum and showed a decreased inhibition or non-inhibition at high concentration (bell-shaped curve). All new compounds have a mixed behavior similar to FQ for a drug:hematin ratio <1 but closer to CQ for a ratio >1. This result was unexpected as the compounds are more lipo-

Table 2
In vitro sensitivities (nM) of *P. falciparum* strains towards the FQ derivatives **1–5**.

Compound	Strain																					
	3D7		D6		8425		Voll		L1		PA		Bres		FCR3		W2		K2		K14		FCM29	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
1	99.8	227.5	81.9	193.2	52.8	84.5	198.2	339.6	211.4	345.1	69.3	118	201.4	336.5	148.3	309	200.9	347.5	200.9	343.6	200	335.7	230.1	365.6
2	188.8	338.8	165.2	319.2	134	289.7	189.7	335	199.1	343.6	194.5	340.4	220.8	354	185.4	336.5	218.3	255.6	212.8	355.6	213.8	354.8	241.6	369.8
3	162.2	319.9	115.6	264.9	123.6	263	180.3	334.2	171.4	322.1	181.6	333.4	173.4	328.1	157.8	314.1	208	350.8	189.2	339.6	169	322.9	157	309
4	51.2	86.7	41.6	74.1	41.4	74.3	51.9	111.7	49.3	83.6	48.6	84.5	49.8	89.1	44.7	68.7	62.7	105.7	53.2	93.1	52.8	104	65.5	124.7
5	176.6	319.2	200.5	343.6	121.1	265.5	185.8	336.5	213.8	349.1	126.8	255.9	143.9	258.2	193.6	335.7	204.6	347.5	237.1	370.7	184.1	328.1	124.7	265.5
CQ	21.3	40.4	23	44.4	25.5	59.2	262.4	316.2	273.5	417.8	330.4	483.1	442.6	613.8	477.5	685.5	485.3	693.4	493.2	912	648.6	1129.8	879	1241.7
FQ	3.5	6.5	5.6	8	6.5	9.4	11	18.8	8	13.8	7	13	1.2	13.7	11.5	16.9	7.1	12.6	9	16.3	12.6	25.6	10	19.3
MQ	52.1	119.4	32.0	49.2	33.3	97.3	30.7	94.2	30.2	51.5	27.5	49.1	44.1	85.3	62.1	111.9	38.0	78.5	35.9	59.2	36.9	63.2	32.5	53.7
DHA	1.9	3.5	2.5	4.2	1.4	2.9	1.8	2.9	1.2	2.0	1.1	2.3	1.6	3.1	1.4	2.4	1.1	2.1	0.9	1.8	1.4	2.2	1.0	1.7

Values are means of 3–5 independent experiments.
Chloroquine (CQ), ferroquine (FQ), mefloquine (MQ) and dihydroartemisinin (DHA).

philic than FQ itself and thus more susceptible to aggregate at high concentration.

2.6. Intramolecular hydrogen bond

In the structure of neutral FQ, formation of an intramolecular hydrogen bond between the hydrogen atom H11 and the nitrogen atom N24 (Scheme 2) appeared crucial for the antimalarial activity. Indeed, whereas FQ and compound **1** had similar physicochemical properties (Table 1), **1** lacks the ability to form such a hydrogen bond and consequently loses 6-fold in β-hematin IC_{50} (Table 3), compared to FQ, accompanied by a decreased in antimalarial activity (Table 2).

The presence of the H-bond was first evidenced in the crystal structure of neutral FQ [22]. For neutral FQ, resonances of N24 and H11 were correlated in the NMR ^{15}N-HMBC experiment confirming the intramolecular hydrogen bond in solution ($CDCl_3$) as observed in the solid state [22]. Moreover, NOESY experiments confirm that neutral FQ adopt a folded up conformation. This special conformation was evidenced by the presence of NOEs between: (i) H5 and the methyl groups, (ii) H3 and the H12, but the absence of NOEs between: (i) H12 and the methyl groups, (ii) H5 and H12, (iii) and H3 and H23 (see Supporting Information, Fig. SI1). This folded up conformation is not observed for diprotonated FQ in deuterated water. ROESY experiments confirm that diprotonated FQ has no fixed conformation. Only ROEs between H3 and H12 as well as between H5 and H12 were observed (Figure SI2).

From these experiments, it can be concluded that the extended structure (Scheme 2) in polar solvent (water) changes to a more compact conformation via an additional intramolecular hydrogen bond in apolar solvent (chloroform). This peculiar shape leads to an increase of its hydrophobicity, and results in the rejection of the bulky ferrocenyl moiety towards the outside. This special conformation should allow the hydrophobic moieties (ferrocenyl and methyl groups) to interact with the lipids of the membrane whereas the quinoline ring could stack with the ferriprotoporphyrin IX. This flip/flop H-bond between the open conformation (charged FQ) and the folded conformation (uncharged FQ) may also help transport from water to the hydrophobic membranes.

To the contrary, compounds **1**–**4** of the current series are not able to establish an intramolecular hydrogen bond and should adopt no fixed conformation. As an example, a complete assignment of the ^{1}H and ^{13}C NMR signals of compound **4** was achieved on the basis of COSY, ^{13}C-HSQC, ^{13}C-HMBC, and NOESY experiments. In $CDCl_3$, NOEs between H5 and H12, H17, H27, H28, H29, and NOEs between H3 and H12, H17, H27, H28, H29 show that the C4-N11 bond is not rigid (Fig. SI3). Compound **4** is able to adopt at least 4 different conformations (Fig. SI3). NOESY experiments have been also carried out in pyridine-D5 at lower concentration and higher temperature (343 K) at 400 MHz. In these experimental conditions, no difference in the NOESY spectra can be observed confirming that the observed NOEs were intramolecular and not

Table 3
In vitro inhibition of β-hematin of 4-*N*-substituted FQ analogues in methanol

Compound	Maximum inhibition reached (%) (at drug:hematin ratio)	IC_{50}
1	87 (3:1)	2.0
2	65 (5:1)	2.2
3	81 (5:1)	1.0
4	97 (3:1)	0.4
5	93 (3:1)	1.2
CQ	71 (2:1)	1.4
FQ	76 (0.75:1)	0.3

