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Insights into the Mechanism of Action of Ferroquine. Relationship between Physicochemical Properties and Antiplasmodial Activity

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Abstract: Ferroquine (FQ) is a 4-aminoquinoline antimalarial which contains a quinoline nucleus similar to chloroquine, but a novel ferrocenic group in its side chain. Previous work has demonstrated that this compound has excellent activity against malaria parasites, both in vitro and in vivo, with especially good activity against chloroquine-resistant parasites, but details of its mechanism of action have not previously been reported. In this study, we have investigated the physicochemical properties of FQ for comparison with chloroquine (CQ). Like CQ, FQ forms complexes with hematin in solution (log $K = 4.95 \pm 0.05$). FQ is an even stronger inhibitor of β-hematin formation than CQ ($IC_{50} = 0.78$ equiv relative to hematin for FQ vs 1.9 for CQ). These data suggest that the mechanism of action of FQ is likely to be similar to that of CQ and probably involves hematin as the drug target and inhibition of hemozoin formation. However, both the basicity and lipophilicity of FQ are significantly different from those of CQ. The lipophilicity of FQ and CQ are similar when protonated at the putative food vacuole pH of 5.2 (log $D = -0.77$ and -1.2 respectively), but differ markedly at pH 7.4 (log $D = 2.95$ and 0.85 respectively). In addition, the pK_a values of FQ are lower (p$K_{a1} = 8.19$ and p$K_{a2} = 6.99$) than those of CQ (10.03 and 7.94, respectively). This suggests that there will be somewhat less vacuolar accumulation of FQ compared with CQ. Single crystal structure determination of FQ shows the presence of a strong internal hydrogen bond between the 4-amino group and the terminal N atom. This, together with the electron donating properties of the ferrocene moiety, probably explains the decreased pK_a. Interestingly, the decreased accumulation arising from the less basic behavior of this compound is partly compensated for by its stronger β-hematin inhibition. Increased lipophilicity, differences in geometric and electronic structure, and changes in the N−N distances in FQ compared to CQ probably explain its activity against CQ-resistant parasites.

Keywords: Malaria; hematin; hemozoin; ferroquine; chloroquine; drug resistance; structure−activity relationships

Introduction

Malaria remains one of the main causes of morbidity and mortality in the world. The burden of malaria has not declined in the tropics, partly because parasites have become resistant to available drugs.[1] The inexpensive mainstay of malaria control, chloroquine (CQ), is now ineffective in

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the majority of areas, and resistance to antifolates has emerged rapidly after large scale deployment. In some areas, there are pockets of multidrug resistance. There is great expectation from artemisinin-type compounds,[2] and the current WHO recommendation is to use them combined with other drugs which will provide mutual protection against the development of resistance. There is however at present a shortage of novel drugs that do not share the same mechanisms of resistance with those that are failing today. The quinoline type compounds continue to attract interest because their mechanisms of action and resistance are independent.

CQ is believed to interfere with the disposal of heme, the toxic byproduct of the digestion of host hemoglobin, which takes place in the *Plasmodium* digestive vacuole. One of the detoxification pathways involves sequestration of free heme into hemozoin. Indeed, at least 95% of the iron released during hemoglobin digestion has been shown to be in the form of hemozoin.[3]

Structural and spectroscopic analysis has revealed that hemozoin has the same structure as β-hematin.[4] The molecules of ferriprotoporphyrin IX (FPPIX) are linked into dimers through reciprocal iron−carboxylate bonds to one of the propionic side chains of each porphyrin, and these dimers form chains linked by hydrogen bonds. The mechanism of hemozoin formation is still a matter of debate. Chloroquine (CQ) is thought to exert its antimalarial effect by preventing the conversion of toxic heme to hemozoin, which finally leads to membrane damage and parasite death.[5−8] The mechanism of this blockade is still unknown, but CQ can bind heme, hence preventing it from being incorporated into the hemozoin crystal.[8] Equally unclear are the mechanisms whereby this event leads to parasite damage and death.[9]

We have previously described how ferroquine (FQ), a ferrocenic analogue of chloroquine, was identified in a collaborative drug discovery project.[10−12] Here, by analogy with CQ, we report the interaction of ferroquine with hematin in order to gain information about the mechanism of action of this drug. Additional questions, such as FPPIX/ferroquine complex formation, the role of pH gradient in drug accumulation and resistance, and the relationship of physicochemical properties and structure to the antimalarial activity, are discussed.

