Structural Specificity of Chloroquine−Hematin Binding Related to Inhibition of Hematin Polymerization and Parasite Growth

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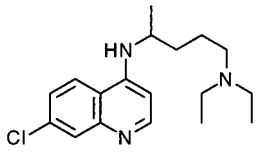
Considerable data now support the hypothesis that chloroquine (CQ)−hematin binding in the parasite food vacuole leads to inhibition of hematin polymerization and parasite death by hematin poisoning. To better understand the structural specificity of CQ−hematin binding, 13 CQ analogues were chosen and their hematin binding affinity, inhibition of hematin polymerization, and inhibition of parasite growth were measured. As determined by isothermal titration calorimetry (ITC), the stoichiometry data and exothermic binding enthalpies indicated that, like CQ, these analogues bind to two or more hematin μ-oxo dimers in a cofacial $\pi-\pi$ sandwich-type complex. Association constants (K_a's) ranged from 0.46 to 2.9 $\times$ 10^5 M^{-1} compared to 4.0 $\times$ 10^5 M^{-1} for CQ. Remarkably, we were not able to measure any significant interaction between hematin μ-oxo dimer and **11**, the 6-chloro analogue of CQ. This result indicates that the 7-chloro substituent in CQ is a critical structural determinant in its binding affinity to hematin μ-oxo dimer. Molecular modeling experiments reinforce the view that the enthalpically favorable $\pi-\pi$ interaction observed in the CQ−hematin μ-oxo dimer complex derives from a favorable alignment of the out-of-plane π-electron density in CQ and hematin μ-oxo dimer at the points of intermolecular contact. For 4-aminoquinolines related to CQ, our data suggest that electron-withdrawing functional groups at the 7-position of the quinoline ring are required for activity against both hematin polymerization and parasite growth and that chlorine substitution at position 7 is optimal. Our results also confirm that the CQ diaminoalkyl side chain, especially the aliphatic tertiary nitrogen atom, is an important structural determinant in CQ drug resistance. For CQ analogues **1**−**13**, the lack of correlation between K_a and hematin polymerization IC_{50} values suggests that other properties of the CQ−hematin μ-oxo dimer complex, rather than its association constant alone, play a role in the inhibition of hematin polymerization. However, there was a modest correlation between inhibition of hematin polymerization and inhibition of parasite growth when hematin polymerization IC_{50} values were normalized for hematin μ-oxo dimer binding affinities, adding further evidence that antimalarial 4-aminoquinolines act by this mechanism.

Introduction

With widespread chloroquine (CQ)-resistant *Plasmodium falciparum* malaria,[1] there is a need to design new antimalarials to take the place of this very important, but unfortunately now less useful, drug.[2] To identify potential alternatives to CQ, a molecular understanding of its mechanism of action would be most helpful. Considerable data now support the hypothesis that CQ and other antimalarial quinolines inhibit parasite growth by binding to hematin[3] leading to inhibition of hematin polymerization[3c,f,4] and death of the parasite by hematin poisoning.[5] Hematin polymerization, a unique parasite mechanism to detoxify hematin, is a nonenzymatic process[4d] in which the hematin released from parasite digestion of hemoglobin is converted to hemozoin or malaria pigment,[6] an insoluble ionic polymer in which adjacent hematin units are linked via a carboxylate−iron(III) coordinate bond. We recently found a positive correlation between inhibition of hematin polymerization and hematin binding affinity for CQ and seven other antimalarial quinolines.[3f] Therefore, it is probable that the interaction of CQ with hematin is an important event in the manifestation of the antimalarial activity of this drug.[3e,f,5a]



Chloroquine (CQ)

NMR,[7] Mössbauer spectroscopy,[8] UV−Vis,[5f,9] and equilibrium dialysis[3a] experiments provide substantial evidence for complex formation between CQ and hematin. Recent isothermal titration calorimetry (ITC) experiments[3f] suggest that CQ binds to hematin in the form of two hematin μ-oxo dimers in a cofacial $\pi-\pi$ sandwich-type complex supporting an original hypothesis by Moreau et al.[7] We hypothesized[3f] that this

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Chart 1. Side Chain-Modified CQ Analogues

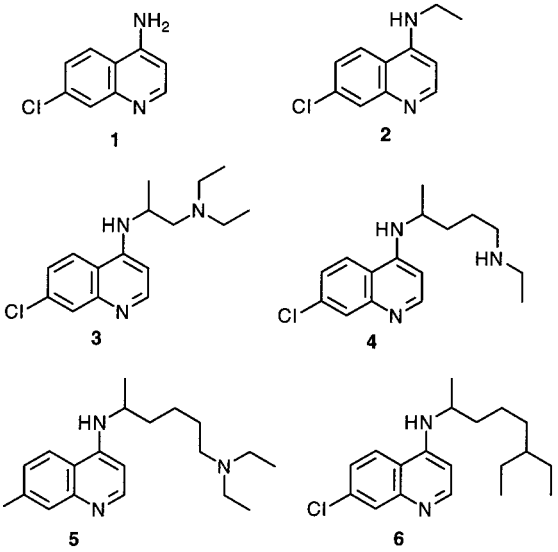
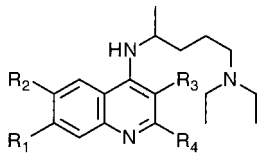


Chart 2. Quinoline Ring-Modified CQ Analogues



Compd	R_1	R_2	R_3	R_4
7	H	H	H	H
8	Br	H	H	H
9	O_2N	H	H	H
10	H_2N	H	H	H
11	H	Cl	H	H
12	Cl	H	H	CH_3
13	Cl	H	CH_3	H

binding produces a forward shift in the equilibrium between hematin monomer and hematin μ-oxo dimer[10] thereby decreasing the concentration of hematin monomer available for incorporation into hemozoin, a molecular mechanism to account for CQ-mediated inhibition of hematin polymerization.

To advance our understanding of the structural specificity of CQ−hematin binding, and to test the hypothesis that hematin can serve as a potential target for the design of new antimalarial agents,[11] we selected a set of 13 CQ analogues for further study (Charts 1 and 2). For these compounds we measured hematin binding affinity, inhibition of hematin polymerization, and inhibition of parasite growth against both CQ-sensitive and CQ-resistant strains of *P. falciparum*. In the course of this work, we also anticipated that useful structural insights related to CQ drug resistance might also be gained.

