Articles

Novel Short Chain Chloroquine Analogues Retain Activity Against Chloroquine Resistant K1 *Plasmodium falciparum*

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A series of short chain chloroquine (CQ) derivatives have been synthesized in one step from readily available starting materials. The diethylamine function of CQ is replaced by shorter alkylamine groups (**4**−**9**) containing secondary or tertiary terminal nitrogens. Some of these derivatives are significantly more potent than CQ against a CQ resistant strain of *Plasmodium falciparum* in vitro. We conclude that the ability to accumulate at higher concentrations within the food vacuole of the parasite is an important parameter that dictates their potency against CQ sensitive and the chloroquine resistant K1 *P. falciparum*.

Introduction

Malaria remains one of the most important diseases of man with over half the world's population at risk of infection and 1−2 million deaths annually. Despite the importance of this disease, investment into the treatment and control of malaria is modest. As a consequence, the number of deaths due to malaria is gradually increasing as available antimalarial drugs become less and less effective due to the advance of multidrug resistance.

Chloroquine (CQ, **1**) and other quinoline antimalarials have been mainstays of malaria chemotherapy for much of the past 40 years. The success of these drugs was based on excellent clinical efficacy, limited host toxicity, ease of use, and simple, cost effective synthesis. However, the use of these drugs has been seriously eroded in recent years, mainly as a result of the development and spread of parasite resistance to CQ and related compounds.[1] Although much of the current effort is directed toward the identification of novel chemotherapeutic targets, we still do not fully understand the mode of action of and the mechanism of resistance to the quinoline compounds, knowledge that would greatly assist the design of novel, potent, and inexpensive quinoline antimalarials.

The malarial parasite resides in an environment rich in hemoglobin, which is pinocytosed primarily by trophozoites, the most metabolically active of the erythrocytic stages.[2] In the food vacuole, the digestion of hemoglobin produces large amounts of free hematin (ferriprotoporphyrin IX, Fe(III)FPIX), a toxic lytic moiety that must be sequestered or degraded for parasite survival. It is in this compartment that CQ is thought to exert its effects.[3,4] Recent evidence suggests that CQ and other 4-aminoquinolines act by interfering with the detoxification of hematin that is released during hemoglobin digestion.[5−9] Normally, free hematin is detoxified either by incorporation into hemozoin crystal,[10,11] a crystalline matrix of coordinated dimers, and/or by degradation of the porphyrin ring system.[12−14] Under drug pressure, however, a drug−hematin complex is formed,[15,16] impairing hematin sequestration and hematin destruction.[13,14,17−20] This leads to a build-up of hematin, which is able to exert a toxic effect resulting in parasite death.

The molecular basis for CQ resistance is not fully understood; however, it is clear that CQ resistant parasites accumulate less drug than sensitive strains.[21] Earlier studies have shown that 4-aminoquinolines containing *N*,*N*-diethylaminoalkyl side chains of altered length retained their activity against CQ resistant strains of *Plasmodium falciparum* malaria.[22] A potential drawback of these derivatives was their ability to undergo side chain dealkylation (i.e., for short chain CQ analogues, deethylation) in vivo.[23] We have previously shown that this metabolic change significantly reduces the lipid solubility of the drug and significantly increases cross-resistance up to and beyond that seen with CQ.[26] However, the effect of incorporating a basic side chain into CQ that cannot be metabolized to dealkylated derivatives has been demonstrated in few examples.[24,25] Amodiaquine (AQ, **2**, Figure 1) analogues containing a metabolically stable side chain have been shown to retain significant antimalarial activity in vitro.[26] Furthermore, the in vivo efficacy of tebuquine (**3**, Figure 1) is thought to be partially due to the replacement of the diethylamine side chain of AQ (**2**) with a *tert*-butylamine functionality thereby inhibiting metabolism of the side chain to metabolites that display cross-resistance.[26,27a]

In light of these observations, we have synthesized a new series of CQ analogues where the diethylamino

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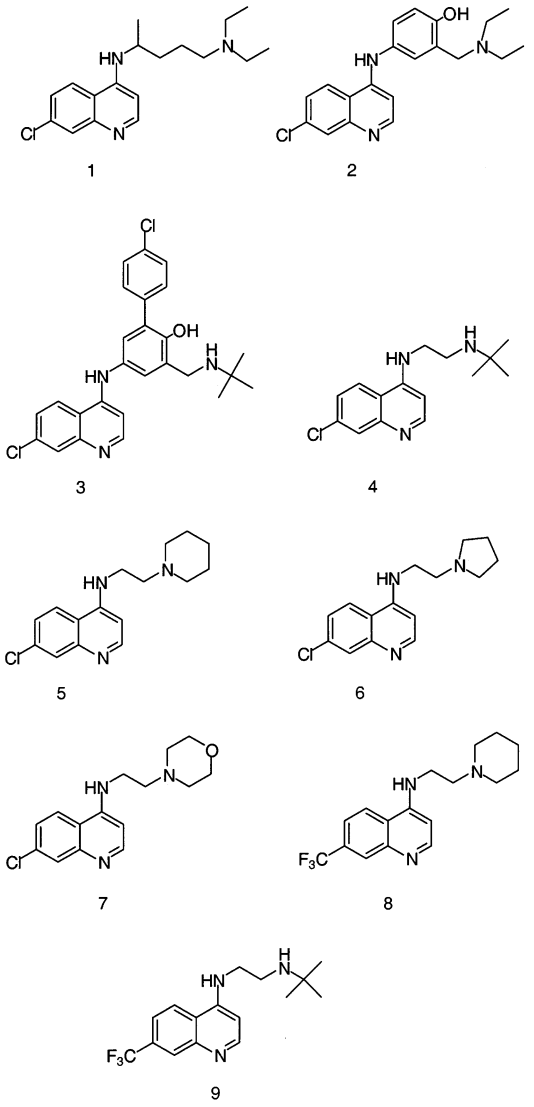
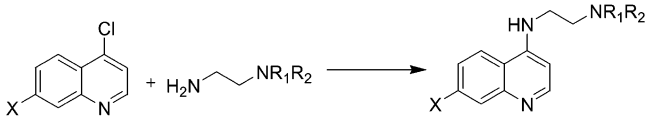


Figure 1. Structures of CQ (**1**), AQ (**2**), tebuquine (**3**), and the short chain CQ derivatives (**4**−**9**).