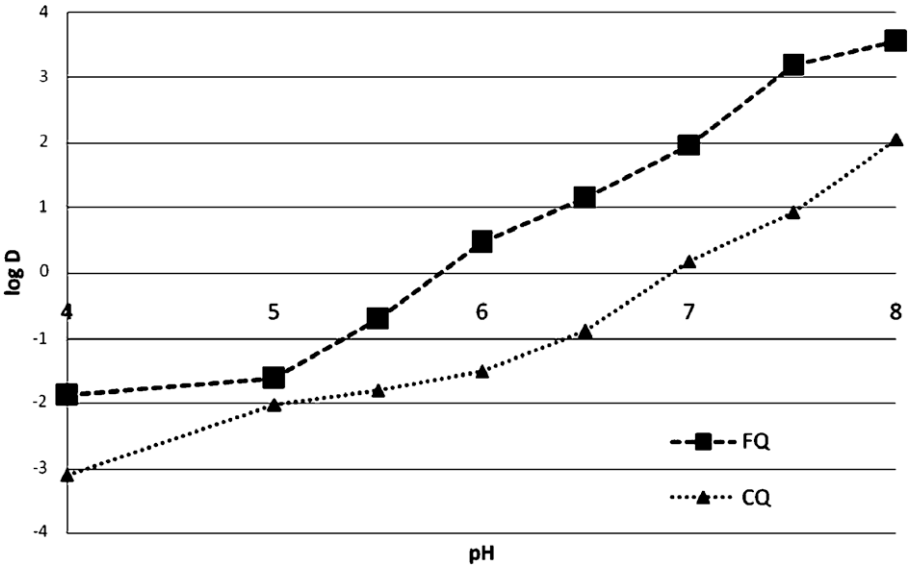


Fig. 1. Partition coefficients for chloroquine (CQ) and ferroquine (FQ) as a function of pH.

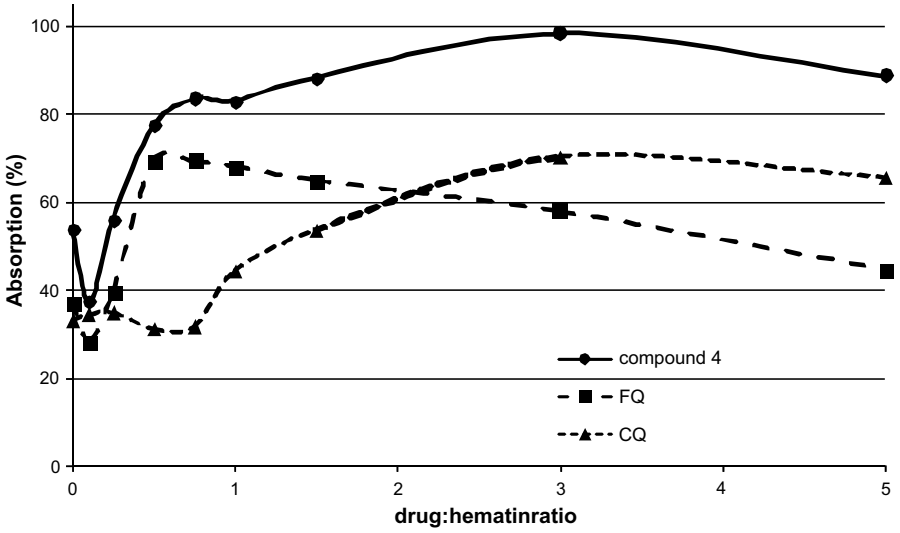
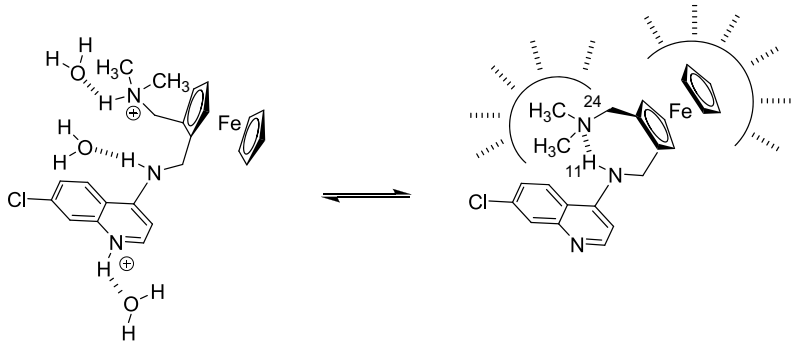


Fig. 2. β-Hematin inhibition curves for chloroquine (CQ), ferroquine (FQ) and compound **4**.



preferred conformation in polar solvent (acidic water)

preferred conformation in hydrophobic media (membrane)

Scheme 2. Flip/flop H-bond may help transport of FQ through the hydrophobic membranes.

intermolecular. The steric hindrance due to the bulky groups should lead to rotation around the C–N bond leading to a loss in coplanarity which disrupts conjugation in the molecule.

3. Discussion

CQ and FQ share some chemical features, but their activity on both laboratory clones and field isolates of *P. falciparum* were shown to be very different in all studies already performed [17–20]. To date, the activity of FQ is quite constant regardless the level of resistance of the strains. This notion was extended to currently used antimalarials (MQ, halofantrine, artesunate, quinine) for which no cross-reactivity was found with FQ under standardized antimalarial test conditions [20]. Two interpretations can be proposed: (i) the mechanism of action of FQ presents specific aspects which are not shared with CQ, (ii) it has been suggested that FQ is expelled from the DV by mechanisms currently involved in resistance to some antimalarials [17]. Molecules **1–5** provide complementary information regarding structure–activity relationship of FQ analogues.

Concerning the mechanism of action, aminoquinolines are known to form complexes with hematin and to inhibit its conversion into hemozoin [36–38]. As of today, it is not well understood whether both phenomena are linked, and how [39,40]. Nevertheless, there is strong evidence that the 4-aminoquinoline ring is required for non covalent interaction (i.e. stacking interaction) with hematin whereas the weak bases assist vacuolar accumulation in the DV of the parasite through pH trapping [41].

Since the discovery of CQ, variations on substituent(s) of the aminoquinoline ring and/or of the lateral side chain have been performed, and numerous CQ-like analogues have been synthesized and tested [42,43]. Only one study is reported about the synthesis of a tertiary basic 4-amino group in CQ, but no precise results concerning their antimalarial activity are available [44].