Results

Chemistry. 4-Amino-7-chloroquinoline (**1**) and 4-(ethylamino)-7-chloroquinoline (**2**) were prepared by reaction of 4,7-dichloroquinoline with ammonia and ethylamine, respectively. The 7-NO_2 analogue of CQ (**9**) was synthesized according to the procedure for the synthesis of 4-aminoquinolines described by Price and Roberts[12] (Scheme 1). Condensation of equimolar amounts of 3-nitroaniline with diethyl ethoxymethylenemalonate yielded the corresponding arylaminomethylene ester **14** which was cyclized to the 7-nitroquinoline ester **15** with high regioselectivity. Alkaline hydrolysis of the ester yielded the corresponding acid **16**, the silver salt of which was decarboxylated in refluxing diphenyl ether to produce 4-hydroxy-7-nitroquinoline (**17**). Chlorination with phosphorus oxychloride produced 7-nitro-4-chloroquinoline (**18**) which was treated with 2-amino-5-(diethylamino)pentane at 165 °C under nitrogen to afford **9**. Catalytic hydrogenation of **9** (H_2, Pd/C, 50 atm) yielded **10**, isolated as its trihydrochloride salt. The 7*H* (**7**)[13] and 7-bromo (**8**)[14] analogues of CQ were synthesized according to the reaction sequence described above.

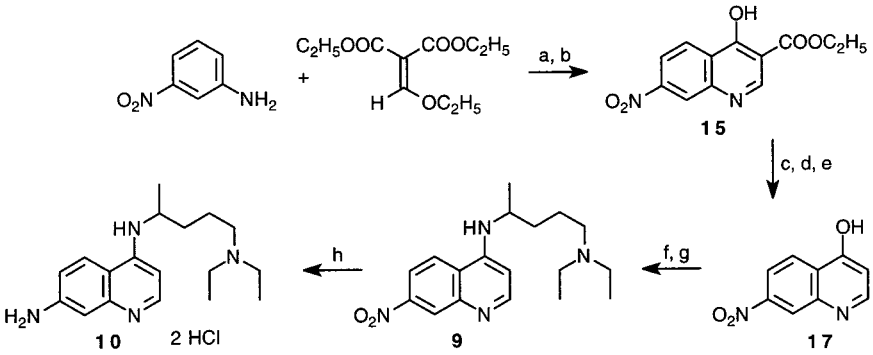
Interaction with Hematin. As we have noted before,[3f] it would be preferable to carry out both drug−hematin binding and hematin polymerization experiments at pH 4.8−5.4, the pH of the parasite food vacuole.[15] However, due to the poor solubility of hematin at low pH, we settled on pH 6.5[3f] as a compromise, because at this pH hematin is fully soluble and hematin polymerization still proceeds reasonably.

The thermodynamic parameters of hematin binding for CQ analogues **1**−**13** are shown in Table 1. In solution hematin exists almost exclusively in the form of its μ-oxo dimer,[16] and this is reflected in the observed binding stoichiometries. With the exception of **6**, all of the CQ analogues bound to at least two hematin μ-oxo dimers. The lower binding stoichiometry for **6** probably derives from the presence of the ethanol cosolvent required to solubilize this compound as organic solvents impede hematin dimerization and aggregation.[17] Conversely, the binding stoichiometry for **7** suggests that this compound binds to an aggregate of five hematin μ-oxo dimers. As a whole, the stoichiometry data support a model proposed by Moreau et al.[7] in which CQ binds to hematin μ-oxo dimers in a cofacial $\pi-\pi$ sandwich-type complex.

CQ analogues **1**−**13** had hematin μ-oxo dimer association constants (K_a's) ranging from 0.46 to 2.9×10^5 M^{-1} (Table 3). Suprisingly, not one of the analogues bound more avidly to hematin μ-oxo dimer than did CQ. The 8−9-fold decrease ($P = 0.0071$) in binding affinities for analogues **1** and **2** suggests that the alkyl side chain of CQ may position the quinoline heterocycle in an optimal stacking orientation for favorable $\pi-\pi$ orbital overlap with hematin μ-oxo dimer. For CQ analogues **3**−**5**, more subtle structural variation in the alkyl side chain of CQ reduced binding affinities only 1.5 to 3-fold ($0.029 < P < 0.249$). However, replacement of the tertiary nitrogen atom of CQ with a methine carbon atom in **6** decreased binding affinity by 5-fold ($P = 0.011$). As addition of ethanol cosolvent was required for the titration of hematin with **6** because of its poor solubility, these data must be considered with caution because polar protic solvents such as ethanol significantly disrupt $\pi-\pi$ ligand−receptor interactions.[18]

For **7**, the 7*H* CQ analogue, binding affinity decreased 5-fold ($P = 0.011$). For **8**−**10**, replacement of the 7-chloro substituent with bromo, nitro, and amino functional groups also lowered binding affinities, but only 1.5−3-fold ($0.019 < P < 0.339$) compared to CQ. Remarkably,

Scheme 1[a]



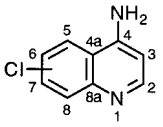
[a] Key: (a) 90 °C; (b) biphenyl ether, 255 °C; (c) 10% aq NaOH then HCl; (d) NH_4OH, $AgNO_3$; (e) biphenyl ether, 255 °C; (f) $POCl_3$, 110 °C; (g) 2-amino-5-(diethylamino)pentane, 120 °C; (h) H_2, Pd/C, EtOH then HCl(g).

Table 1. Thermodynamic Characterization of CQ Analogue−Hematin μ-Oxo Dimer Binding

compd[a]	n[b]	1/n	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
1	0.23 ± 0.02	4.4	−6.7 ± 0.1	−9.1 ± 1.0	−2.4 ± 1.2
2[c]	0.20 ± 0.01	5.0	−6.6 ± 0.2	−9.4 ± 0.5	−2.8 ± 0.6
3	0.21 ± 0.01	4.8	−7.7 ± 0.2	−8.7 ± 0.3	−1.0 ± 0.5
4	0.21 ± 0.01	4.8	−7.4 ± 0.2	−9.7 ± 0.3	−2.3 ± 0.5
5[c]	0.15 ± 0.01	6.7	−7.3 ± 0.2	−9.7 ± 0.4	−2.4 ± 0.4
6[d]	0.43 ± 0.03	2.3	−6.9 ± 0.3	−6.3 ± 2.0	0.6 ± 0.1
7	0.10 ± 0.01	10	−7.0 ± 0.1	−13 ± 2	−6.0 ± 2.4
8	0.28 ± 0.04	3.6	−7.2 ± 0.1	−6.1 ± 0.2	1.1 ± 0.3
9	0.18 ± 0.01	5.6	−7.7 ± 0.2	−12 ± 1	−4.3 ± 1.2
10	0.13 ± 0.04	7.7	−7.7 ± 0.3	−10 ± 2	−2.3 ± 1.8
12	0.25 ± 0.03	4.0	−7.3 ± 0.1	−10 ± 1	−2.7 ± 0.3
13	0.17 ± 0.03	5.9	−7.0 ± 0.1	−6.0 ± 0.6	1.0 ± 0.7
CQ[e]	0.24 ± 0.03	4.2	−7.9 ± 0.4	−10 ± 1	−2.1 ± 1.2