Materials and Methods

Partition Coefficients: log *D* (pH 7.4 or pH 5.2). The relative log *D* (pH 7.4 or 5.2) in this study was assessed by the micro-HPLC method.[13] These determinations were performed with a chromatographic apparatus (Spectra Series, San Jose) equipped with a model P100 pump, a model UV 150 ultraviolet detector (λ = 330 nm), and a ChromJet data module integrator (ThermoFinnigan, San Jose). A reversed-phase column was used: a Waters XTerra MS C_{18} (3.9 × 150 mm; 5 μm particle size) with a mobile phase consisting of acetonitrile−phosphate buffer (pH = 7) (60:40, v/v (FQ), and 20:80, v/v (CQ)). The compounds were partitioned between 1-octanol (HPLC grade) and phosphate buffer (pH = 5.2 or 7.4). Octanol was presaturated with buffer, and vice versa. An amount of 1 mg of each compound was dissolved in an adequate volume of methanol in order to achieve 1 mg/mL stock solutions. Then an appropriate aliquot of these methanolic solutions was dissolved in buffer to obtain a final concentration of 100 μg/mL. Under the above-described chromatographic conditions, 20 μL of this aqueous phase was injected into the chromatograph, leading to the determination of a peak area before partitioning (W_0).

In screw-capped tubes, 500 μL of the aqueous phase (V_{aq}) was then added to 100 μL of *n*-octanol (V_{oct}) when working at pH = 5.2; V_{aq} = 2000 μL and V_{oct} = 10 μL for determination at pH = 7.4. The mixture was shaken by mechanical rotation for 30 min. Then the centrifugation was carried out at 3000 rpm for 15 min. An amount of 20 μL of

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Table 1. Crystallographic Data for Neutral and Diprotonated Ferroquine

parameter	FQ	FQH_2^{2+}
formula	$C_{23}H_{24}ClFeN_3$	$C_{23}H_{34}Cl_3FeN_3O_4$
MW (g mol^{-1})	433.75	578.73
a [Å]	7.595(3)	6.9045(10)
b [Å]	12.825(6)	24.690(4)
c [Å]	20.487(9)	15.415(2)
α [deg]	90.00	90.00
β [deg]	92.567(9)	95.585(3)
γ [deg]	90.00	90.00
V [Å^3]	1993.7(15)	2615.4(7)
Z	4	4
crystal system	monoclinic	monoclinic
space group	$P2_1/n$	$P2_1/n$
μ (cm^{-1})	0.904	0.917
ρ (g cm^{-3})	1.445	1.470
F(000)	904	1208
θ limits [deg]	1.87−31.42	2.66−28.90
T (K)	100	100
no. of data collected	5838	6074
$R = \sum\|\|F_o\| - \|F_c\|\|/\sum\|F_o\|$	0.0480	0.0552
R_w	0.1142	0.1225
goodness of fit	1.085	0.989
$\Delta\rho_{min}$ [e Å^{-3}]	−0.437	−0.441
$\Delta\rho_{max}$ [e Å^{-3}]	1.266	0.655

the lower phase was injected into the chromatograph column. This led to the determination of a peak area after partitioning (W_1). The log *D* was determined by the formula

$$\log D = \log \frac{(W_0 - W_1)V_{aq}}{W_1 V_{oct}}$$

X-ray Crystal Structure Determination of Neutral and Diprotonated FQ. Suitable crystals were mounted on a Bruker SMART CCD area-detector diffractometer with Mo Kα ($\lambda = 0.71073$ Å). The structures were solved by using SHELXS97 and refined by using SHELXL97.[14] All non-hydrogen atoms were anisotropically refined. Hydrogen atoms were introduced in calculated positions in the last refinements, and they were allocated one overall isotropic thermal parameter. Major crystallographic data and collection details are summarized in Table 1. CCDC 262108 and CCDC 262109 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Inhibition of *β*-Hematin Formation and Association of Hematin with FQ. The association constant of FQ with hematin was measured as described previously.[15] Briefly, a stock solution of the iron porphyrin was prepared by dissolving 8.0 mg of hemin in DMSO. A working solution was freshly prepared from this stock solution consisting of 40% v/v DMSO, 0.020 M HEPES pH 7.5, and 4.0 μM iron porphyrin. This was then titrated with a solution of 2.0 mM FQ in 40% v/v DMSO, 0.020 M HEPES pH 7.5. Temperature was maintained at 25 °C using a water circulating bath, and absorbance readings were recorded at 402 nm on a Varian Cary 100 spectrophotometer. The reference cell containing 40% v/v DMSO, 0.020 M HEPES pH 7.5 was also titrated with FQ in order to blank out the absorbance of the drug. Absorbance data were corrected for dilution and then fitted to a 1:1 association model using nonlinear least-squares fitting.