To date, pharmacomodulations of the FQ skeleton mainly focused on the terminal nitrogen atom (numbered N24) [24,25]. No systematic variation of the subsituents on the anilino nitrogen atom has been envisaged. The new compounds were designed to explore the influence of the substitution patterns on the 4-*N* atom on the quinoline ring of FQ and the resulting structural specificity. It is assumed that the unprotonated (or the monoprotonated) drug(s) should predominate in the lipid rich membrane interface where the hemozoin formation occurs [45]. These modifications were envisaged here in order to modify the electron density of the quinoline ring and its shape, and therefore modulate the specific non-covalent interaction with the porphyrin ring. Moreover, such modifications should influence their basicity (pK_a) and their lipophilicity (log *D*), and to the same extent the transport in the parasite.

The syntheses were easily achieved in a five-step procedure starting from low cost reagents. The structures of the analogues **1–5** were unambiguously determined by classical laboratory techniques. From these experiments, relevant conclusions were drawn:

(i) In terms of basicity, introduction of an alkyl group on the 4-*N* atom of FQ did not alter significantly the pK_a of the quinolyl N atom. The second titratable amino group is much more affected but remains in the same range as for FQ.

(ii) The lipophilicity of the 4-*N*-substituted FQ analogues is increased and correlates with the length of the side chain. Compared to CQ, an improved activity on CQ-resistant parasites was noted as previously observed for all molecules incorporating the ferrocene core in their side chain [24,25]. Taken together, all these data reinforce the conclusion that the ferrocene moiety has to be covalently flanked by a 4-aminoquinoline and an alkylamine.

(iii) No direct relationship between the level of β-hematin formation inhibition and antiplasmodial activities could be established. Nevertheless, when restricting the analysis to the present five new alkyl 4-*N*-substituted analogues of FQ (i.e. compounds **1**–**5**), the best inhibitors of β-hematin formation were the best antimalarial compounds. In this series, the IC_{50} value for the inhibition β-hematin formation is a discriminating parameter of the resulting antimalarial activity.

(iv) Replacement of the hydrogen atom by the methyl group on the amine at the 4-position of the quinoline ring did not alter significantly the physicochemical properties (see Section 2.6.) but led to significant reduction in activity against CQ-susceptible and CQ-resistant strains. The presence of a hydrogen bonding interaction in the lateral side chain of FQ should contribute to the antiplasmodial activity.

Plots of VAR versus IC_{50} (Fig. 3) allow a direct visualization of the influence of structural parameters and physicochemical properties. The IC_{50} were not correlated with the VAR. Moreover, the absence of a clear difference between the most active compound **4** and the other ones (**1**, **2**, **3** and **5**) indicates that the VAR, as defined here, is not a discriminating property in the current series. The higher antimalarial activity of compound **4** should be due to its preferential interaction with hematin and its inhibition of hemozoin formation. In the case of CQ, two clusters were observed corresponding to the CQ-susceptible (Fig. 3, crosses) and CQ-resistant strains (Fig. 3, open circles). Interestingly, for all the ferrocenic compounds (including FQ), no discrimination could be established between both kinds of strains. Indeed, only one cluster is observed. In other words, the selective activity on CQ-resistant strains does not depend on the VAR but on the presence of a ferrocene moiety in the lateral side chain of CQ as precised above.

Moreover, our results confirm that this family of analogues of FQ may not interact with known transport proteins involved in resistance mechanisms of *P. falciparum,* based on sequence

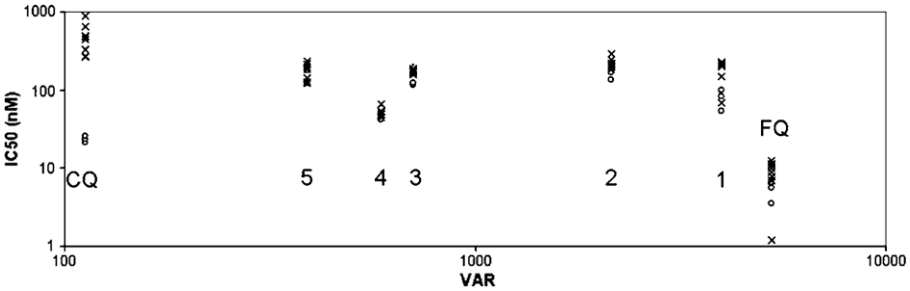


Fig. 3. Relationship between VAR and IC_{50} of CQ, FQ, and compounds **1–5**.

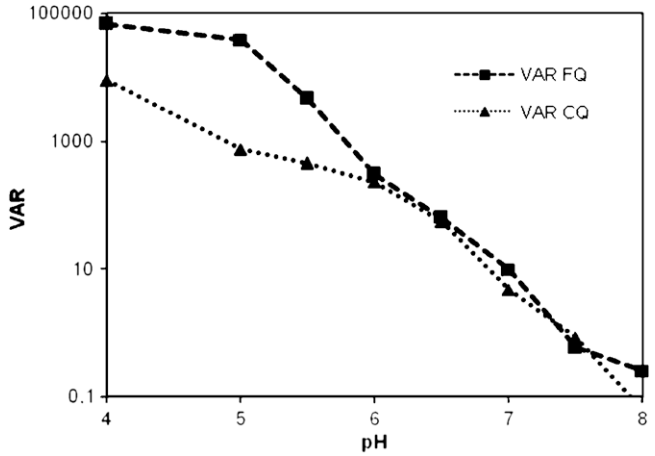


Fig. 4. Vacuolar Accumulation Ratios (VAR) of CQ and FQ as a function of internal pH. The cytosolic pH value was set to 7.4.

polymorphism of the proteins involved in resistance in the tested clones [19].

3.1. Re-investigation of the localization of ferroquine

With regard to the previous conclusions, we re-investigated the behavior of CQ and FQ as a function of pH of the media. Plots of log *D* versus pH (Fig. 1) allow a direct visualization of the accumulation of CQ and FQ in each phase (i.e. lipid and water). FQ displays a more strongly lipophilic character than CQ even at pH values around 5. This pH value, in the range 5.2–5.5 according to the methods used and the authors, is supposed to reflect the acidic pH value of the digestive vacuole [46]. The determination of the precise DV pH value is subject to controversy. Nevertheless, it seems that no significant differences could be detected between CQ-susceptible and CQ-resistant strains. Thus DV pH does not play a primary role in CQ resistance [47,48].