[a] Analogue **11** was insoluble in phosphate buffer, pH 6.5, but it was sufficiently soluble in 20 mM MES buffer, pH 5.6.[26] Under these conditions, no measurable binding enthalpy was detectable by ITC for analogue **11**. [b] Binding stoichiometry. [c] Analogues **2** and **5** were first dissolved in 3−4 drops of 200 mM acetate buffer, pH 4.0 and then made up to volume with 250 mM phosphate buffer, pH 6.5 [d] Analogue **6** was not soluble in 250 mM phosphate buffer, so it was first dissolved in EtOH (0.5 mL) and then diluted with H_2O (9.5 mL) to a concentration of 2 mM. [e] Data from Dorn et al.[8]

Table 2. Atomic Electrostatic Charge Densities of 7-Chloro-4-aminoquinoline (**1**) and 6-Chloro-4-aminoquinoline Calculated at the 6-31G** Level



atom	7-chloro	6-chloro
N1	−0.75	−0.79
C2	+0.48	+0.45
C3	−0.70	−0.78
C4	+0.64	+0.90
C4a	−0.31	−0.66
C5	−0.13	−0.10
C6	−0.23	−0.09
C7	+0.20	+0.09
C8	−0.38	−0.55
C8a	+0.66	+0.92
NH_2	−0.90	−1.09
Cl	−0.14	−0.13

we were not able to measure any significant interaction between hematin μ-oxo dimer and **11**, the 6-chloro analogue of CQ. This result indicates that the 7-chloro substituent in CQ is a critical structural determinant in its binding affinity to hematin μ-oxo dimer. For CQ analogues **12** and **13**, methyl substitution at the 2 and 3 positions of the quinoline heterocycle decreased binding affinity 3- and 4-fold ($P = 0.012$), respectively. This suggests that the methyl groups sterically prevent the approach of the quinoline heterocycle to hematin μ-oxo dimer, and thus for **12** and **13**, the efficiency of the enthalpically favorable $\pi-\pi$ stacking interaction may decrease.

As for CQ, drug−hematin μ-oxo dimer binding was primarily enthalpy-driven for each of the CQ analogues excepting **11** (Table 1). We attribute this exothermic binding enthalpy largely to $\pi-\pi$ interactions between the compounds and hematin μ-oxo dimer, although release of perturbed water molecules from the amphiphilic surfaces[19] of hematin μ-oxo dimer and these CQ analogues could also contribute to this favorable exothermic enthalpy and drive this CQ analogue−hematin μ-oxo dimer association. For these CQ analogues, a slope of 315.5° (compensating temperature) from an enthalpy−entropy compensation plot[20] (data not shown) is diagnostic of the participation of hydrophobic or structural water in the interaction between these compounds and hematin μ-oxo dimer. The observed linear enthalpy−entropy compensation also indicates similar complex geometry. This correlates with results from molecular modeling experiments (vide infra).

For **6**, **8**, and **13**, hematin μ-oxo dimer binding was also entropy-driven. Except that **8** is more hydrophobic than CQ, there is no obvious structurally based explanation for the albeit modest favorable binding entropy (9−15%) for these three CQ analogues. Binding entropies, whether favored or disfavored, largely reflect net changes in the hydrogen-bonded network of water[19,21,22] in the hydrophobic hydration shell of the CQ analogue−hematin μ-oxo dimer complexes vs free CQ analogue and free hematin μ-oxo dimer assuming a similar unfavorable entropy due to the bimolecular ordering of the complex. It is interesting to note that although **7** and **9** had significantly higher enthalpies (ΔH) of hematin μ-oxo dimer binding than did CQ, the entropic penalty ($T\Delta S$) for this binding also increased such that the free energy (ΔG) of binding changed little due to enthalpy−entropy compensation.[23] This suggests that even though the $\pi-\pi$ interactions between hematin μ-oxo dimer and **7** and **9** were probably more favorable as reflected in their higher binding enthalpies, there may be an upper limit to 4-aminoquinoline−hematin μ-oxo dimer binding affinity and CQ may be near this barrier.

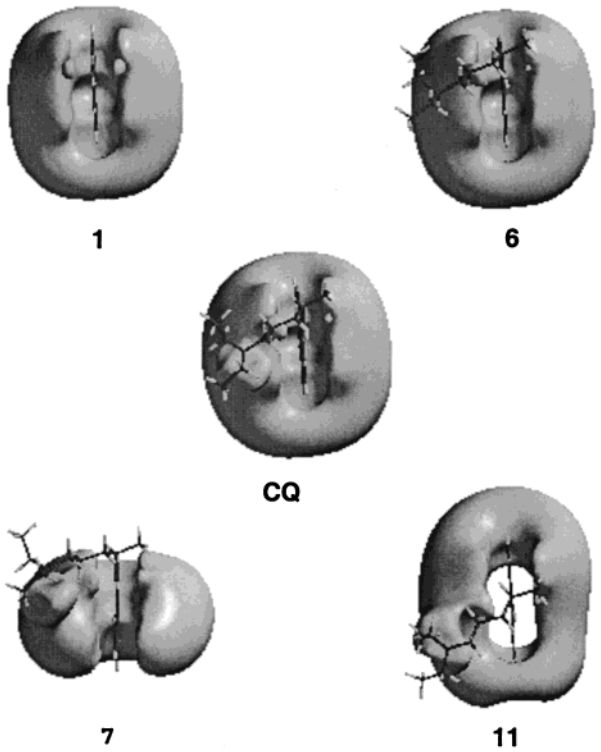


Figure 1. Three-dimensional isopotential contours of molecular electrostatic potentials at −5 kcal/mol for CQ and CQ analogues **1**, **6**, **7**, and **11**.

Molecular Modeling. Results of our molecular modeling studies are also consistent with the experimental observations of the present investigation. Structures for CQ and CQ analogues **1**, **6**, **7**, and **11** were generated using ab initio calculations at the 3-21G* level to more fully understand the structural aspects of CQ−hematin μ-oxo dimer binding and to shed light on the underlying basis for the complete lack of binding interaction between hematin μ-oxo dimer and **11**, the 6-chloro analogue of CQ. Three-dimensional electrostatic potential profiles (Figure 1) of CQ and four CQ analogues distinguish **11** from **1**, **6**, **7**, and CQ in terms of their hematin μ-oxo dimer binding affinities, substantiating the view that $\pi-\pi$ interactions predominate in CQ−hematin μ-oxo dimer binding.