The ability of FQ to inhibit β-hematin formation was determined as described previously.[16] A solution of hematin was prepared by dissolving 15 mg of hemin in 3.0 mL of 0.1 M NaOH. To this solution was added 3 molar equiv of solid FQ, and then the NaOH was neutralized with 0.30 mL of 1.0 M HCl. After 10 min of preincubation at 60 °C, 1.74 mL of 12.9 M sodium acetate/acetic acid, pH 5 was added to give a final solution pH of 4.5. Addition of acetate causes immediate precipitation of hematin. This slurry was heated with stirring for 60 min, after which it was cooled on ice and then filtered using an 8 μm cellulose nitrate filter disk and washed with water. A control reaction was also performed under identical conditions, but no FQ was added. These solids were dried over P_4O_{10} and silica gel. After 48 h, KBr disks were prepared using 2 mg of sample and 250 mg of dry KBr and infrared spectra were recorded.

Quantification of Inhibition of *β*-Hematin Formation. The quantitative BHIA (β-hematin inhibitory activity) assay is based on the different solubility of hemin and β-hematin in DMSO and NaOH solution, respectively.[17] The method determines a 50% inhibitory concentration for β-hematin inhibition in equivalents of compounds to hemin ($BHIA_{50}$). A total of 50 μL of an 8 mM solution of hemin is dissolved in DMSO and distributed in 96-well U-bottom microplates (0.4 μmol/well); 50 μL of different compounds in water, in different doses ranging between 0.25 and 2 molar equiv relative to hemin, are added to triplicate test wells. Control wells receive 50 μL of water. Water-insoluble compounds are solubilized in 25 μL of DMSO and then added to hemin prepared at 16 mM and distributed into the wells in 25 μL aliquots. The final concentration of DMSO/well is kept constant at 25%. β-Hematin formation is initiated by the addition of 100 μL of 8 M acetate buffer (pH 5) and the assay developed after an overnight incubation, as detailed previously.[17,18]

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Molecular Modeling. All energy calculations were carried out using the Gaussian 03 suite of programs.[19] In a first step the DFT B3LYP parameter hybrid method (which corresponds to Becke's three-parameter hybrid exchange[20] and to Lee, Yang, and Parr's correlation functionals[21]) was employed to optimize the crystallographic structures of FQ and CQ at the pseudopotential LANL2DZ and polarized split-valence 6-31G(d,p) basis set level, respectively. Molecular electrostatic potential (MEP) surfaces were drawn using the CUBEGEN utility. The molecular electrostatic potential maps (MEPs) isoenergy contours were generated in the range of +60 to +220 kcal/mol and superimposed onto the total electron density surface (0.005 e/au^3). As the B3LYP/LANL2DZ calculations suggests that ferrocene has a singlet ground state, this reference state was also considered in the calculations performed on ferroquine. The visualization of all calculation results was performed with Gaussview.

Results and Discussion

Recent studies on 4-aminoquinoline analogues of CQ[22,23] have suggested a plausible structure−activity relationship (SAR) for this class of compound. The 4-aminoquinoline nucleus appears to be essential for strong complex formation with Fe(III)PPIX, while the 7-chloro group is required for inhibition of β-hematin formation. The tertiary amino group in the side chain and the quinoline N, both of which are basic, appear to be important for drug accumulation in the acidic parasite food vacuole through pH trapping resulting from their basicity. Thus, physicochemical properties, including association with Fe(III)PPIX, strength of inhibition of β-hematin formation, pK_a, and possibly also lipophilicity, may be related to biological activity in this class of compound. In order to investigate the mode of action of FQ, we determined some of these physicochemical properties.

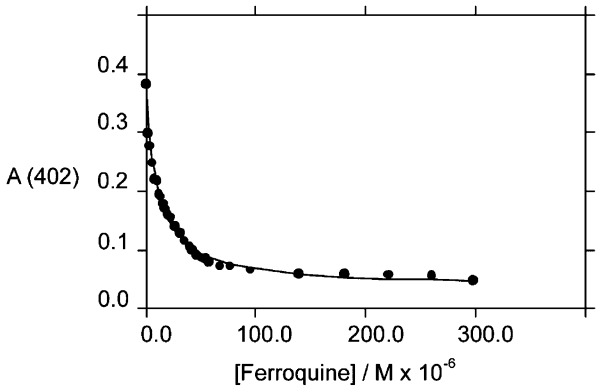

Figure 1. Variation in absorbance of Fe(III)PPIX at 402 nm as a function of ferroquine concentration.