At these values, the concentration of neutral or monoprotonated forms is 10 times larger for FQ than for CQ. As a result, whereas CQ should be concentrated in the aqueous regions, FQ should be concentrated in the lipid regions (or at least at the interface between lipids and water). These results should be replaced in view of the recent advances in understanding the process of hemozoin formation. Indeed, there are now pieces of evidences for heme crystallization within lipids in diverse parasites [49–52]. The enhanced antimalarial activity of FQ compared to CQ could be mainly justified by its preferential localization at the site of crystallization of hemozoin. Indeed, the accumulation of FQ close to the DV membrane could induce two distinct phenomena: first FQ could inhibit the self assembly of the hemozoin crystal, and second, FQ could generate ROS formation and lipid peroxidation.

As discussed above, compared to CQ, a greater accumulation of FQ is expected in the DV. Nevertheless, as special transport mechanism(s) could not be excluded and are even strongly suspected (the binding to free heme should contribute to uptake)[53,54], the calculation of VAR could only be estimated. Between pH values of 6 and 8, the VAR of CQ and FQ are very similar (Fig. 4). The behavior of their VARs is roughly linear as a function of the internal pHs. A striking difference is observed starting from pH 6 where FQ should accumulate far more than CQ. Around pH 5, FQ is even expected to concentrate 50-fold more than CQ.

4. Conclusion and prospects

We have reported the synthesis, physicochemical properties, β-hematin inhibition properties and antimalarial activities of a series of 4-*N*-substituted analogues of ferroquine. All compounds were more active than CQ against a large panel of CQ-resistant strains. They displayed a more lipophilic behavior than FQ (and CQ) at cytosolic and vacuolar pH. Their structural modifications did not alter dramatically their pK_a values. All these observations implied that there was no simple relationship between activity, drug accumulation, and inhibition of β-hematin formation. The antimalarial activity is the result of a subtle mixture between transport (membrane(s) penetration), localization (heme-targeting) and inhibition of hemozoin formation. We have however evidenced that the ferrocenic compounds escape the mechanism of resistance of CQ.

These data in combination with recent bibliographic references about the nucleation site of hemozoin formation promote us to re-investigate the physicochemical behavior of FQ as a function of pH.

In this regard, localization for FQ at the interface between lipids of the membrane and water could mainly justify its enhanced antiplasmodial activity compared to CQ. This specific targeting should allow a stronger inhibition of hemozoin formation and a direct proximity to the membranes resulting in a more efficacious lipid peroxidation.

Identification of the targeting site will constitute the next step in elucidating the mechanism of action of FQ. Here again, the specificity of the FQ could be exploited. The ruthenium analogue of FQ, known as ruthenoquine [55,56], could be used as a tracer in electron microscopy. Chemically, ruthenoquine is close to FQ and possesses similar in vitro antimalarial activity [55,56]. Previous experiments supported its use for subcellular localization [57].

5. Experimental

5.1. General remarks

Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded at room temperature on a Bruker AC 300 spectrometer. TMS was used as an internal standard and $CDCl_3$ as the solvent. ^{1}H NMR analyses were obtained at 300 MHz (s: singlet, d: doublet, t: triplet, dd: double doublet, m: multiplet); whereas ^{13}C NMR analyses were obtained at 75.4 MHz. The chemical shifts (δ) are given in parts per million relative to TMS (δ = 0.00). Mass spectra were recorded by means of a Waters Micromass Quattro II triple quadrupole LC mass spectrometer equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources. Melting points were determined on a Köfler apparatus and are uncorrected. Column chromatography, carried out on silica gel (Merck Kieselgel 60) was used for the purification of compounds. Reactions were monitored by thin-layer chromatography (TLC) using coated silica gel plates, detection by UV lamp. The purity (P_{HPLC}) of the compounds was checked by two types of high pressure liquid chromatography (HPLC) columns, a Macherey-Nagel C18 Nucleosil column (4 × 300 mm, 5 μm, 100 Å) or Macherey-Nagel EC 250/4.6 Nucleodur 100-5 CN-RP (4 × 300 mm, 5 μm, 100 Å). Analytical HPLC was performed on a Spectra system equipped with a UV detector set at 254 nm. Compounds were dissolved in acetonitrile and injected through a 50 μL loop. The following solvent systems were used: eluent (A): 0,05% trifluoroacetic acid (TFA) in H_2O, eluent (B) 100% CH_3CN. HPLC retention times (HPLC t_R) were obtained, at flow rates of 1 mL/min, using the following conditions: 100% eluent A for 5 min, then a gradient run to 100% eluent B over the next 20 min.

5.2. Synthesis

5.2.1. General procedure for the preparation of the secondary amines ***7–11***

Aldehyde 6 (316 mg, 1 mmol) was dissolved in anhydrous THF (20 mL). To this solution was added the corresponding primary

amine ($MeNH_2$, $EtNH_2$, $PrNH_2$, $BuNH_2$ or $iBuNH_2$, 7 mmol). The mixture was stirred at room temperature for 1.5 h. The solvent was evaporated under reduced pressure and the resulting oil was dissolved in anhydrous MeOH (10 mL).

After cooling at 0 °C, sodium borohydride (380 mg, 10 mmol) was added. The initially red solution turned to yellow. The mixture was poured into water. The product was then extracted with diethyl ether. The organic phase was dried over Na_2SO_4, filtered and evaporated. The crude product was chromatographed on silica gel column using ethylacetate/triethylamine 9:1 as eluent to afford the secondary amines **7**–**11**.

*5.2.1.1. (2-Dimethylaminomethylferrocenyl)-methylamine (**7**).* Brown oil (70% yield): ^{1}H NMR: δ 4.22 (s, 1H), 4.14 (s, 1H), 4.07 (s, 5H), 4.06 (s, 1H), 3.80 (d, 1H, J = 13.2 Hz), 3.65 (d, 1H, J = 12.3 Hz), 3.34 (d, 1H, J = 13.4 Hz), 2.85 (d, 1H, J = 12.2 Hz), 2.38 (s, 3H), 2.14 (s, 6H). ^{13}C NMR: δ 85.0 (C^{IV}), 83.6 (C^{IV}), 71.0 (CH), 70.1 (CH), 69.0 (5CH), 66.1 (CH), 58.0 (CH_2), 49.5 (CH_2), 44.9 ($2CH_3$), 35.1 (CH_3). MS (EI): *m/z*: 287 [MH^+].