The contiguous three-dimensional electrostatic potential surfaces of CQ, **1**, and **6** were almost identical; for **7** this electrostatic potential surface was somewhat smaller which illustrates the effect of the 7-chloro substituent on the electron distribution in the π orbitals of the quinoline heterocycle. Conversely for **11**, the 6-chloro CQ analogue, there was a substantial gap in the same electrostatic potential surface at the N1−C8 edge of the quinoline heterocycle, a region which appears to be critical for binding with the hematin μ-oxo dimer. Subsequent ab initio calculations at the 6-31G** level for 7-chloro-4-aminoquinoline (**1**) and 6-chloro-4-aminoquinoline, structurally simplified surrogates for CQ and **11**, respectively, allowed us to more accurately determine atomic electrostatic charge densities for these molecules (Table 2). As illustrated by these data, shifting the chlorine atom from the 7 to the 6 position significantly altered the π-electron density distribution at positions C4, C4a, C8, and C8a. The striking loss of binding affinity for **11** reinforces the view that the enthalpically favorable $\pi-\pi$ interaction observed in the CQ−hematin μ-oxo dimer complex derives from a favorable alignment of the out-of-plane π-electron density in CQ and hematin μ-oxo dimer at the points of intermolecular contact.[24]

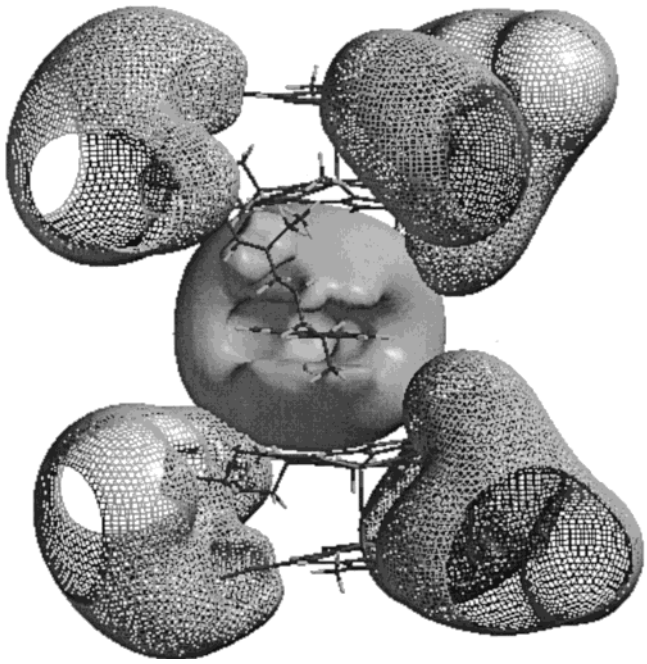


Figure 2. Model of a 1:2 CQ−hematin μ-oxo dimer $\pi-\pi$ sandwich-type complex.

These results support a model originally proposed by Moreau et al.[7] in which CQ forms a $\pi-\pi$ sandwhich-type complex with two hematin μ-oxo dimers (Figure 2). As is evident in this model, the electrostatic potential surface of the hematin μ-oxo dimer indicates an electron-deficient region around the iron atom on both sides of the dimer. We conclude that enthalpy-driven CQ−hematin μ-oxo dimer binding derives largely from attractive electrostatic[24] interactions between the quinoline and porphyrin rings of CQ and hematin μ-oxo dimer, respectively.

Inhibition of Hematin Polymerization. Inhibition of hematin polymerization and inhibition of plasmodial growth for CQ analogues **1**−**13** are shown in Table 3. CQ analogue **4**, the *N*-desethyl metabolite of CQ, **8**, the 7-bromo analogue of CQ, and **12** and **13**, the two methyl-substituted CQ analogues, were as effective as CQ in inhibition of hematin polymerization in vitro. Remarkably, CQ analogue **1**, which contains an unsubstituted 4-amino functional group, also shared this distinction. The other CQ analogues **2**, **3**, **5**, and **6** with modified alkyl side chains were somewhat less potent. Replacement of the 7-chloro substituent of CQ with a 7-nitro (**9**) decreased potency by 6-fold ($P = 0.0001$). However, replacement of the chlorine with a hydrogen (**7**) or amino group (**10**) or shifting the chlorine from the 7 to the 6 position (**11**) abolished hematin polymerization inhibitory activity altogether, results that illustrate the predominating influence of the quinoline heterocycle structure on inhibition of hematin polymerization. This is reinforced by noting that **1**, **6**, and CQ, with nearly identical electrostatic isopotential surfaces (Figure 1), are potent inhibitors of hematin polymerization, whereas **7** and **11**, with substantially different electrostatic

Table 3. Hematin μ-Oxo Dimer Binding Affinities (K_a) and IC_{50} Values for Inhibition of Hematin Polymerization and Parasite Growth for CQ Analogues **1**−**13**

compd	K_a (10^5 M^{-1})	hematin polymerization IC_{50} (μM)	*P. falciparum* IC_{50} (nM)[a] NF54	K1
1	0.52 ± 0.11	16 ± 2	1700	1000
2	0.46 ± 0.14	70 ± 1	820	870
3	2.7 ± 0.9	18 ± 1	41	93
4	1.8 ± 0.5	15 ± 1	45	1300
5	1.5 ± 0.4	60 ± 1	24	420
6	0.79 ± 0.31	30 ± 7	54	200
7	0.85 ± 0.07	>2500	350	8000
8	1.2 ± 0.2	18 ± 2	93	1400
9	2.9 ± 0.8	100 ± 10	15	68
10	2.9 ± 1.1	>2500	2600	4300
11	NA	>2500	170	4900
12	0.91 ± 0.20	17 ± 1	150	1100
13	1.5 ± 0.1	15 ± 1	20	59
CQ	4.0 ± 1.7b	15 ± 5	13	350

[a] Average of $n = 2$. [b] Data from Dorn et al., 1998.

isopotential surfaces (Figure 1), are completely inactive. For 4-aminoquinolines related to CQ, these data suggest that electron-withdrawing functional groups at the 7 position of the quinoline ring are required for activity against hematin polymerization and that chlorine substitution at position 7 is optimal.