Association of FQ with Hematin and Inhibition of β-Hematin Formation. The interaction of ferroquine with monomeric hematin was performed in 40% aqueous DMSO (pH 7.5) at 25 °C. Previous studies[24] have shown that association constants between hematin and quinoline antimalarials (like CQ, quinine, or mefloquine) in this solvent system closely mirror those in acidic aqueous solution as reported by titration calorimetry.[6] So, the association constant determined for FQ is likely to be a reasonable estimate of that in acidic aqueous solution and hence under physiological conditions.

The Soret band of hematin at 402 nm was monitored as a function of ferroquine concentration, after corrections for dilution and the absorbance of FQ at this wavelength (Figure 1). Fitting of the data to a 1:1 association model yielded the association constant, log $K = 4.95 \pm 0.05$. This value is lower than that previously reported for CQ, log $K = 5.52 \pm 0.05$, but still in the same range.[22] Association between monomeric hematin and a 4-aminoquinoline decreases as water is replaced by organic solvents.[25] Considering that the system is buffered and that there is still a significant aqueous component, FQ probably remains largely protonated, as it

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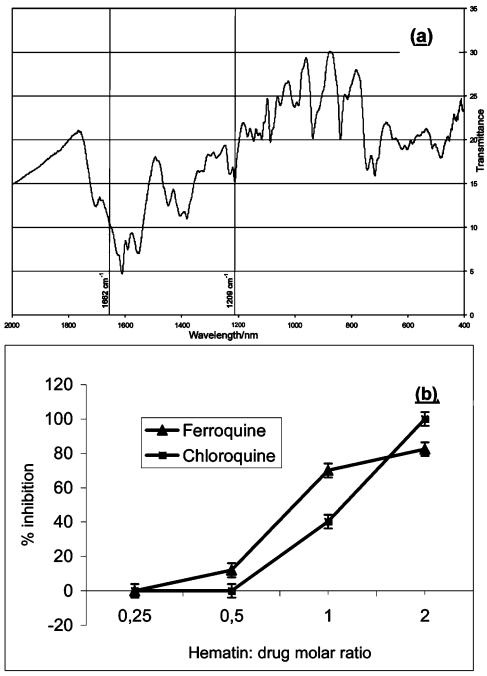

Figure 2. (a) Infrared spectra of hematin in the presence of ferroquine (3 equiv) after heating at 60 °C at pH 4.5 in 4.5 M acetate solution. (b) Inhibition of β-hematin formation by different molar equivalents of ferroquine compared to chloroquine. The results refer to a representative experiment out of two conducted in triplicate according to the protocol of the BHIA assay described in ref 17.

would be at the food vacuole pH. Furthermore, it is noteworthy that the association of both drugs with hematin is insensitive to ionic strength. So, these results, when interpreted in the light of the results of previous studies on CQ,[25] suggest that hydrophobic effects probably drive 4-aminoquinoline/hematin interaction, while electrostatic interactions (possibly including cation$-\pi$ interactions) may govern the specificity and geometry of the interaction. This bears similarities with protein−protein interaction, which is a dynamic process.

When hematin was subjected to conditions used in the preparation of β-hematin,[16] β-hematin formation was inhibited by FQ. This is demonstrated by the infrared spectrum in which the peaks at 1662 and 1209 cm^{-1} characterizing β-hematin are absent (Figure 2a). This clearly shows that ferroquine is a strong inhibitor of β-hematin formation.

The inhibition of β-hematin formation by diprotonated FQ was also quantitated and related to that of chloroquine using the BHIA assay.[17] As shown in Figure 2b, a dose-dependent inhibition of β-hematin formation was seen. Whereas the maximal inhibition (100%) of β-hematin formation was not reached for FQ, its $BHIA_{50}$ (that is, the hematin:drug molar ratio inhibiting β-hematin formation by 50%) was 0.78, whereas the $BHIA_{50}$ of chloroquine in the same set of experiments was 1.9. This confirms that FQ is a strong inhibitor of β-hematin formation, and more potent than CQ as measured in terms of IC_{50} in the BHIA assay. This increase in β-hematin inhibitory activity will be expected to partly compensate for decreased vacuolar accumulation arising from the weaker basicity of FQ (see below).