*5.2.1.2. (2-Dimethylaminomethylferrocenyl)-ethylamine (**8**).* Brown oil (59% yield): ^{1}H NMR: δ 4.18 (s, 1H), 4.09 (s, 1H), 4.03 (s, 5H), 4.03 (s, 1H), 3.79 (d, 1H, J = 13.0 Hz), 3.65 (d, 1H, J = 12.6 Hz), 3.39 (d, 1H, J = 13.0 Hz), 2.84 (d, 1H, J = 12.6 Hz), 2.62 (m, 2H), 2.14 (s, 6H), 1.09 (t, 3H, J = 7.1 Hz). ^{13}C NMR: δ 85.6 (C^{IV}), 83.5 (C^{IV}), 70.9 (CH), 70.0 (CH), 68.9 (5CH), 65.9 (CH), 57.9 (CH_2), 47.3 (CH_2), 44.7 ($2CH_3$), 42.9 (CH_2), 14.8 (CH_3). MS (EI): *m/z*: 301 [MH^+].

*5.2.1.3. (2-Dimethylaminomethylferrocenyl)-propylamine (**9**).* Brown oil (50% yield): ^{1}H NMR: δ 4.18 (s, 1H), 4.10 (s, 1H), 4.04 (s, 6H), 3.76 (d, 1H, J = 12.8 Hz), 3.65 (d, 1H, J = 12.4 Hz), 3.35 (d, 1H, J = 13.0 Hz), 2.82 (d, 1H, J = 12.6 Hz), 2.51 (m, 2H), 2.13 (s, 6H), 1.48 (m, 2H), 0.88 (t, 3H, J = 7.2 Hz). ^{13}C NMR: δ 86.1 (C^{IV}), 83.7 (C^{IV}), 70.9 (CH), 70.1 (CH), 68.9 (5CH), 65.8 (CH), 58.0 ($2CH_2$), 51.0 (CH_2), 47.9 (CH_2), 44.9 ($2CH_3$), 22.9 (CH_2), 11.8 (CH_3). MS (EI): *m/z*: 315 [MH^+].

*5.2.1.4. (2-Dimethylaminomethylferrocenyl)-butylamine (**10**).* Brown oil (42% yield): ^{1}H NMR: δ4.18 (s, 1H), 4.09 (s, 1H), 4.04 (s, 5H), 4.01 (m, 1H), 3.73 (d, 1H, J = 13.1 Hz), 3.63 (d, 1H, J = 12.5 Hz), 3.32 (d, 1H, J = 13.1 Hz), 2.82 (d, 1H, J = 12.5 Hz), 2.54 (m, 2H), 2.12 (s, 6H), 1.43 (m, 2H), 1.31 (m, 2H), 0.89 (t, 3H, J = 7.1 Hz). ^{13}C NMR: δ 85.8 (C^{IV}), 83.9 (C^{IV}), 71.8 (CH), 71.5 (CH), 69.6 (5CH), 67.0 (CH), 57.8 (CH_2), 46.4 (CH_2), 45.3 (CH_2), 44.3 ($2CH_3$), 28.9 (CH_2), 20.0 (CH_2), 13.6 (CH_3). MS (EI): *m/z*: 329 [MH^+].

*5.2.1.5. (2-Dimethylaminomethylferrocenyl)-isobutylamine (**11**).* Brown oil (42% yield): ^{1}H NMR ($CDCl_3$) δ 4.16 (s, 1H), 4.07 (s, 1H), 4.01 (s, 5H), 3.99 (s, 1H), 3.70 (d, 1H, J = 12.8 Hz), 3.60 (d, 1H, J = 12.4 Hz), 3.30 (d, 1H, J = 13.0 Hz), 2.80 (d, 1H, J = 12.4 Hz), 2.30 (m, 2H), 2.10 (s, 6H), 1.71 (m, 1H), 0.87 (d, 3H, J = 3.0 Hz), 0.84 (d, 3H, J = 3.0 Hz). ^{13}C NMR ($CDCl_3$) δ 86.6 (C^{IV}), 83.7 (C^{IV}), 70.8 (CH), 70.1 (CH), 68.7 (5CH), 65.6 (CH), 58.0 (CH_2), 57.6 (CH_2), 48.2 (CH_2), 45.0 ($2CH_3$), 28.3 (CH), 20.7 ($2CH_3$). MS (EI): *m/z*: 329 [MH^+].

*5.2.2. General procedure for the preparation of the FQ derivatives **1**–**5***

A mixture of the corresponding amine **7–11** (2 mmol), 4,7-dichloroquinoline (2 g, 10 mmol), triethylamine (2 mL, 14.4 mmol), and K_2CO_3 (0.4 g, 2.9 mmol) in *N*-methyl-2-pyrrolidinone (7 mL) was stirred under nitrogen at 135 °C for 4 h and, after cooling to room temperature, diluted with CH_2Cl_2 (50 mL). The reaction mixture was washed with brine (10 × 50 mL) and dried over Na_2SO_4. The organic phase was then reduced under vacuum, and the resulting oil was chromatographed on silica gel column with ethylacetate/triethylamine, 99:1 as eluent to afford the FQ derivatives **1**–**5**.

*5.2.2.1. (7-Chloro-quinolin-4-yl)-(2-dimethylaminomethylferrocenyl)-methylamine (**1**).* Brown solid (60% yield): HPLC (C18 Nucleosil) P_{HPLC} 98% t_R 14.5 min; HPLC (CN-RP) P_{HPLC} 98% t_R 13.5 min.