Scheme 1. One Step Synthesis of CQ Analogues



*iso*pentyl function of the CQ side chain has been replaced by shorter side chains, containing metabolically more resilient terminal secondary and tertiary alkylamino groups.[27b] The ability of each compound within the series (**4**−**9**) (Scheme 1) to arrest the growth of CQ sensitive and CQ resistant strains of *P. falciparum* in vitro has been measured in the anticipation that these derivatives may circumvent the mechanism of drug resistance. In addition, the ability of these derivatives (**4**−**9**) to compete for the same intraparasitic receptor as CQ, Fe(III)FPIX, has been investigated to gain insight into their mode of action and potential mechanism of resistance.[27c,28a]

Table 1. Antimalarial Activity of Short Chain CQ Derivatives (**4**−**9**)

compd	resistance factor (K1/HB3)	antimalarial activity HB3 (nM)	antimalarial activity K1 (nM)
CQ	10	19.0 ± 2.1 (2)	190 ± 15 (3)
4, 7-Cl-*tert*-butyl (C2Bu)	3.56	4.3 ± 1.3 (2)	15.3 ± 2.9 (2)
5, 7-Cl-piperidyl (C2Pip)	1.90	6.8 ± 0.8 (4)	13.0 ± 2.3 (4)
6, 7-Cl-pyrrolidino (C2Pyr)	1.94	6.8 ± 1.2 (3)	13.2 ± 1.1 (3)
7, 7-Cl-morpholino (C2Mor)	1.56	150 ± 41 (3)	240 ± 29 (3)
8, 7-CF_3-piperidyl (F2Pip)	2.09	10.6 ± 0.5 (3)	22.2 ± 3.5 (3)
9, 7-CF_3-*t*-butyl (F2Bu)	1.78	5.5 ± 0.7 (2)	9.8 ± 0.6 (2)

Results

Antimalarial Activity. All of the six short chain CQ derivatives prepared (**4**−**9**) inhibited growth of both the CQ sensitive (HB3) and the CQ resistant (K1) parasites in vitro (Table 1) at concentrations in the nanomolar range. Replacement of the diethylamino function of CQ with a more metabolically inert basic side chain group, such as *tert*-butyl, piperidyl, or pyrrolidino, led to a substantial increase in antimalarial activity against the CQ resistant strain. The derivatives that contained a *tert*-butyl terminal amino group in the basic side chain were found to be the most active. There was more than a 4-fold increase in activity against CQ sensitive strain for C2Bu (**4**, 4.3 nM) as compared with CQ (19 nM). Importantly, all derivatives displayed greatly reduced cross-resistance to CQ, with resistant factors between 1.5 and 3.6 as compared to 10 for CQ. In fact, the F2Bu analogue (**9**) exhibited almost a 20-fold increase in activity against the CQ resistant strain with the remaining derivatives exhibiting a 12-fold increase.

Our data (Table 1) suggest that replacing the 7-chloro substituent with a trifluoromethyl group did not significantly alter the antimalarial activity against either strain. Conversely, when the *tert*-butyl terminal group was replaced with a morpholino group, a substantial reduction in drug activity was observed. Activity decreased from 4.2 (**4**) to 150 nM (**7**) for the CQ sensitive isolate and from 15.3 (**4**) to 240 nM (**7**) for the CQ resistant strain.

Short Chain CQ Analogues Bind to the Same Binding Site as CQ in *P. falciparum*. All of the short chain CQ derivatives inhibited [^{3}H]CQ uptake into intact parasites in a dose-dependent manner (Table 2). The C2Bu (**4**) derivative was the most potent inhibitor of CQ binding to this intraparasitic receptor site and was more potent than unlabeled CQ in inhibiting the uptake of [^{3}H]CQ into intact parasites (CQ, 0.95 mM, as compared to C2Bu, 0.71 mM, Table 2). The C2Pip (**5**) analogue was equipotent to CQ (0.91 mM). It was not possible to determine the 50% inhibitory concentration for the C2Mor (**7**) compound; at the highest drug concentration tested, 10 mM, uptake was inhibted by only 15%. The remaining derivatives, C2Pyr, F2Bu, and F2Pip (**6**, **8**, and **9**) exhibited similar inhibitory potency (IC_{50} values between 1.65 and 2.09 mM), greater than that observed for unlabeled CQ. Similarly, these short chain CQ derivatives displaced prebound [^{3}H]CQ from Fe(III)FPIX-loaded ghosts in a concentration-dependent manner (Table 2). The most potent compound was C2Bu (**4**, 3.9 mM), while the least potent was C2Mor (**7**, 25 mM).

Short Chain CQ Analogues Bind to Fe(III)FPIX and Prevent Incorporation of Fe(III)FPIX into

Table 2. Inhibitory Values for Fe(III)FPIX Uptake, CQ Uptake in Intact Parasites, and CQ Displacement from Fe(III)FPIX-Loaded Membranes for CQ and Short Chain CQ Derivatives

compd	inhibition of CQ uptake from intact parasites IC_{50} values (mM)	displacement of chloroquine from Fe(III)FPIX-loaded membrane IC_{50} values (mM)	β-hematin formation IC_{50} values (mM)
CQ	0.95 ± 0.02	1.76 ± 2.23	38 ± 5
4, 7-Cl-*tert*-butyl (C2Bu)	0.71 ± 0.01	3.94 ± 1.61	70 ± 12
5, 7-Cl-piperidyl (C2Pip)	0.91 ± 0.21	7.65 ± 2.30	86 ± 6
6, 7-Cl-pyrrolidino (C2Pyr)	1.62 ± 0.05	5.94 ± 2.20	78 ± 4
7, 7-Cl-morpholino (C2Mor)	N/A	24.9 ± 6.50	102 ± 10
8, 7-CF_3-piperidyl (F2Pip)	2.03 ± 0.46	8.39 ± 2.92	65 ± 2
9, 7-CF_3-*t*-butyl (F2Bu)	2.09 ± 0.25	4.96 ± 2.0	71 ± 8

***β*-Hematin Polymer.** All of the short chain CQ derivatives (**4**−**9**) inhibited β-hematin formation (Table 2) in a concentration-dependent manner. None of the new derivatives were more potent than CQ. With the exception of C2Mor (**7**), all of the new derivatives displayed a similar ability to inhibit Fe(III)FPIX crystallization as demonstrated by their respective 50% inhibitory concentrations (between 65 and 86 mM). The least potent inhibitor was C2Mor (**7**) with an inhibitory concentration of 102 mM.