Molecular Modeling. The molecular electrostatic potential (MEP) surfaces have been computed for diprotonated FQ and CQ. Optimized geometries of charged FQ and CQ were generated in a vacuum at the DFT-B3LYP level using the polarized split-valence 6-31G(d,p) basis set for CQ and using the pseudopotential basis set LANL2DZ for FQ. DFT takes into account the concept of electronic correlation in the calculations and tends to favor a homogenized electronic structure with delocalized π electrons. The MEP map of the charged ferroquine (Figure 3) exhibits a profile with values of potential ranging from +60 kcal/mol (orange) to +220 kcal/mol (blue). The less positive potential is located on the position occupied by the chlorine atom and the cyclopentadienyl rings. The area around the three nitrogen atoms presents the most positive electrostatic potential. Note that FQ and CQ MEPs show a considerable similarity in the quinoline area and are complementary to the previously described receptor whose calculated potential is extremely flat.[26−28] Hematin binding and β-hematin inhibition in CQ analogues appear to be largely independent of the side chain. In light of these theoretical aspects, we suggest a similar mode of interaction between these active drugs (FQ or CQ) and hematin. They are likely to form parallel stacked complexes in aqueous solution. Indeed, the positively charged quinoline moiety could favorably approach the partially negative central zone of the porphyrin ring giving a favorable London dispersion interaction. The geometry of the interaction is likely to be influenced by the electrostatic component associated with the quadrupole moment of hematin and/or by HOMO−LUMO charge-transfer interaction. Finally, as previously noted for the benzene dimer,[29,30] the binding interaction in water is probably complicated by additional hydrophobic effects, in agreement with the experimental investigations described above. Further experiments (e.g., relaxation experiments using NMR spectroscopy) will be now needed to elucidate the structural and energetic parameters of FQ/FPPIX noncovalent association and to compare them to those for CQ.[31]

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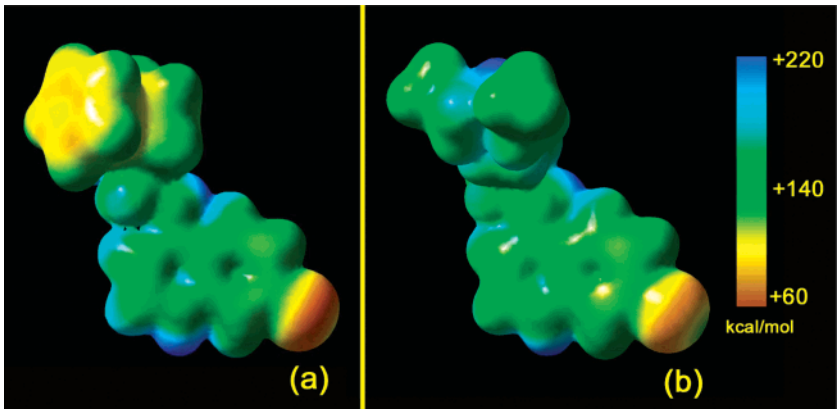

Figure 3. Molecular electrostatic potentials plotted onto the total electron density surface (0.005 e/au^3) for (a) FQ and (b) CQ. The orange color corresponds to a potential of +60 kcal/mol, and the blue color corresponds to a potential of +220 kcal/mol. The molecules are oriented with the quinoline moiety in the plane of the page.