^{1}H NMR ($CDCl_3$) δ 8.63 (d, 1H, J = 5.2 Hz), 8.05 (d, 1H, J = 9.1 Hz), 8.02 (d, 1H, J = 2.0 Hz), 7.38 (dd, 1H, J = 2.2, 8.9 Hz), 6.79 ppm (d, 1H, J = 5.3 Hz), 4.39 (m, 2H), 4.22 (m, 2H), 4.11 (m, 1H), 4.05 (s, 5H), 3.31 (d, 1H, J = 12.9 Hz), 3.10 (d, 1H, J = 12.9 Hz), 2.91 (s, 3H), 2.08 (s, 6H). ^{13}C NMR ($CDCl_3$) δ 157.3 (C^{IV}), 151.6 (CH), 150.6 (C^{IV}), 134.8 (C^{IV}), 128.8 (CH), 126.1 (CH), 125.4 (CH), 121.9 (C^{IV}), 108.8 (CH), 83.4 (C^{IV}), 83.2 (C^{IV}), 71.1 (CH), 70.1 (CH), 69.6 (5CH), 67.1 (CH), 57.7 (CH_2), 54.1 (CH_2), 45.2 ($2CH_3$), 40.5 (CH_3). MS (EI): *m/z*: 450 [MH^+ ^{37}Cl], 448 [MH^+ ^{35}Cl], 405 [M ^{37}Cl – NMe_2^+], 403 [M ^{37}Cl – NMe_2^+]. Anal. Calc. for $C_{24}H_{26}ClFeN_3$: C, 64.37; H, 5.85; N, 9.38. Found: C, 64.46; H, 5.81; N, 9.38%.

*5.2.2.2. (7-Chloro-quinolin-4-yl)-(2-dimethylaminomethylferrocenyl)-ethylamine (**2**).* Brown oil (58% yield): HPLC (C18 Nucleosil) P_{HPLC} 98% t_R 14.6 min; HPLC (CN-RP) P_{HPLC} 98% t_R 13.5 min.

^{1}H NMR ($CDCl_3$) δ 8.62 (d, 1H, J = 5.2 Hz), 8.07 (d, 1H, J = 9.0 Hz), 8.02 (d, 1H, J = 2.0 Hz), 7.38 (dd, 1H, J = 2.2, 9.0 Hz), 6.89 (d, 1H, J = 5.2 Hz), 4.43 (d, 1H, J=14,7 Hz), 4.32 (d, 1H, J=14,7 Hz), 4.18 (m, 2H), 4.04 (s, 1H), 4.03 (s, 5H), 3,41 (q, 2H, J=7.0 Hz), 3.33 (d, 1H, J = 12.8 Hz), 3.03 (d, 1H, J = 12.8 Hz), 2.08 (s, 6H), 1.09 (t, 3H, J= 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ 155.9 (C^{IV}), 151.3 (CH), 150.5 (C^{IV}), 134.6 (C^{IV}), 128.6 (CH), 125.9 (CH), 125.5 (CH), 122.9 (C^{IV}), 111.0 (CH), 83.7 (C^{IV}), 83.5 (C^{IV}), 70.6 (CH), 69.7 (CH), 69.4 (5CH), 66.5 (CH), 57.7 (CH_2), 50.6 (CH_2), 46.4 (CH_2), 45.2 (2 CH_3), 11.8 (CH_3). MS (EI): *m/z*: 464 [MH^+ ^{37}Cl], 462 [MH^+ ^{35}Cl], 419 [M ^{37}Cl – NMe_2^+], 417 [M ^{37}Cl – NMe_2^+]. Anal. Calc. for $C_{25}H_{28}ClFeN_3$: C, 65.02; H, 6.11; N, 9.10. Found: C, 65.10; H, 6.02; N, 9.15%.

*5.2.2.3. (7-Chloro-quinolin-4-yl)-(2-dimethylaminomethylferrocenyl)-propylamine (**3**).* Brown oil (40% yield): HPLC (C18 Nucleosil) P_{HPLC} 98% t_R15.0 min; HPLC (CN-RP) P_{HPLC} 98% t_R 13.9 min.

^{1}H NMR ($CDCl_3$) δ 8.61 (d, 1H, J = 5.1 Hz), 8.09 (d, 1H, J = 9.1 Hz), 8.02 (d, 1H, J = 2.2 Hz), 7.39 (dd, 1H, J = 2.2, 9.1 Hz), 6.85 ppm (d, 1H, J = 5.1 Hz), 4.46 (d, 1H, J = 14.6 Hz), 4.34 (d, 1H, J = 14.6 Hz), 4.16 (m, 2H), 4.13 (m, 1H), 4.01 (s, 5H), 3.30 (d, 1H, J = 12.5 Hz), 3.30 (t, 2H, J = 7.5 Hz), 2.98 (d, 1H, J = 12.7 Hz), 2.06 (s, 6H), 1.56 (m, 2H), 0.82 (t, 3H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ 156.1 (C^{IV}), 151.3 (CH), 150.6 (C^{IV}), 134.6 (C^{IV}), 128.6 (CH), 125.9 (CH), 125.4 (CH), 122.9 (C^{IV}), 111.0 (CH), 83.6 ($2C^{IV}$), 70.6 (CH), 69.7 (CH), 69.3 (5CH), 66.6 (CH), 57.7 (CH_2), 53.3 (CH_2), 51.5 (CH_2), 45.2 ($2CH_3$), 19.8 (CH_2), 11.5 (CH_3). MS (EI): *m/z*: 478 [MH^+ ^{37}Cl], 476 [MH^+ ^{35}Cl], 433 [M ^{37}Cl – NMe_2^+], 431 [M ^{37}Cl – NMe_2^+]. Anal. Calc. for $C_{26}H_{30}ClFeN_3$: C, 65.63; H, 6.35; N, 8.83. Found: C, 65.58; H, 6.29; N, 8.80%.

*5.2.2.4. (7-Chloro-quinolin-4-yl)-(2-dimethylaminomethylferrocenyl)-butylamine (**4**).* Brown oil (56% yield): HPLC (C18 Nucleosil) P_{HPLC} 98% t_R 15.3 min; HPLC (CN-RP) P_{HPLC} 98% t_R 14.2 min.