Molecular Modeling Studies. A number of studies have indicated that a key feature of CQ's antimalarial activity is its interaction with hematin in the digestive vacuole of the parasite. Recently, Vippagunta has employed isothermal titration calorimetry studies (ITC) to derive association constants for CQ−hematin binding.[28b] From this work, it was shown that CQ binds to two μ-oxo dimers in a sandwich arrangement originally proposed by Moreau.[28c] Importantly, these studies demonstrate that CQ−hematin binding is independent of ionic strength suggesting that an interaction between the charged side chain terminal nitrogen of CQ with the carboxylates of hematin does not play a major role in complex formation. Studies on CQ−hematin binding by Egan and co-workers employed 40% dimethyl sulfoxide (DMSO) water, conditions that maintain hematin in a monomeric state.[28d,f] Stoichiometries of CQ binding to hematin, in this work, were recorded to be 1:1. The most recent investigation employed fluorescence spectroscopy in aqueous buffered solutions. A stoichiometry of 1:2 (CQ:hematin) probably suggested that the binding involves CQ complexed to one of the faces of a μ-oxo dimer.[28e] Because it is still not clear whether CQ interacts primarily with hematin monomers, μ-oxo dimers, or other forms of hematin in the food vacuole, for simplicity, we have carried out modeling on a 1:1 complex of CQ:hematin in line with our previous work.[24] We also note that other works have suggested that CQ may inhibit hemozoin formation by blocking the growing face of the hemozoin crystal by a capping effect.[28f] In this case and in the interactions with a monomer, because the bonding interactions are cofacial, we would anticipate that the interaction energies emerging from this study would also be representative of such a capping effect.

The ability of the new series of CQ analogues to form a complex with Fe(III)FPIX was investigated by molecular modeling studies both in vacuo and solvated with water (Tables 3 and 4). The binding potential was investigated using diprotonated short chain CQ derivatives as it is this form that is likely to play a role in Fe(III)FPIX:drug complex in the acidic food vacuole of the malarial parasite. There was no correlation observed between antimalarial activity and the Fe(III)FPIX binding energies or the intermolecular distances of the short chain CQ analogues under either set of conditions. In the solvated water model, all of the short chain CQ derivatives exhibited shorter bond lengths between the intermolecular nitrogen of the side chain and the carbonyl oxygen of Fe(III)FPIX when compared to the in vacuo model. However, the intramolecular nitrogen to nitrogen distance for each drug in the water model was similar to those values predicted in vacuo.

From our earlier work,[24] we suggested that the principle bonding interactions between hematin and CQ and AQ were $\pi-\pi$-stacking interactions of the quinoline ring over the porphyrin with a possible additional weak electrostatic interaction between the protonated ammonium function and the carboxylate groups of hematin. We could find no evidence, in this earlier work, to support a H-bonding interaction between the protonated nitrogen of CQ or AQ and the carboxylates of the hematin porphyrin side chain. From Table 3, it can clearly be seen that the protonated nitrogen of CQ in the CQ−hematin complex is 3.70 Å from the carboxy-

Table 3. Fe(III)FPIX Binding Energies and Intermolecular Distances of the Short Chain CQ Analogues In Vacuo

compd	N−N (Å)[a]	NH−O (Å)[b]	E_I (kcal)[c]
CQ	9.41	3.701	−42.56
4, C2Bu	7.315	2.602	−41.82
5, C2Pip	7.479	2.74	−46.03
6, C2Pyr	7.003	2.82	−42.3
7, C2Mor	7.093	2.595	−42.91
8, F2Pip	7.521	2.55	−54.04
9, F2Bu	6.791	2.456	−55.26

[a] Intramolecular distance (in Angstoms) between the terminal nitrogen of the side chain and the nitrogen at the 4-position of the quinoline ring. [b] Intermolecular distance (in Angstoms) between the protonated terminal nitrogen of the side chain and the nearest carbonyl oxygen atom from a carboxylic acid group of Fe(III)FPIX. [c] The interaction energy for the Fe(III)FPIX−drug complex (see main text for details).

Table 4. Fe(III)FPIX Binding Energies and Intermolecular Distances of the Short Chain CQ Analogues in a Water Model

compd	NH−O (Å)[a]	E_I (kcal)
4, C2Bu	2.553	−100.52
5, C2Pip	2.967	−127.86
6, C2Pyr	2.734	−70.12
7, C2Mor	2.609	−107.15
8, F2Pip	2.701	−143.71
9, F2Bu	2.905	−77.44

[a] Intermolecular distances of the nitrogen atom in the side chain and the carboxyl oxygen. [b] Intermolecular distance (in Angstoms) between the protonated terminal nitrogen of the side chain and the nearest carbonyl oxygen atom from a carboxylic acid group of Fe(III)FPIX. [c] The interaction energy for the Fe(III)FPIX−drug complex within a solvated sphere.