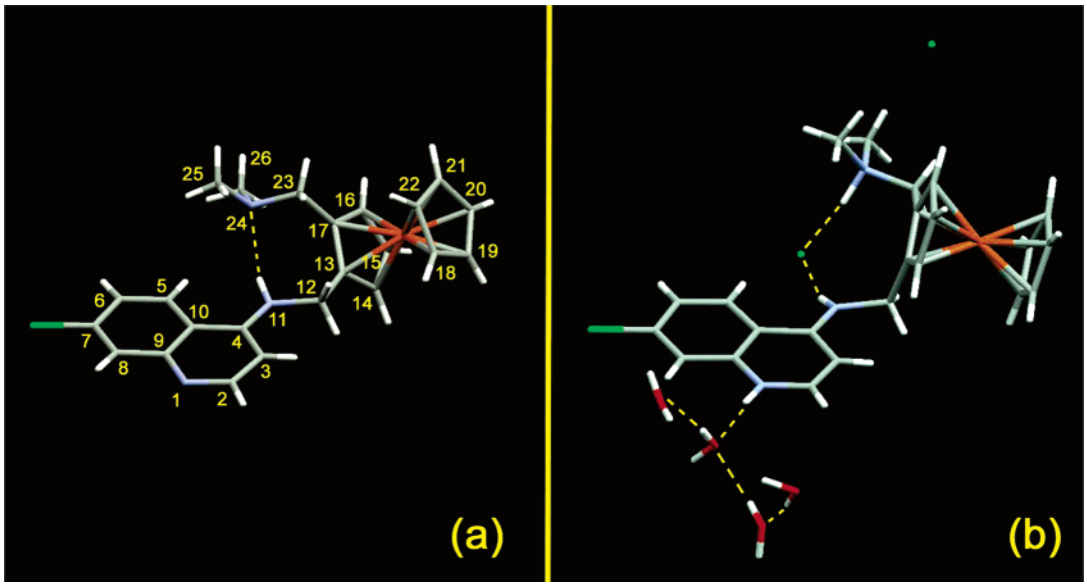

Figure 4. View of the molecular structure of (a) neutral and (b) diprotonated ferroquine with atomic numbering scheme. The carbon atoms are colored in gray, the hydrogen atoms in white, the chloride atoms in green, the nitrogen atoms in blue, and the iron atoms in orange. Hydrogen bonding is depicted by a yellow dashed line. The images were generated using Mercury.

Structure of Neutral and Diprotonated Ferroquine. Crystallization of neutral and diprotonated ferroquine from AcOMe and MeOH/H_2O produced yellow crystals which were suitable for X-ray structural determination. Ferroquine dichloride was crystallized in the dicationic form with protonation on the quinoline ring nitrogen atom and on the terminal amino group. Both neutral and diprotonated ferroquine crystallize in the monoclinic space group $P2_1/n$. Crystallographic data are collected in Table 1. The molecular structures of both compounds are shown in Figure 4.

The solid state structure of neutral ferroquine is stabilized by a strong intramolecular hydrogen bond between the anilino N(11) and the tertiary amino N(24), giving a N(24)···(N11) distance of 2.95 Å. In the diprotonated form, this distance becomes 4.07 Å due to electrostatic repulsion between the charged H−N(24) and partially charged H−N(11) hydrogen atoms. By comparison, this value for chloroquine is 5.39 Å.[32] The length of the side chain and the distance between the two exocyclic nitrogen atoms may be factors involved in resistance against 4-aminoquinolines by *Plasmodium falciparum*; 4-aminoquinolines with shorter (two or three carbon atoms) or longer side chains (10 or 12 carbon atoms) than CQ are more active against CQ-resistant *P. falciparum*.[33,34] These authors propose that these molecules have a N−N spacing which is less suited for binding with the putative chloroquine transporter, and are therefore less efficiently extruded from the food vacuole. Thus, this

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shortened distance in FQ may be a second important factor (in addition to lipophilicity, see below) in its activity against CQ-resistant parasites. The strong intramolecular hydrogen bond is probably also an important factor in lowering the pK_a of the side-chain nitrogen atom.

The value of the torsion angle in the unprotonated form along the N(11)−C(12) bond is 179.0° while it is decreased to 82.9° in the charged FQ due to electrostatic repulsion between both heteroatomic groups. As the side chain is coplanar with the quinoline ring in the neutral FQ, the distance between the diastereotopic protons of the C(12) and the H(3) of the quinolic ring is quite similar: 2.38 and 2.48 Å (*pro-R* and *pro-S*, respectively). This distance is shifted to 3.49 and 2.18 Å in the diprotonated form, as the side chain is almost orthogonal to the quinoline plane. The ferrocene has adopted an eclipsed conformation in both structures. The iron distances to the two cyclopentadienyl rings differ slightly (less than 0.1 Å) in the charged and uncharged FQs. Interestingly, the packing diagram of the charged FQ shows that the quinoline rings are $\pi-\pi$ stacked in a slipped antiparallel configuration, with a phenyl−phenyl distance of 3.32 Å. This orientation is mainly influenced by the position of the chlorine atom of one ferroquine almost above the secondary amino group of the antiparallel ferroquine (Supporting Information, Figure SI 1) to minimize the interactions between both compounds (see the FQ MEP, Figure 3). This interaction is absent in the neutral form. Moreover, a hydrogen-bonding network exists in this crystal between the protonated endocyclic nitrogen atom, four water molecules, and four chloride anions (Supporting Information, Figure SI 1).

Lipophilicity and Basicity. In the absence of special transport mechanisms, the accumulation of weak bases like CQ and FQ in a particular organelle can be described in terms of physicochemical behavior through the partitioning theory.[13,35] This involves the influence of pK_a and lipophilicity of CQ and FQ.[36] Indeed the lipophilic, diprotic CQ is believed to cross membranes in its unprotonated form by passive diffusion and then to accumulate after protonation in the food vacuole of the parasite.[37−39] Some studies indicate that association with Fe(III)PPIX may play a major part in CQ accumulation.[40,41] However, it has been pointed out that accumulation through pH trapping probably remains critical to drive this equilibrium process, even if it represents only a small fraction of the overall accumulation.[42] So, the pH gradient between the food vacuole and the cytosol probably plays an important role in CQ accumulation and could likewise be important in FQ activity. Consequently, in order to evaluate drug accumulation in the food vacuole, we determined the apparent partition coefficient (log *D*) at vacuolar and cytosolic pHs (5.2 and 7.4 respectively) of CQ and FQ by HPLC. The in silico vacuolar accumulation ratios (VAR) based on a weak-base model were then calculated.[43]