Antimalarial Activity. Each CQ analogue was screened against the CQ-sensitive NF54 strain and the CQ-resistant K1 strain of *P. falciparum* in vitro (Table 3). The 4-amino (**1**) and 4-ethylamino (**2**) analogues of CQ had very little activity against either clone, but for these two analogues, the differences in potency between the two clones was insignificant. This result is consistent with observations[25] that the structure of the CQ alkyl side chain is an important determinant in CQ drug resistance. The excellent activity of **3** (Ro 48-0346), the Roche short-chain CQ analogue, against both *P. falciparum* strains is consistent with previous IC_{50} data[13,25a] for this compound. Analogue **4**, the *N*-desethyl metabolite of CQ, had very little activity against the K1 strain, data consistent with previous observations.[26] CQ analogue **5**, in which the length of the alkyl side chain was extended by one carbon, was only slightly less potent than CQ against the two *P. falciparum* clones. For CQ analogue **6**, in which the tertiary nitrogen atom of CQ was replaced with a methine carbon atom, potency against the NF54 strain decreased 4-fold, whereas potency against the K1 strain increased 1.7-fold, implicating the aliphatic tertiary nitrogen atom of CQ as a structural feature associated with parasite drug resistance.

With the exception of **13**, the 3-methyl analogue of CQ (sontoquine), and **9**, the 7-nitro analogue of CQ, the remaining CQ analogues that differed from CQ in quinoline ring substitution were substantially less potent than CQ against the NF54 strain. In this series, the 7-amino analogue of CQ (**10**) was notable for its very weak activity against the NF54 strain. Conversely, **9** and **13** were more potent than CQ against the K1 strain of the parasite, and each had a much lower resistance index than did CQ. Replacement of the chlorine atom at position 7 with a hydrogen (**7**) or moving the chlorine to position 6 (**11**) decreased the antimalarial activity by an order of magnitude compared to CQ, but the resistance index was largely unchanged, data consistent with

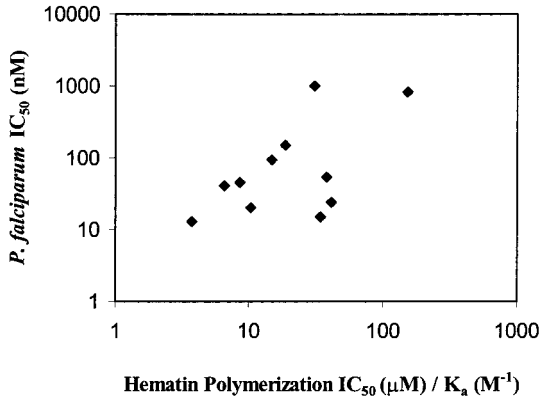


Figure 3. Correlation of normalized hematin polymerization IC_{50} values and inhibition of parasite growth. Data points for **7**, **10**, and **11** were not included as these CQ analogues were inactive against hematin polymerization in vitro.

that reported by De et al.[13] for **7** and **11**. Although this decline in antimalarial potency was less marked for **8**, the 7-bromo analogue of CQ, these results confirm that a 7-chloro substituent confers optimal antimalarial potency for 4-aminoquinolines active against *P. falciparum* in vitro.[13,27] In contrast to a recent study[28] in which the degree of drug resistance to antimalarial quinolines correlated with relatively general physicochemical properties such as lipophilicity and molar refractivity, and not on closely defined structural features, the antimalarial data for these 13 CQ analogues illustrate that small structural changes can produce dramatic effects in relative drug resistance.

Correlations. First, it is instructive to note that the three CQ analogues (**7**, **10**, **11**) that did not inhibit hematin polymerization also had the weakest activity against the two *P. falciparum* clones (average IC_{50} values between 2500 and 5000 nM). For CQ and the remaining CQ analogues, no correlations exist between either hematin μ-oxo dimer binding affinities and hematin polymerization IC_{50} values ($r = 0.34$, $P = 0.27$) or hematin polymerization and *P. falciparum* IC_{50} values ($r = 0.065$, $P = 0.85$). This result demonstrates that a compound with excellent activity against hematin polymerization in vitro does not necessarily possess antimalarial properties, and, as we have previously observed[3f] for primaquine, hematin μ-oxo dimer binding affinity does not always predict inhibition of hematin polymerization. Yet, if we normalized the inhibition of polymerization IC_{50} data by dividing by K_a, a significant but modest correlation ($r = 0.61$, $P = 0.046$) with the *P. falciparum* IC_{50} data was evident (Figure 3). This correlation improved ($r = 0.93$, $P < 0.0001$) when the data for CQ analogue **1** was omitted. Additional refinements in this correlation analysis would have to consider the relative accumulation of these CQ analogues in the parasite food vacuole as elegantly demonstrated by Hawley et al.[29] for CQ, amodiaquine, and several amodiaquine analogues.

Discussion

This work provides an experimental test of some theoretical predictions to emerge from a recent molecular modeling study[30] probing the structural specificity of CQ−hematin binding. In the two-point attachment model proposed for CQ−hematin binding by O'Neill et

al.,[30] hydrogen bonding and ionic interactions between the two carboxylates of hematin and the charged tertiary nitrogen atom of CQ were thought to be important. However, we have demonstrated[31] that hydrogen bonding and ionic interactions probably contribute little to CQ−hematin μ-oxo dimer binding as this binding is pH-independent and is nearly unaffected by high salt concentrations. That replacement of the tertiary nitrogen atom of CQ with a methine carbon atom (**6**) decreases hematin μ-oxo dimer binding affinity only 5-fold further supports this interpretation.

Koh et al.[32] demonstrated that CQ can adopt a conformation with an inter-nitrogen separation (N_{quin}−$N_{diethyl}$) of 8.38 Å,[33] closely matching the distance of 8.20 Å between the central iron atom and the carboxylates of hematin[30] as calculated from the X-ray structure of hemin. However hematin μ-oxo dimer binding data for **3**, the short-chain analogue of CQ, and for **5**, the CQ analogue with an extra carbon atom in the side chain, show that hematin μ-oxo dimer binding affinity is largely independent of this inter-nitrogen separation. In addition, NMR data[7,34] suggest that no iron coordination occurs between the hematin iron atom and the three nitrogen atoms of chloroquine, and if hematin μ-oxo dimer is considered, iron coordination *trans* to the Fe−O−Fe bonds would be minimal as the iron atoms are displaced some 0.5 Å out of the porphyrin planes toward the bridging oxygen atom.[35]

Instead, our experimental and molecular modeling data both suggest that enthalpy-driven CQ−hematin μ-oxo dimer binding derives largely from an energetically favorable $\pi-\pi$ molecular recognition[24,36,37] interaction between the quinoline heterocycle of CQ and the metalloporphyrin ring of hematin μ-oxo dimer. For this binding interaction, Egan et al.[3b] suggest that the hydrophobicity of the aromatic ring of the drug may play a predominant role. This interpretation is supported by our enthalpy−entropy compensation temperature of 315.5.[20] Yet, replacement of the 7-chloro substituent in CQ with a 7-amino or 7-nitro substituent decreased binding affinity only marginally, despite the marked differences in polarity and electronic nature of the amino and nitro functional groups. We also found that **8**, the more hydrophobic 7-bromo analogue of CQ, bound 3-fold less strongly to hematin μ-oxo dimer than did CQ. Most significantly **11**, the 6-chloro analogue of CQ, displayed no measurable affinity for hematin μ-oxo dimer. Hence, our data suggest that this $\pi-\pi$ binding interaction, which is apparently optimal for 4-aminoquinolines with a 7-chloro substituent, derives from an energetically favorable alignment of the out-of-plane π-electron density in CQ and hematin μ-oxo dimer at the points of intermolecular contact.[24] As shown by the minimal decrease in hematin binding affinity for **1** and **2** vs CQ, this $\pi-\pi$ interaction is largely independent of the structure of the 4-alkylamino side chain.