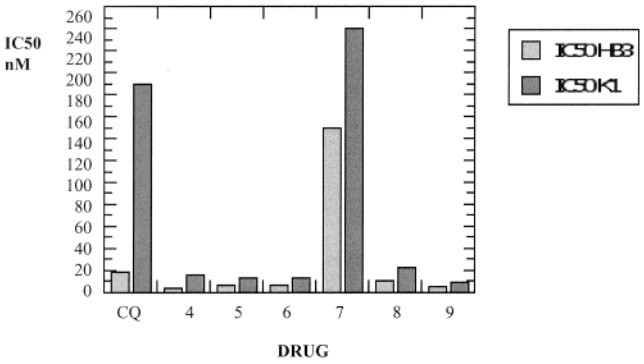


Figure 2. Bar chart showing the IC_{50} values for the short chain compounds and CQ against CQ sensitive and resistant parasites.

lates in hematin. Thus, this interaction probably contributes little to the overall interaction energy of the drug complex. In line with this, and as noted above, Vippagunta has clearly shown experimentally by ITC studies that this interaction probably contributes little to drug−hematin binding.[28b,28g]

In comparison to CQ, the shorter chain analogues of CQ have a significantly reduced distance for the protonated charged ammonium nitrogen to the carboxylates in hematin (<2.80 Å). Figure 3 shows the orientation of CQ with Fe(III)FPIX where the most important interaction is the $\pi-\pi$-stacking of the quinoline ring over the porphyrin macrocycle. In Figure 4, modeling suggests that for compound **4**, C2Bu, the principal interactions are $\pi-\pi$-stacking of the quinoline ring with an additional potential for an interaction of the protonated ammonium function with the hematin carboxylates (C2Bu, 2.6 Å for NH to O in hematin). (Although it would appear that such an interaction is feasible, it is also clear from the interaction energies (E_i) in Table 3 that this interaction does not significantly alter the drug−hematin interaction energy when compared with CQ, first two entries in Table 3.) With further appropriate ITC experiments on these short chain analogues, it remains to be seen whether the principal interactions in the hematin drug complex are the same as those in the CQ−hematin complex with an absence of electrostatic interactions as suggested in these modeling studies.

As can be seen from Table 4, although the drug compounds show good binding interactions with Fe(III)-

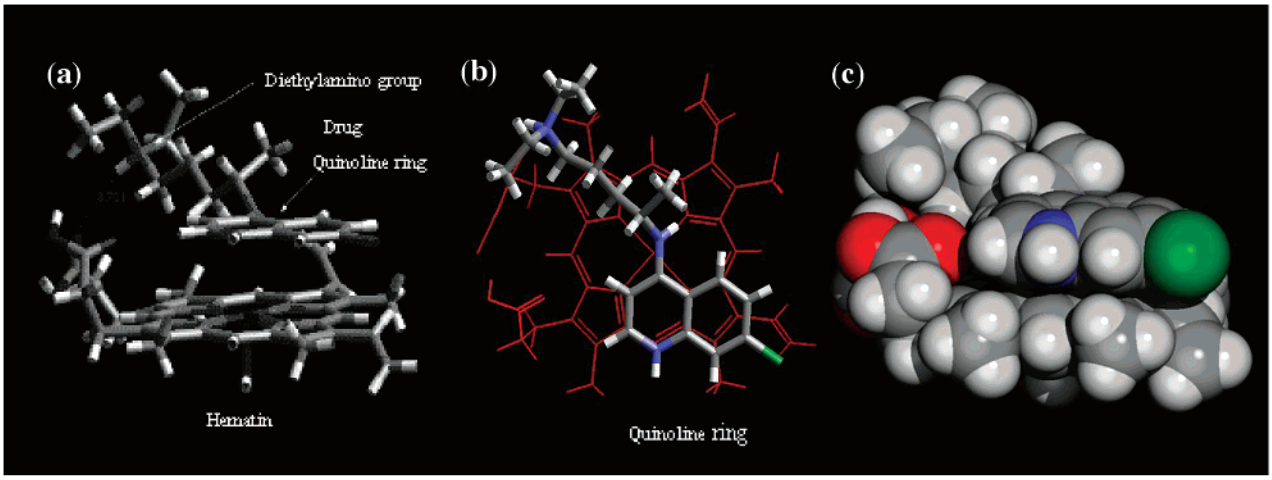


Figure 3. (a) Lowest energy complex of hematin (Fe(III)FPIX) (in vacuo) with CQ showing the π-stacking interaction between the porphyrin ring of hematin and the quinoline ring of CQ. The figure on the left is the side on view (a) with the top down view on the right (b). For panel b, all atoms of hematin are displayed in red for clarity. The space-filling model for the complex is shown on the right (c). Carbon atoms are represented in gray. Hydrogens are white, nitrogens are blue, oxygens are light red, chlorine is green, and iron is dark red.

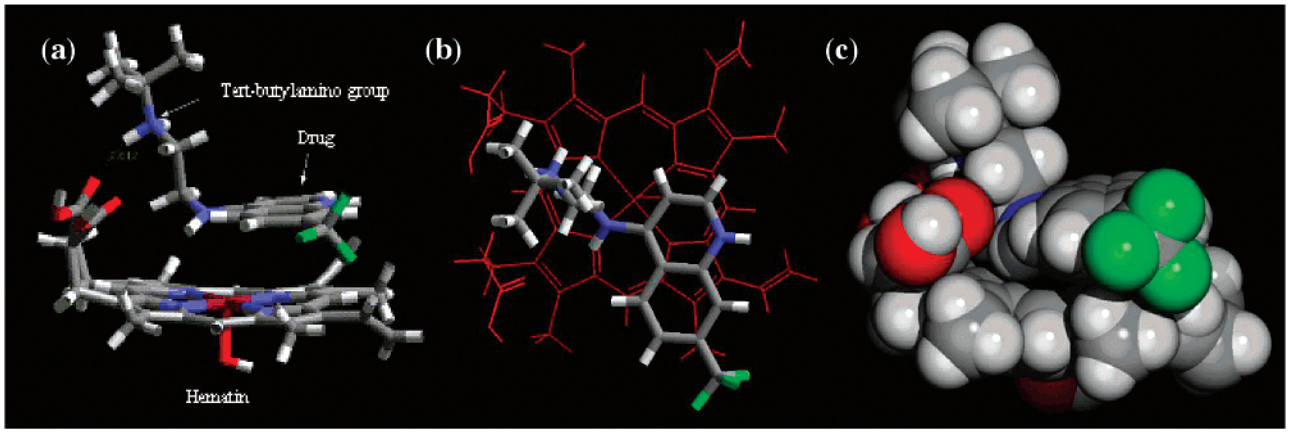


Figure 4. (a) Lowest energy complex of Fe(III)FPIX (in vacuo) with F2Bu (**9**) showing the $\pi-\pi$-stacking interaction between the porphyrin ring of hematin and the quinoline ring of CQ and a potential electrostatic interaction between the carboxylic acid group and the protonated amino side chain of the drug. The complex viewed from the top down direction is shown in panel b. The space-filling model for the complex is shown on the right (c). Atom labels are as in Figure 3.