The log *D* values of CQ and FQ have been determined by HPLC at pHs 5.2 and 7.4. The measured *D* values show a considerably larger lipophilicity at cytosolic pH for FQ compared to CQ (more than 100-fold), whereas only a slight difference in lipophilicity can be noticed at vacuolar pH (about 3-fold). In terms of basicity, published pK_as of the two drugs indicate that FQ (pK_{a1} = 8.19 and pK_{a2} = 6.99) is less basic than CQ (pK_{a1}= 10.03 and pK_{a2}= 7.94).[44] Consequently, in the absence of special transport mechanisms, FQ, as a weaker base, would be expected to accumulate in the food vacuole less than CQ (VAR (FQ) = 6402, VAR (CQ) = 19521, Table 2). The variation between these two values reflects large differences in the protonation of CQ and FQ.

One main structural difference between FQ and CQ consists of the introduction of a ferrocenyl moiety into the side chain. This ferrocenyl entity possesses lipophilic and electron-donating properties.[45] Consequently, the presence of a ferrocenyl group in the FQ system produces a large

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Table 2. Experimental pK_a, log *P*, log *K*, IC_{50}, and log *D* at Two Different pHs Reflecting the Cytosolic and Vacuolar Environments and in Vitro Antimalarial Activity against the CQ-Senstive Strain (HB3) and the CQ-Resistant Strain (Dd2)

				log *D*				⟨IC_{50}/nM⟩	
compd	pK_{a1}	pK_{a2}	log *P*	pH 5.2	pH 7.4	VAR	log *K*	HB3	Dd2
CQ	10.03[a]	7.94[a]	4.63	−1.20	0.85	19521	5.52 ± 0.03[b]	26[c]	137[c]
FQ	8.19[a]	6.99[a]	5.1	−0.77	2.95	6402	4.95 ± 0.05	24[c]	27[c]

[a] From ref 44. [b] From ref 15. [c] From ref 53.

increase in lipophilicity and a modification in basicity. Each compound in its neutral form has similar lipophilicity: the partition coefficients log *P* are 4.65 and 5.1 for CQ and FQ, respectively.[46] Changes affecting the pK_as of CQ and FQ lead to differences in protonation equilibria at both pH 5.2 and 7.4. These differences in equilibria are also reflected in the measured lipophilicities. At vacuolar pH, FQ and CQ are both hydrophilic with negative log *D* and seem to be prevalently present in the diprotonated form. At cytosolic pH, there are distinct differences between the protonation equilibria of the two drugs and interestingly their lipophilicity behaviors are also different. Within each compartment, the different behaviors of CQ and FQ are thus mainly owing to the influence of their pK_as and the pH of that compartment.

Structure Activity Relationship. There is now clear evidence that FQ shares with CQ some similarities in their mechanism of action. On the other hand, there is also evidence that the mechanism of resistance to FQ is different from that of CQ.

It is interesting to consider whether FQ biological activity can be related to that of CQ via the QSAR reported for short chain analogues of CQ.[23] Two factors should be borne in mind in attempting such a comparison. First, the QSAR was determined only for compounds with an ethyl side chain and diethylamino terminus. Second, the strain of parasite was different. Nevertheless, the relationship between the accumulation normalized IC_{50} and $BHIA_{50}$ values, combined with the predicted accumulation ratios described above, leads to predicted IC_{50} values against CQ-sensitive parasites of 17 and 27 nM for CQ and FQ, respectively. Experimental values observed with the HB3 strain are 25.8 ± 2.7 and 23.6 ± 5.5 nM respectively (Table 2.). Thus, the predicted values lie within 1 and 2 standard deviations of the experimental values, respectively. Thus, with respect to inherent biological activity FQ appears to be very similar to CQ.

The most striking differences arise from the facts that (i) IC_{50} variations for field isolates were found unrelated to mutations occurring in *P. falciparum* chloroquine resistance transporter (PfCRT) protein (suspected to be the drug efflux system) or its level of expression, and (ii) resistance of the malarial parasite (W2 strain) to FQ could not be induced under drug pressure.[47] Clearly, the main structural difference between FQ and CQ arises from their side chains, namely, the ferrocene nucleus and the alkyl group.