In a study with **7**, the 7*H* analogue of CQ, Veignie and Moreau[27] showed that the weak base properties of CQ are insufficient to account for its accumulation in the parasite food vacuole, the organelle in which hematin polymerization takes place. They found that **7** (pK_a's of 8.8 and 10.2) apparently accumulates 20−50-fold less than does CQ (pK_a's of 8.1 and 10.2)[38] in the parasite, despite the very similar weak base properties of **7** and CQ. Notwithstanding any difference in food vacuole accumulation between **7** and CQ, **7** has no effect on hematin polymerization, suggesting that it exerts its antimalarial activity, feeble as it is, by another mechanism. Nonetheless, if hematin binding contributes to the specific accumulation of 4-aminoquinolines in the parasite food vacuole,[3a,e,39,40] it is conceivable that the diminished accumulation and much weaker antimalarial potency of **7** vs CQ is partly a function of its 5-fold weaker hematin binding affinity. Similar conclusions were drawn by Hawley et al.[41] from data where parasite accumulation of 4-aminoquinolines correlated with inhibition of parasite growth.

For CQ analogues **1**−**13**, the lack of correlation between K_a and hematin polymerization IC_{50} values suggests that other properties of the CQ−hematin μ-oxo dimer complex, rather than its association constant alone, play a role in the inhibition of hematin polymerization. For example, CQ−hematin μ-oxo dimer complexation decreases hematin solubility and enhances hematin μ-oxo dimer aggregation,[3a,42] both of which may also contribute to the CQ-mediated inhibition of hematin polymerization. Moreover, Sullivan et al.[3d] suggest that inhibition of hematin polymerization by CQ may be a function of its binding interaction with both hematin and hemozoin. Finally, one might also consider that CQ analogues **7**, **10**, and **11** had almost no activity either as hematin polymerization inhibitors or as antimalarials, limiting the utility of a correlation analysis.

For this series of CQ analogues, inhibition of hematin polymerization correlated with observed antimalarial potencies against the CQ-sensitive NF54 strain of *P. falciparum* when hematin polymerization IC_{50} values were normalized for hematin μ-oxo dimer binding affinities. For a compound to inhibit hematin polymerization, that compound must accumulate to sufficiently high concentration in the parasite food vacuole.[43] Whether CQ analogues **1**−**13** meet this criterion is not known, as the structural modifications in the CQ analogues may have affected the physicochemical properties relevant to their cellular accumulation in the parasite.[30] If differences in the CQ analogue food vacuole accumulation were known and considered, the correlation between inhibition of parasite growth and inhibition of hematin polymerization would likely improve.[29]

Our in vitro antimalarial data clearly support the well-known observation that the 7-chloro substituent is associated with optimal antimalarial activity, although we did find that **9**, the 7-nitro CQ derivative, was equipotent to CQ and had a much lower resistance index. The low in vitro antimalarial activity of **7**, the 7*H* analogue of CQ, supports existing data[13,27,44] for this compound. In contrast, our data show that **11**, the 6-chloro analogue of CQ, was 13-fold less potent than CQ in vitro, although it is as active as CQ in *P. gallinaceum*-infected chicks.[45] Similarly **8**, the 7-bromo analogue of CQ, was 4−7-fold less potent than CQ in vitro, although it is as effective as CQ in *P. berghei*-infected mice.[46] Our data also indicate that replacement of the tertiary aliphatic nitrogen atom of CQ with a methine carbon atom (**6**) does not completely abolish antimalarial activity as predicted by Thompson and Werbel.[45] Although there was no correlation between IC_{50} values against *P. falciparum* in vitro and compara-

tive quantitative antimalarial activities against *P. gallinaceum*-infected chicks for CQ and CQ analogues **4**, **7**, **8**, **12**, and **13** (data not shown), this vitro antimalarial data and that of De et al.[13] reinforce the assertion by Thompson and Werbel[45] that chemical modification of the quinoline heterocycle of CQ tends to have a more pronounced impact on vivo antimalarial activity than do modifications of the side chain.

In summary, as determined by isothermal titration calorimetry (ITC), the stoichiometry data and exothermic binding enthalpies indicate that CQ and these 13 CQ analogues bind to two or more hematin μ-oxo dimers in a cofacial $\pi-\pi$ sandwich-type complex. Remarkably, we were not able to measure any significant interaction between hematin μ-oxo dimer and **11**, the 6-chloro analogue of CQ. This result indicates that the 7-chloro substituent in CQ is a critical structural determinant in its binding affinity to hematin μ-oxo dimer. For 4-aminoquinolines related to CQ, our data suggest that electron-withdrawing functional groups at the 7-position of the quinoline ring are required for activity against both hematin polymerization and parasite growth and that chlorine substitution at position 7 is optimal. For this set of CQ analogues, inhibition of hematin polymerization did correlate with inhibition of parasite growth when hematin polymerization IC_{50} values were normalized for hematin μ-oxo dimer binding affinities, adding further evidence that antimalarial 4-aminoquinolines act by this mechanism. This work also confirms that the CQ alkyl side chain, especially the aliphatic tertiary nitrogen atom, is an important structural determinant in CQ drug resistance. Some of these CQ analogues may be useful probes to elucidate the structural basis of CQ drug resistance in future work with proteins encoded by CQ-resistance associated genes.[47,48] Moreover, these data also support an assertion by Ridley and Hudson[49] that a new 4-aminoquinoline active against drug-resistant malaria could be identified.