FPIX, there is no direct correlation between antimalarial activity for the short chain compounds and the Fe(III)FPIX interaction energies calculated within the water model. It should be noted that changes in structural functionality as seen in the current series of analogues will lead to altered physicochemical properties for the drug molecule, which will influence cellular accumulation within the food vacuole. It is these physiochemical properties that will ultimately dictate the potency of a drug against the malarial parasite. All of the short chain compounds display good and essentially equivalent potential to interact with Fe(III)FPIX; the superior antimalarial activity of these drugs results from their enhanced cellular accumulation within the parasite attributed to enhanced membrane lipid solubility. In fact, the C2Mor (**7**) derivative is able to interact with Fe(III)FPIX as well as the other derivatives in the series even though it is almost 8-fold less potent than CQ against CQ sensitive isolates, which clearly confirms the view that additional factors influence drug efficacy.

Discussion

Malaria parasites accumulate CQ to a greater extent than any other type of eukaryotic cell.[31] It has been assumed that CQ, a diprotic weak base, accumulates in the acidic compartments of the parasite facilitated by a pH gradient.[32−34] However, passive diffusion cannot be the only mechanism driving CQ accumulation, as CQ uptake is more pronounced in *P. falciparum* as compared to other eukaryotic cells that contain large acidic compartments.[31] In the 1970s, Fitch[35] and co-workers demonstrated that CQ uptake into *P. falciparum* was saturable, energy-dependent, and could be inhibited by a number of compounds.[36] These studies suggest that malaria parasites possess an additional CQ concentrating mechanism acting in concert with passive diffusion and proton trapping. Our own recent studies have concluded that free Fe(III)FPIX acts as an intraparasitic receptor, and it is this moiety that allows CQ and other 4-aminoquinolines to accumulate to millimolar concentrations inside the malaria parasite.[9,25]

By schizogony, the malarial parasite has digested large amounts of host hemoglobin, producing vast quantities of free Fe(III)FPIX, a toxic moiety that can become incorporated into hemozoin (malaria pigment).[37] CQ and other quinolines readily form a complex with free Fe(III)FPIX in vitro,[10,15] and the idea that this interaction could contribute to the specific accumulation of CQ within the malaria parasite is well-established.[38,39] Previously, it was thought that the amount and rate of Fe(III)FPIX production by the parasite was insufficient to account for the total CQ uptake by the infected cell.[40] More recently, however, we have demonstrated that the uptake of drug into the parasite exhibits both a saturable and a nonsaturable component. Moreover, because the saturable component of drug uptake is relevant to antimalarial activity, we have shown that there is more than enough Fe(III)FPIX produced by the parasite to account for all of the saturable CQ accumulation.[41] Furthermore, recent evidence obtained using cell-free systems implicates CQ−Fe(III)FPIX binding rather than active uptake or proton trapping as the predominant mechanism responsible for this saturable uptake of CQ.[9,41]

Previously, our research has shown that CQ uptake was dependent on free Fe(III)FPIX concentration. To demonstrate that free Fe(III)FPIX is also the intraparasitic receptor driving the accumulation of these short chain CQ derivatives, we have shown that they can compete with CQ for the same binding site using purified ghost membranes preloaded with Fe(III)FPIX. Interestingly, there was no correlation between the IC_{50} for CQ uptake into intact parasites and displacement of CQ from Fe(III)FPIX-loaded ghost membranes suggesting that other factors, such as membrane permeability, can influence the potential for Fe(III)FPIX:drug binding with the intact parasite.

The predicted therapeutic benefits of these short chain CQ compounds are 2-fold. In the first instance, there is improved activity and reduced cross-resistance with all compounds studied, with the exception of C2Mor. In addition, the inclusion of a metabolically more resilient terminal nitrogen means that these pharmacological characteristics should be retained in vivo.

It has previously been reported that CQ analogues with diethylamino alkane side chains containing 2−3 or 10−12 carbon atoms show equal activity against CQ sensitive and resistant isolates.[23a] From such evidence, it was proposed that resistant parasites contain a CQ efflux protein with two negatively charged residues that specifically target CQ analogues containing side chains between four and six carbon atoms long.[23b] However, it has been shown that short chain diethylamino-CQ derivatives, which would be expected to circumvent this resistance mechanism, undergo substantial in vivo dealkylation to metabolites that display a large cross-resistance factor in vitro, despite retaining the same internitrogen distance.[22] This single observation confirms that the CQ resistance mechanism is unconnected to the internitrogen distance but rather must rely on the lipophilicity of the molecule or the charge state characteristics of the terminal nitrogen.

In line with our earlier studies and with more recent molecular modeling studies,[28g] the principal interaction of these short chain CQ analogues involves $\pi-\pi$-stacking interactions of the quinoline ring with the porphyrin ring system with the potential for a second weak electrostatic interaction of the charged ammonium group with the carboxyl groups of hematin. (This latter interaction for CQ itself has been ruled out on the basis of ITC studies.) With the exception of C2Mor, the short chain CQ compounds show excellent antimalarial activity against a drug resistant and a sensitive isolate of *P. falciparum*. The improved potency of these compounds in vitro cannot be directly correlated to an enhanced binding effect of the drug to Fe(III)FPIX. It is believed that the superior activity observed for these compounds as compared to CQ is more reliant on their ability to accumulate within the food vacuole of the parasite at higher concentrations. We believe that these new analogues may offer a cost effective therapeutic solution to CQ resistant *P. falciparum* malaria. Future work will include an investigation of these analogues against other CQ resistant strains and a full assessment of the drug metabolism of lead compounds **4** and **9**, analogues that cannot undergo detrimental P450 N-terminal dealkylation to metabolites cross-resistant with CQ.