^{1}H NMR ($CDCl_3$) δ 8.62 (d, 1H, J = 5.2 Hz), 8.08 (d, 1H, J = 9.1 Hz), 8.02 (d, 1H, J = 1.9 Hz), 7.39 (dd, 1H, J = 1.9, 8.9 Hz), 6.85 ppm (d, 1H, J = 5.1 Hz), 4.53 (d, 1H, J = 14.4 Hz), 4.38 (d, 1H, J = 14.4 Hz), 4.16 (m, 1H), 4.12 (m, 1H), 4.03 (m, 1H), 4.01 (s, 5H), 3.30 (m, 3H), 2.96 (d, 1H, J = 12.9 Hz), 2.07 (s, 6H), 1.50 (m, 2H), 1.24 (m, 2H), 0.85 (t, 3H, J = 7.3 Hz). ^{13}C NMR ($CDCl_3$) δ 156.1 (C^{IV}), 151.3 (CH), 150.7 (C^{IV}), 134.9 (C^{IV}), 128.7 (CH), 125.9 (CH), 125.4 (CH), 122.9 (C^{IV}), 111.1 (CH), 83.6 ($2C^{IV}$), 70.6 (CH), 69.7 (CH), 69.3 (5CH), 66.6 (CH), 57.7 (CH_2), 51.7 (CH_2), 51.2 (CH_2), 45.2 ($2CH_3$), 28.7 (CH_2), 20.3 (CH_2), 13.9 (CH_3). MS (EI): *m/z*: 492 [MH^+ ^{37}Cl], 490 [MH^+ ^{35}Cl], 447 [M ^{37}Cl – NMe_2^+], 445 [M ^{37}Cl – NMe_2^+]. Anal. Calc. for $C_{27}H_{32}ClFeN_3$: C, 66.20; H, 6.58; N, 8.58. Found: C, 66.29; H, 6.50; N, 8.58%.

*5.2.2.5. (7-Chloro-quinolin-4-yl)-(2-dimethylaminomethylferrocenyl)-isobutylamine (**5**).* Brown oil (36% yield): HPLC (C18 Nucleosil) P_{HPLC} 98% t_R 15.2 min; HPLC (CN-RP) P_{HPLC} 98% t_R 14.1 min.

^{1}H NMR ($CDCl_3$) δ 8.62 (d, 1H, J = 5.2 Hz), 8.16 (d, 1H, J = 8.9 Hz), 8.04 (d, 1H, J = 2.1 Hz), 7.44 (dd, 1H, J = 2.1, 9.0 Hz), 6.80 ppm (d, 1H, J = 5.2 Hz), 4.54 (d, 1H, J = 14.4 Hz), 4.37 (d, 1H, J = 14.4 Hz), 4.15 (m, 1H), 4.12 (m, 1H), 4.05 (m, 1H), 3.97 (s, 5H), 3.25 (d, 1H, J = 13.0 Hz), 3.12 (m, 1H), 2.93 (m, 1H), 2.88 (d, 1H, J = 13.0 Hz), 2.02 (s, 6H), 1.03 (m, 1H), 0.86 (d, 3H, J = 4.6 Hz), 0.83 (d, 3H, J = 4.6 Hz). ^{13}C NMR ($CDCl_3$) δ 156.3 (C^{IV}), 151.2 (CH), 150.7 (C^{IV}), 134.6 (C^{IV}), 128.7 (CH), 126.0 (CH), 125.4 (CH), 123.1 (C^{IV}), 111.4 (CH), 83.8 (C^{IV}), 83.3 (C^{IV}), 70.6 (CH), 69.7 (CH), 69.3 (5CH), 66.7 (CH), 57.7 (CH_2), 57.6 (CH_2), 53.3 (CH_2), 45.1 ($2CH_3$), 26.0 (CH), 20.6 (CH_3), 20.5 (CH_3). MS (EI): *m/z*: 492 [MH^+ ^{37}Cl], 490 [MH^+ ^{35}Cl], 447 [M ^{37}Cl – NMe_2^+], 445 [M ^{37}Cl – NMe_2^+]. Anal. Calc. for $C_{27}H_{32}ClFeN_3$: C, 66.20; H, 6.58; N, 8.58. Found: C, 66.27; H, 6.52; N, 8.50%.

5.3. Partition coefficients – log D (pHs 7.4 and 5.2)

In this study, the relative log *D*s were assessed at pHs 7.4 and 5.2 by the micro-HPLC method. Determinations were performed with a chromatographic apparatus (Spectra Series, San Jose, USA) equipped with a model P1000XR pump and a model SCM 1000 vacuum membrane degasser, a model UV 150 ultraviolet detector (λ = 330 nm) and a ChromJet data module integrator (ThermoFinnigan, San Jose, USA). The reversed phase column used was a Waters XTerra™MS C_{18} (3.9 × 150 mm; 5 μm particle size) with a mobile phase consisting of acetonitrile – potassium dihydrogenophosphate 6.24×10^{-2} M [KH_2PO_4/K_2HPO_4] (pH = 6) (35:65, v/v (**1–3**)), (40:60, v/v (**4,5**)). The compounds were partitioned between *n*-octanol (HPLC grade) and phosphate buffer (pH = 7.4 or 5.2). Octanol was presaturated with the adequate phosphate buffer (1%), and conversely. An amount of 1 mg of each compound was dissolved in an adequate volume of methanol in order to produce 1 mg/mL stock solutions. Then, an appropriate aliquot of these methanolic solutions was dissolved in buffer to obtain final concentration of 50 μg/mL. Under the above-described chromatographic conditions, 50 μL of aqueous phase was injected into the chromatograph, leading to the determination of a peak area before partitioning (W_0). In screw-capped tubes, 4000 μL of the aqueous phase (V_{aq}) were then added to 10 μL of *n*-octanol (V_{oct}) when measuring at pH = 7.4 or $V_{aq} = V_{org}$ = 500 μL when working at pH = 5.2. The mixture was shaken by mechanical rotation during 20 min, followed by centrifugation at 3000 rpm during 10 min. An amount of 50 μL of the lower phase was injected into the chromatograph column. This led to the determination of a peak area after partitioning (W_1). For each compound, the log *D* value was calculated using the formula:

$$\log D = \log[(W_0 - W_1)V_{aq}/W_1V_{oct}] \quad (3)$$

5.4. Potentiometric titration – pK_a determination

5×10^{-5} mole of compound was dissolved in a mixture of 25 mL of hydrochloric acid (5×10^{-3} M) and 25 mL of dioxane [58]. Potassium nitrate 0.1 M was added to this solution to adjust the ionic strength. The solution was stirred and connected to a thermostated water bath and allowed to equilibrate thermally. After which the initial pH was recorded using a potentiometric glass pH meter. The solution was then titrated using aliquots of a known volume of 0.01 M NaOH. The pH of the solution was measured ten seconds after each addition of NaOH.