Experimental Section

Chemistry. CQ analogues **4** (7-chloro-4-(4-(ethylamino)-1-methylbutyl)aminoquinoline dioxalate),[50] **5** (7-chloro-4-(5-(diethylamino)-1-methylamyl)aminoquinoline),[51] **6** (7-chloro-4-(5-ethyl-1-methylheptyl)aminoquinoline hydrochloride), **8** (7-bromo-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline),[14] **11** (6-chloro-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline),[51] **12** (7-chloro-2-methyl-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline),[52,53] and **13** (7-chloro-3-methyl-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline)[54] were obtained from the Division of Experimental Therapeutics inventory at the Walter Reed Army Institute of Research. CQ analogue **3** (7-chloro-4-(4-(diethylamino)-1-methylethyl)aminoquinoline)[55] was obtained from the Hoffmann−La Roche inventory. Other chemicals and reagents were obtained from Aldrich or Sigma Chemical Co. Melting points were determined on a Mel-Temp apparatus and are uncorrected. NMR spectra were recorded using a Varian XL-300 spectrometer. Elemental analysis were performed by M−H−W Laboratories, Phoenix, AZ. Thin-layer chromatography was carried out on precoated Analtech silica gel coated plates.

4-Amino-7-chloroquinoline (1). 4,7-Dichloroquinoline (19.8 g, 0.10 mol) was dissolved in phenol (100 g) with stirring and heating at 165 °C. Ammonia was bubbled through this mixture for 15 min. The temperature was then raised to 200 °C and the bubbling of ammonia was continued for 2.5 h. After cooling, the reaction mixture was treated with 0.2 M acetate buffer, pH 4.0 (400 mL), and ether (250 mL). The layers were separated, and the aqueous layer was washed with fresh ether and basified with 2 N NaOH to afford **1** (15.6 g, 88%) as an off-white solid. Crystallization from benzene yielded **1** as white needles: mp 147−148 °C (lit.[56] mp 148.5−149.5 °C); ^{1}H NMR ($CDCl_3$) δ 4.86 (br s, 2H), 6.6 (d, J = 5.1 Hz, 1H), 7.4 (dd, J = 9.0 Hz, 2.1 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 8.0 (d, J = 2.1 Hz, 1H), 8.53 (d, J = 5.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 103.94, 117.1, 121.71, 125.65, 128.74, 135.23, 149.48, 149.64, 151.74.

7-Chloro-4-(ethylamino)quinoline (2). A mixture of 4,7-dichloroquinoline (5.94 g, 30 mmol) and ethylamine (22.0 g) were heated with stirring in a bomb at an oil bath temperature of 90−95 °C for 64 h. After cooling, excess ethylamine was allowed to evaporate; 1 N KOH (200 mL) was added and an off-white solid (5.97 g, 96%) was obtained after filtration and washing with water and cold ether. Recrystallization from ether afforded **2** as a white crystalline solid: mp 172−173 °C; ^{1}H NMR ($CDCl_3$) δ 1.4 (t, J = 7.3 Hz, 3H), 3.3−3.4 (m, 2H), 5.1 (br s, 1H), 6.4 (d, J = 5.1 Hz, 1H), 7.32 (dd, J = 9.0, 1.5 Hz, 1H), 7.7 (d, J = 9.0 Hz, 1H), 7.95 (d, J = 1.5 Hz, 1H), 8.53 (d, J = 5.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 14.15, 37.83, 98.99, 117.07, 120.99, 125.13, 128.65, 134.72, 149.04, 149.65, 151.97.

Ethyl α-Carbethoxy-β-(3-nitroanilino)acrylate (14). 3-Nitroaniline (50.0 g, 0.362 mol) and diethyl ethoxymethylenemalonate (78.3 g, 0.362 mol) were heated in a three-necked flask for 1 h at 90 °C. On cooling and recrystallization from 95% EtOH, **14** (107.5 g, 96%) was obtained as a yellow solid: mp 81−82 °C (lit.[57] mp 81−82 °C); ^{1}H NMR ($CDCl_3$) δ 1.3−1.4 (m, 6H), 4.26−4.33 (q, J = 7.2 Hz, 4H), 7.4 (d, J = 8.1 Hz, 1H), 7.6 (tt, J = 7.9, 2.6 Hz, 1H), 8.0 (br d), J = 9.4 Hz, 2H), 8.5 (dd, J = 13.2, 2.7 Hz, 1H).

3-(Ethoxycarbonyl)-4-hydroxy-7-nitroquinoline (15). **14** (107.5 g, 0.35 mol) dissolved in biphenyl ether (150 mL) was refluxed at 255 °C for 0.5 h during which time the liberated ethanol was collected by distillation. The reaction mixture was cooled and filtered and the solid reaction residue was washed thoroughly with acetone to give a light yellow solid. Recrystallization from DMSO afforded **15** (28.1 g, 31%) as a pale yellow crystalline solid: mp 290−296 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.3 (t, J = 7.1 Hz, 3H), 4.2 (q, J = 7.2 Hz, 2H), 8.1 (dd, J = 9.0, 2.3 Hz, 1H), 8.4 (d, J = 8.8 Hz, 1H), 8.5 (d, J = 2.2 Hz, 1H), 8.8 (s, 1H).

3-Carboxy-4-hydroxy-7-nitroquinoline (16). **15** (20.0 g, 0.076 mol) was stirred in 10% aqueous NaOH (150 mL) for 0.5 h at 100 °C. The reaction mixture was then cooled and neutralized with HCl to obtain **16** (11.4 g, 64%) as a reddish brown solid: mp 274−275 °C dec (lit.[57] mp 287 °C); ^{1}H NMR (DMSO-d_6) δ 8.3 (dd, J = 8.94, 2.2 Hz, 1H), 8.5 (d, J = 8.9 Hz, 1H), 8.7 (d, J = 2.3 Hz, 1H), 9.1 (s, 1H).

4-Hydroxy-7-nitroquinoline (17). **16** was decarboxylated by way of its silver salt as previously reported.[57,58] To **16** (11.4 g, 49 mmol) suspended in boiling water was added concentrated NH_4OH (0.82 g, 49 mmol) to obtain a clear solution. To this clear solution was added saturated aqueous silver nitrate (8.23 g, 49 mmol) to obtain a light gray solid. The reaction mixture was refluxed for 0.5 h and then allowed to cool to afford the silver salt of **16** (16.3 g, 99%). To the dried silver salt was added biphenyl ether (100 mL) and the reaction mixture was heated at 255 °C for 1 h. On cooling, the reaction solid was filtered and washed thoroughly with hexane. This crude reaction product was dissolved in ethanol (300 mL) and refluxed for 6 h to obtain after cooling **17** (3.14 g, 34%) as a light yellow solid: mp 314−317 °C dec (lit.[57] mp 317−320 °C); ^{1}H NMR (DMSO-d_6) δ 6.2 (d, J = 7.4 Hz, 1H), 8.1 (dd, J = 8.9, 2.2 Hz, 1H), 8.12 (d, J = 7.5 Hz, 1H), 8.3 (d, J = 8.7 Hz, 1H), 8.5 (d, J = 2.2 Hz, 1H).