Such metabolic cleavage of the *N*,*N*-diethylamino function in short chain analogues is proposed to be a major drawback in terms of development of this class of 4-aminoquinoline analogue.

Experimental Section

Antimalarial Activity. The capacity of CQ (**1**) and the short chain derivatives (**4**−**9**) to arrest the growth of CQ sensitive (19 nM for CQ) and CQ resistant (190 nM for CQ) strains of *P. falciparum* was determined.

Culturing of *P. falciparum* and Drug Sensitivity Assays. The CQ sensitive strain (HB3) and CQ resistant strain (K1) of *P. falciparum* used in this study were originally from South America and Thailand, respectively. Parasites were maintained in continuous culture using the method of Trager and Jenson.[29] Cultures were grown in culture flasks containing human erythrocytes (2−5%) with a parasitaemia in the range of 1−10%, suspended in RPMI 1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, and 10% human serum (complete medium). Cultures were gassed with a mixture of 3% O_2, 4% CO_2, and 93% N_2.

Antimalarial activity was assessed using an adaption of the 48 h sensitivity assay of Desjardins et al.,[30] using [^{3}H]-hypoxanthine incorporation as an assessment of parasite growth. Stock drug solutions were prepared in 100% DMSO and diluted to the appropriate concentration using complete medium. Assays were performed in sterile 96 well microtiter plates, and each plate contained 200 mL of parasite culture (2% parasitaemia, 0.5% Fe(III)FPIXatocrit) with or without 10 mL of drug dilution. Each drug was tested in triplicate, and parasite growth was compared to control wells (which constituted 100% parasite growth). After they were incubated for 24 h at 37 °C, 0.5 mCi [^{3}H]hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto filter-mats and dried for 1 h at 55 °C, and the radioactivity was counted using a Wallac 1450 Microbeta Trilux Liquid scintillation and luminescence counter. IC_{50} values were calculated from log dose−response curves using the four point logistic model (Grafit-Erithacus Software).

Measurement of Inhibition of [H^3]CQ Uptake by Intact Parasitized Erythrocytes. The inhibition of [H^3]CQ uptake into intact parasitized erythrocytes by the short chain CQ derivatives was performed according to the method of Bray et al.[9] Essentially, infected erythrocytes were suspended in RPMI-1640 buffer containing [H^3]CQ (5 nM), at a parasitemia of 1−2%, Fe(III)FPIXatocrit of 0.5%. CQ uptake was terminated by centrifugation of cells (14 000 rpm, 2 min) through silicon oil followed by processing for scintillation counting.[26] The inhibitory effect of the short chain CQ derivatives (**4**−**9**) on steady state accumulation of CQ was measured after incubation for 1 h at 37 °C in the presence of specific concentrations of the short chain CQ analogues (10−0.01 mM). Data are represented as the concentration required to inhibit steady state CQ uptake by 50% (IC_{50}).

Determination of the Effect of Short Chain CQ Derivatives on Fe(III)FPIX Incorporation into Hemozoin Crystal.[43] Measurement of the inhibition of hemozoin formation by the short CQ derivatives was performed by a modification of the procedure described by Raynes et al.[18] Parasitized erythrocytes (1.8 mL of packed erythrocytes, approximately 15% trophozoite, HB3 strain) were washed with phosphate-buffered saline, pH 8 (PBS). The pellet was mixed with 0.05× vol of 1% saponin and incubated at room temperature for 10 min. The parasites were pelleted (2000 g, 5 min) and washed three times in PBS. The washed pellet (approximately 60 mL) was suspended in 2 mL of sodium acetate buffer (500 mM, pH 5.2), titurated with a 27G needle at room temperature, and the parasite lysate was stored at −70 °C until use.

An aliquot of trophozoite lysate (100 mL) and hematin (100 mL of 3 mM hematin in 0.1 M NaOH) was mixed with an aliquot of 1 M HCl (10 ml) and sodium acetate (500 mM, pH 5.2) to give a volume of 900 mL in each tube. A series of drug concentrations (0.001−1 mM) were prepared in ethanol, and 100 mL of each was added to the appropriate samples. The effect of ethanol on the crystallization process was assessed by the addition of 100 mL of ethanol to the control samples. Samples were mixed and incubated for 12 h, with occasional mixing. After they were incubated, samples were centrifuged (14 000 rpm, 15 min, 21 °C) and the hemozoin pellet was repeatedly washed with 2% w/v sodium dodecyl sulfate (SDS) with sonication (30 min, 21 °C, bath sonicator, Decon FS100 ultrasonics Ltd, U.K.) until the supernatant was clear (usually 3−4 times). After the final wash, the supernatant was removed and the pellet was resuspended in 1 mL of 0.1 M NaOH and incubated for a further 1 h at room temperature; samples were then mixed by aspiration with a pipet. The hemozoin content was determined by measuring the absorbance at 400 nm (Beckmann DU640 spectrophotometer) using a 1 cm quartz cuvette. The amount of hemozoin formed during the incubation was corrected for preformed hemozoin (the amount of preformed hemozoin in the parasite extract was determined from a sample containing extract, but no substrate, which was incubated and repeatedly washed with 2% SDS as described above). The concentration of drug required to produce 50% inhibition of hemozoin crystallization (IC_{50}) was determined.