In CQ-resistant strains, we can hypothesize that incorporation of the bulky lipophilic metallocenic moiety in the skeleton of CQ does not allow the "masked" chloroquine-like part of the molecule to interact with the transporter and to be effluxed from the digestive vacuole. A significant correlation between the hydrophobicity of a series of CQ-like compounds and their antiplasmodial activity against chloroquine-resistant parasites has been previously reported.[48,49] Lipophilic compounds are thus believed to block the PfCRT pore. Moreover, PfCRT mutations that result in CQ resistance (including Lys76→Thr (K76T)) also confer verapamil (another lipophilic compound) reversibility.[50,51] So, ferroquine may be considered to retain the antimalarial properties of chloroquine, while simultaneously blocking PfCRT through its lipophilic properties like a resistance reversing agent.

The result will be maintainance of the concentration of toxic drug within the vacuole. Both differences in N−N distances in FQ relative to CQ and increased lipophilicity relative to CQ may account for increased activity against CQ-resistant parasites. The proposed structure−activity relationship in ferroquine is shown in Figure 5. A recent study by Cheruku et al.[52] on carbon isosteres of chloroquine has suggested that the pyridine substructure of the 4-aminoquinoline nucleus is in fact the critical component for hematin binding. The 4-amino-7-chloroquinoline has been confirmed as critical for β-hematin inhibition and hence biological activity. This study also suggests that the side

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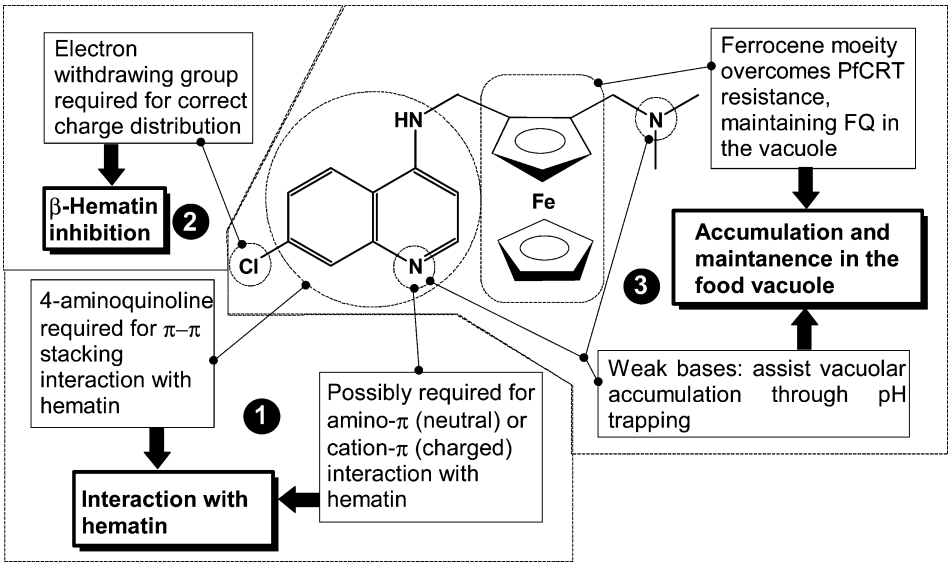

Figure 5. Proposed structure−activity relationships for ferroquine.

chain plays little role in either hematin binding or β-hematin inhibition.

Conclusion

Ferroquine is a lipophilic weak base and should concentrate itself in the parasites' food vacuole. Due to its physicochemical properties, the detailed contributions of the postulated mechanisms (protonation, active uptake, and/or specific receptor) should be clearly different from those of chloroquine.

As CQ is thought to exert its action by interfering with heme metabolism, more precisely by binding to hematin preventing its aggregation into hemozoin, the data obtained here (association of FQ with hematin and inhibition of β-hematin formation) have allowed the establishment of a similar mechanism of action for ferroquine.

In comparison to CQ, the presence of the ferrocene moiety with different (i) shape, (ii) volume, (iii) lipophilicity, (iv) effects on basicity, and (v) electronic profile dramatically modifies the pharmacological behavior of the parent drug. Therefore, FQ appears to present reduced affinity for the postulated transporter linked to CQ resistance, which seems to be extremely structure specific. This may partially explain the remarkable activity of FQ against CQ-resistant strains or isolates.

Additional experiments will be needed to determinate the exact form of FQ (neutral, monoprotonated, or diprotonated) interacting with hematin and to probe the nature of the interaction(s) in the drug−heme complex ($\pi-\pi$ stacking, cation$-\pi$, or other).

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Supporting Information Available: Packing diagram of diprotonated ferroquine (Figure SI 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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