5.4.1. Plasmodium falciparum cultures and in vitro assay

Twelve parasite strains or isolates from a wide panel of countries, Africa (3D7), Brazil (Bre), Cambodia (K2 and K14), Cameroon (FCM29), Djibouti (Voll), the Gambia (FCR3), Indochina (W2), Niger (L1), Senegal (8425), Sierra Leone (D6), and Uganda (PA), were maintained in culture in RPMI 1640 (Invitrogen, Paisley, United Kingdom), supplemented with 10% human serum (Abcys S.A. Paris, France) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$. Parasites were grown in A-positive human blood (Centre de Transfusion des Armées, Toulon, France) under controlled atmospheric conditions that consist of 10% O_2, 5% CO_2, and 85% N_2 at 37 °C with a humidity of 95%.

FQ base was obtained from Sanofi-Aventis (France). CQ diphosphate and DHA were purchased from Sigma (Saint Louis, MO). MQ was obtained from Hoffman-LaRoche (Bale, Switzerland). FQ and synthetic compounds were resuspended and then diluted in RPMI-DMSO (99v/1v) to obtain final concentration ranging from 0.125 and 500 nM. CQ was resolubilized in water in concentrations ranging between 5 and 3200 nM. MQ and DHA were resolubilized in methanol and then diluted in water to obtain concentrations ranging from 3.12 to 400 nM and 0.1 to 100 nM, respectively.

The isotopic, micro drug susceptibility test used was based on the microdilution radioisotope technique of Desjardins. For *in vitro* isotopic microtests, 25 μL/well of antimalarial drug and 200 μL/well of the parasitized red blood cell suspension (final parasitemia, 0.5%; final hematocrit, 1.5%) were distributed into 96 well plates. Parasite growth was assessed by adding 1 μCi of tritiated hypoxanthine with a specific activity of 14.1 Ci/mmol (PerkinElmer, Courtaboeuf, France) to each well at time zero. The plates were then incubated for 48 h in a controlled atmospheric condition. Immediately after incubation, the plates were frozen and thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B; Perkin–Elmer) and washed using a cell harvester (Filter-Mate Cell Harvester; Perkin–Elmer). Filter microplates were dried, and 25 μL of scintillation cocktail (Microscint O; Perkin–Elmer) was placed in each well. Radioactivity incorporated by the parasites was measured with a scintillation counter (Top Count; Perkin–Elmer).

The IC_{50}, the drug concentration able to inhibit 50% of parasite growth, was assessed by identifying the drug concentration corresponding to 50% of the uptake of tritiated hypoxanthine by the parasite in the drug-free control wells. The IC_{50} and IC_{90} values were determined by non-linear regression analysis of log-based dose-response curves (Riasmart™, Packard, Meriden, USA). The cut-off values, defined statistically (>2SD above the mean with or without correlation with clinical failures), for *in vitro* resistance or reduced susceptibility to CQ was 100 nM.

5.5. In vitro assay of β-hematin inhibition

Drug solutions (89.1 mM, 53.5 mM, 26.7 mM, 17.8 mM, 13.4 mM, 8.9 mM, 4.5 mM, 1.8 mM and 0 mM) were prepared by dissolving the drug in MeOH. Hematin stock solution (1.68 mM) was prepared by dissolving bovine hemin (0.64 mg) in 0.1 M NaOH (980 μL). The solution was incubated at room temperature for 60 min. In a series of plastic microtubes 2.0 μL of 1 M HCl and 2.0 μL of drug (or solvent for the blank) solution were dispensed. The microtubes were placed in an incubator at 60 °C and then, 12.9 M sodium acetate solution, pH 5.0, (11.7 μL) preincubated at 60 °C was added. The β-hematin formation process was initiated by addition of hematin stock solution (20.2 μL) prepared above. The final hematin concentration was 1 mM, the final drug concentrations were 5 mM, 3 mM, 1.5 mM, 1 mM, 0.75 mM, 0.5 mM, 0.25 mM, 0.1 mM and 0 mM and the final solution pH was 4.5. The reaction mixtures were incubated at 60 °C for 60 min. After incubation, the reaction mixtures were quenched at room temperature by adding 900 μL of 200 mM HEPES 5% (v/v) pyridine solution, pH 8.2, to adjust the final pH of the mixtures to a value between 7.2 and 7.5. Then, 20 mM HEPES 5% (v/v) pyridine

solution, pH 7.5 (1100 μL) was added. The microtubes were shaken and the precipitate of β-hematin was scraped from the walls of the microtubes to ensure complete dissolution of hematin. The β-hematin mixture was allowed to stand at room temperature for at least 15 min. The supernatant was carefully transferred to a cuvette without disturbing the precipitate and absorption was measured at 405 nm.

5.6. NMR experiments

NMR spectra were recorded at room temperature in $CDCl_3$ for neutral FQ and in D_2O for diprotonated FQ on Bruker Avance 300 MHz spectrometer. ^1H and ^{13}C spectra were referenced to internal TMS for FQ and DSS for FQ.2HCl or the residual proton signals of $CDCl_3$ (δ^1H = 7.24 ppm and $\delta^{13}C$ = 77.2 ppm). COSY, ^{13}C-HSQC and ^{15}N-HMBC experiments were performed using the standard sequences. The UDEFT experiments were recorded as described [59]. Two-dimensional NOESY (mixing time = 300 ms), ^{15}N-HMBC (for neutral FQ) and ROESY (for diprotonated FQ, mixing time = 400 ms) experiments were recorded at 280 K on Bruker Avance 400 MHz spectrometer. For compound **4**, two-dimensional NOESY experiments were recorded at 300 and 323 K on Bruker Avance 400 MHz spectrometer. ROESY experiments were used when the rotational correlation time of the compound led to small and null NOEs.

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Appendix A. Supporting information available

Chemical numbering of proton atoms and observed intramolecular NOEs (or ROEs) of neutral FQ (Fig. SI1), of diprotonated FQ (Fig. SI2) and compound 4 (Fig. SI3). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2008.09.033.

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