Displacement of CQ Bound to Fe(III)FPIX-Loaded Ghost Membranes. Displacement studies were performed as previously reported.[9] Erythrocyte ghost membranes were prepared by lysing washed human erythrocytes with ice-cold 5 mM sodium phosphate (pH 8.0) followed by exhaustive washing in the same buffer. Membranes (0.27 mg protein) were loaded with Fe(III)FPIX (5 μM) and washed in 0.2 M HEPES buffer. Samples of Fe(III)FPIX-loaded membrane (0.01 mg protein) were suspended in 1 mL of 0.2 M HEPES containing 50 nM [^{3}H]CQ and incubated in the absence or presence of the short chain CQ derivatives for 1 h at 37 °C. The membrane suspension was centrifuged (14 000 rpm, 2 min), the supernatant was removed, and the pellet was washed once in ice-cold 0.2 M HEPES buffer without [^{3}H]CQ. The remaining pellet was solubilized and processed for scintillation counting.

Chemistry. *N*-*tert*-Butyl-*N*′-(7-chloro-quinolin-4-yl)-ethane-1,2-diamine (4, C2Bu). To a stirred solution of 4,7-dichloroquinoline (1.0 g, 5 mmol) in ethanol (10 mL) was added (2-*tert*-butylamino)ethylamine (0.81 g, 7 mmol). The reaction was heated at reflux for 18 h, after which time the solvent was removed in vacuo. The resultant solid was purified by column chromatography (silica gel) using dichloromethane/methanol (4:1) as eluent to give the required product as a pale yellow solid (62%). MS [M + H$^+$], *m*/*z* 278. ^{1}H NMR ($CDCl_3$): δ 1.1 (9H, s, tBu), 2.8 (2H, m, CH_2), 3.2 (2H, m, CH_2), 6.3 (1H, d, ArH, J = 5 Hz), 7.3 (1H, dd, ArH, J = 3 Hz), 7.65 (1H, d, ArH, J = 8 Hz), 7.94 (1H, d, ArH, J = 3 Hz), 8.45 (1H, d, ArH, J = 5 Hz). Anal. ($C_{15}H_{20}N_3Cl$) C, H, N.

The remaining derivatives were synthesized according to the above method, where 4,7-dichloroquinoline was replaced with 4-chloro-7-trifluoromethylquinoline for compounds **8** and **9** and the appropriate basic amine side chains were substituted for (2-*tert*-butylamino)ethylamine.

(7-Chloro-quinolin-4-yl)-(2-piperidin-1-yl-ethyl)-amine (5, C2Pip). A light brown solid (65%). MS [M + H$^+$], *m*/*z* 290. ^{1}H ($CDCl_3$): δ 1.45 (2H, m, CH_2), 1.6 (4H, m, $2CH_2$), 2.5 (4H, m, $2CH_2$), 2.7 (2H, m, CH_2), 3.2 (2H, m, CH_2), 6.25 (1H, bs, NH), 6.3 (1H, d, ArH, J = 8 Hz), 7.3(1H, dd, ArH, J = 8 Hz), 7,7 (1H, d, ArH, J = 9 Hz), 8.0 (1H, s, ArH), 8.5 (1H, d, ArH, J = 7 Hz). Anal. ($C_{16}H_{21}N_3Cl$) C, H, N.

(7-Chloro-quinolin-4-yl)-(2-pyrrolidin-1-yl-ethyl)-amine (6, C2Pyr). An off-white solid (71%). MS [M + H$^+$], *m*/*z* 276. ^{1}H ($CDCl_3$): δ 1.5 (4H, m, $2CH_2$), 2.4 (4H, m, $2CH_2$), 2.7 (2H, m, CH_2), 3.2 (2H, m, CH_2), 6.3 (1H, d, ArH, J = 7 Hz), 7.3 (1H, dd, ArH, J = 8 Hz), 7.6 (1H, d, ArH, J = 8 Hz), 8.1 (1H, s, ArH), 8.5 (1H, d, ArH, J = 8 Hz). Anal. ($C_{15}H_{19}N_3Cl$) C, H, N.

(7-Chloro-quinolin-4-yl)-(2-morpholin-4-yl-ethyl)-amine (7, C2Mor). A fawny solid (72%). MS [M + H$^+$], *m*/*z* 292. ^{1}H ($CDCl_3$): δ 1.9 (4H, m, $2CH_2$), 2.6 (4H, m, $2CH_2$), 2.8 (2H, m, CH_2), 3.4 (2H, m, CH_2), 6.3 (1H, d, ArH, J = 7 Hz),

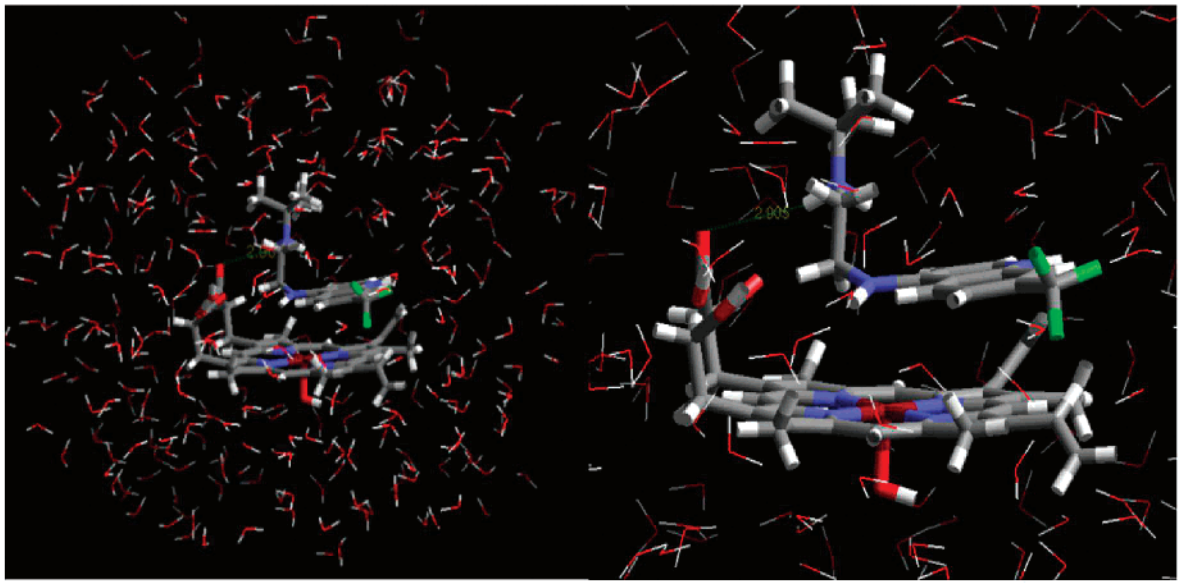


Figure 5. Lowest energy complex of Fe(III)FPIX with F2Bu (**9**) within a solvated aqueous environment. The figure illustrates how the binding interactions between drug and receptor are maintained within an aqueous sphere.

7.3 (1H, dd, ArH, $J = 4$ Hz), 7.6 (1H, d, ArH, $J = 7$ Hz), 8.1 (1H, s, ArH), 8.5 (1H, d, ArH, $J = 8$ Hz). Anal. ($C_{15}H_{18}N_3OCl$) C, H, N.

7-Trifluoromethyl-4-[(2′-piperidyl)ethyl]aminoquinoline (8, F2Pip). A fawny solid (75%). MS [M + H^+], *m/z* 324. ^{1}H ($CDCl_3$): δ 1.2 (2H, m, CH_2), 1.6 (4H, m, $2CH_2$), 2.5 (4H, m, $2CH_2$), 2.7 (2H, m, CH_2), 3.3 (2H, m, CH_2), 6.3 (1H, d, ArH, $J = 8$ Hz), 7.3 (1H, dd, ArH, $J = 6$ Hz), 7.7 (1H, d, ArH, $J = 8$ Hz), 8.1 (1H, s, ArH), 8.6 (1H, d, ArH). Anal. ($C_{17}H_{21}N_3F_3$) C, H, N.

***N-tert*-Butyl-*N′*-(7-trifluoromethyl-quinolin-4-yl)ethane-1,2-diamine (9, F2Bu).** A yellow solid (62%). MS (EI) [M + H^+], *m/z* 312. ^{1}H NMR (200 MHz) ($CDCl_3$): δ 1.16 (9H, s, tBu), 1.8 (1H, bs, NH), 3.05 (2H, m, CH2), 3.36 (2H, m, CH2), 6.18 (1H, bs, NH), 6.45 (1H, d, ArH, $J = 5$ Hz), 7.51 (1H, dd, ArH, $J = 9$ Hz), 7.81 (1H, d, ArH, $J = 9$ Hz), 8.23 (1H, s, ArH), 8.61 (1H, d, ArH, $J = 5$ Hz). Anal. ($C_{15}H_{20}N_3Cl$) C, H, N.

Molecular Modeling. The ability of these derivatives to form a complex with Fe(III)FPIX was investigated by molecular modeling studies. Energy-minimized molecular conformations of the short chain CQ analogues were constructed, and their specific interaction with Fe(III)FPIX was investigated. Molecular modeling experiments concentrated on the diprotonated forms of the compounds, as it is these forms that are predicted to exist within the acidic environment of the parasitic food vacuole. To examine the dynamic effects in the presence of solvent, we have also carried out simulations with a discrete water model for drug−Fe(III)FPIX complexes and the isolated drug molecules alone.

Simulations were carried out on CQ and the short chain analogues. Each molecule was energy-minimized from the "as constructed" conformation using the Universal force field. The Universal force field was able to give a greater range of parameters for a wider range of atom types than earlier force fields.

Energy minimization alone is only able to find the nearest energy minimum to the starting conformation of a given system. To generate a wider representative set, we have employed the method of simulated annealing, similar to that recently employed by Milne.[42] Molecular dynamics runs at 298 K for a simulation time of 5 ps were performed for each drug molecule, and the resulting structure was energy-minimized to gain a low-energy conformation. This process was repeated until 10 structures per molecule had been generated. For each molecular dynamic calculation, the last minimized structure in the set was used as the starting conformation for the next molecular dynamic simulation.

All molecular modeling was carried out on an O2 R5000 silicon graphics workstation. Computational results were obtained using software programs from Molecular Simulations Inc. Dynamics calculations were done with the Open Force Field (OFF) Cerius[2] program, using the Universal 1.02 force field parameter set. Graphical displays were printed from the Cerius[2] molecular modeling system.

For each drug molecule obtained after energy minimization−dynamics simulation, the lowest energy conformation was chosen to generate a complex with Fe(III)FPIX in the following manner. We have previously found that the quinoline ring of the short chain compounds can π-stack with the π-system of the porphyrin ring. With this restriction, each drug molecule was positioned above the porphyrin model in such a way that the proton on the quaternary side chain nitrogen was within 3 Å of a carboxylic acid oxygen on the Fe(III)FPIX molecule. From this starting point, the dimers were then energy-minimized using the Universal parameter set. Each drug molecule was docked in this way in addition to investigation of several alternative starting points. The interaction energy, E_I, for each Fe(III)FPIX complex was then calculated from the equation:

$$E_I = E_D - (E_M + E_H)$$

where E_D is the lowest energy complex obtained and E_M and E_H are the lowest energies calculated for any conformation of the drug molecule and Fe(III)FPIX unit in isolation. The lowest interaction energy for each molecule is shown in Table 3.

Following these initial studies, we then examined the effect of solvent by conducting longer molecular dynamics runs (40 ps) in the presence of a water solvent model. The complex structures generated in the vacuum were solvated by generating a sphere of water around the dimer, centered on the iron atom in the middle of the molecular pair, sufficient to give a layer of solvent molecules of at least 5 Å around both molecules. These structures were then energy-minimized followed by an annealing molecular dynamics equilibration period of 5 ps at 298 K. The resulting complexes were then subjected to a further 40 ps molecular dynamics run at 298 K followed by minimization (Figure 5). In Table 4, we include the relative interaction energy of the drug−Fe(III)FPIX adducts, which is derived from the difference in total energy of the Fe(III)FPIX−drug complex and the solvated drug molecule.

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