4-Chloro-7-nitroquinoline (18). **17** (3.1 g, 0.016 mol) was treated with phosphorus oxychloride (7.0 mL) and the mixture was refluxed at 110 °C for 45 min under N_2. Ice water was added slowly to the cooled reaction mixture until evolution of HCl ceased. Basification with 10% aqueous NaOH afforded a light yellow precipitate (3.34 g, 98%): mp 156−160 °C (lit.[57] mp 171−173 °C); ^{1}H NMR (DMSO-d_6) δ 8.04 (d, J = 4.7 Hz, 1H), 8.48 (d, J = 1.7 Hz, 2H), 8.9 (br d, 1H), 9.1 (d, J = 4.7 Hz, 1H). Anal. ($C_9H_5ClN_2O_2$) C, H, N, Cl.

7-Nitro-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline (9). A mixture of **18** (0.5 g, 2.4 mmol) and 2-amino-5-(diethylamino)pentane (1.9 g, 12 mmol) was heated at 120 °C for 7 h under N_2; 20% aqueous NaOH (20 mL) and $CHCl_3$ (50 mL) were added to the crude reaction product. The $CHCl_3$ layer was washed thoroughly with 20% aqueous NaOH (4 × 30 mL) and water (100 mL) and dried over anhydrous sodium bicarbonate. Removal of the $CHCl_3$ in vacuo afforded a gummy residue which was dissolved in 10% acetic acid and filtered to remove unreacted 4-chloro-7-nitroquinoline. The filtrate was neutralized with 10% aqueous NaOH and extracted with $CHCl_3$ (3 × 10 mL), and the combined $CHCl_3$ extracts were dried over K_2CO_3. The residue was chromatographed on alumina (Brockmann, Grade 1) eluting with dry EtOAc. Removal of the EtOAc in vacuo afforded **9** (220 mg, 28%) as a yellow solid: mp 92−96 °C; ^{1}H NMR δ ($CDCl_3$) 1.02 (t, J = 7.0 Hz, 6H), 1.34 (d, J = 6.4 Hz, 3H), 1.6−1.8 (m, 4H), 2.4−2.58 (m, 6H), 3.7−3.75 (m, 1H), 5.8 (d, J = 5.9 Hz, 1H), 6.5 (d, J = 5.5 Hz, 1H), 7.9 (d, J = 9.1 Hz, 1H), 8.13 (dd, J = 9.2, 2.3 Hz, 1H), 8.64 (d, J = 5.2 Hz, 1H), 8.8 (d, J = 2.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.2, 19.8, 23.7, 34.3, 46.7, 48.4, 52.3, 100.9, 117.1, 121.9, 122.5, 125.7, 147.6, 147.9, 148.9, 152.9. Anal. ($C_{18}H_{26}N_4O_2$) C, H, N.

7-Amino-4-(4-(diethylamino)-1-methylbutyl)aminoquinoline (10). To **9** (85 mg, 0.25 mmol) dissolved in EtOH (10 mL) was added 10% Pd/C (8.4 mg) and the mixture was hydrogenated at 50 atm H_2 for 3 h. The reaction solution was filtered through Celite and the solvent was removed in vacuo to produce a reddish brown oily residue. The crude reaction product was dissolved in dry ether (4 mL), cooled to 0 °C, and exposed to a stream of HCl gas for 5 min to afford a yellow solid. Excess HCl gas was removed with a stream of N_2 and the ether was decanted to yield the trihydrochloride salt of **10** (60 mg, 58%): mp 58−64 °C; ^{1}H NMR ($CDCl_3$) δ 1.04 (t, J = 7.2 Hz, 6H), 1.3 (d, J = 6.4 Hz, 3H), 1.6−1.8 (m, 4H), 2.4−2.7 (m, 6H), 3.7−3.75 (m, 1H), 4.2−4.4 (br s, 1H), 5.1 (d, J = 5.9 Hz, 1H), 6.2 (d, J = 6.1 Hz, 1H), 6.8 (dd, J = 9.0, 2.3 Hz, 1H), 7.2 (d, J = 2.1 Hz, 1H), 7.7 (d, J = 9.0 Hz, 1H), 8.32 (d, J = 6.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.3, 20.3, 23.7, 34.6, 46.7, 48.1, 52.5, 97.0, 110.3, 111.9, 115.7, 120.6, 147.1, 149.0, 150.1, 151.1. Anal. ($C_{18}H_{31}N_4Cl_3$) C, H, N.

Isothermal Titration Calorimetry (ITC). All ITC binding experiments were conducted in 250 mM phosphate buffer, pH 6.5 at 37 °C, using a MCS titration calorimeter from Microcal Inc. (Northampton, MA). An aliquot of a 1 mM stock solution of hematin in 0.01 N NaOH was diluted up to the required concentrations (0.1−0.3 mM) in the phosphate buffer. Hematin was titrated with 0.5−5 mM solutions of CQ analogues **1**−**13** using a 100-μL syringe rotating at 400 rpm. Titration experiments consisted of 15−30 injections of 2−6 μL each, and three to four experiments were performed for each analogue. Heats of dilution and mixing were obtained by 15−30 injections of each analogue into the buffer medium. After having first subtracted heats of dilution and mixing from each injection heat pulse, the binding isotherms were fitted for a model of a single set of identical binding sites by nonlinear least-squares fitting of the titration data using Origin software. Association constants (K_a's), enthalpy change (ΔH), and stoichiometry (n) were obtained from binding isotherms using the Marquardt algorithm.[59]

Inhibition of Hematin Polymerization. As previously described[3f,4d] purified hemozoin from the food vacuole of the malarial parasite *P. falciparum*[60] was used to initiate hematin polymerization at pH 6.5. Compounds were added to the reaction mixture as DMSO solutions up to a maximum DMSO concentration of 10%. The dpm values obtained from the assay were expressed as percent inhibition relative to hemozoin formation in a drug-free control. The values of triplicate assays were plotted semilogarithmically (CA-Cricket Graph III 1.5.2) and the IC_{50} values (μM) calculated graphically ±SD (standard deviation).

Inhibition of Parasite Growth. *P. falciparum* strains NF54 and K1 were cultured according to the method of Trager and Jensen[61] with minor variations as described by Dorn et al.[4d] Compound inhibition of parasite growth was determined by a semiautomated microdilution assay using asynchronous stock cultures.[25a,62]

Molecular Modeling. Gaussian 94, revision A.1 (Gaussian, Inc.), on a SGI Power Indigo R8000 workstation and Spartan 5.0 (Wavefunction, Inc.) on a SGI Indigo Extreme R4000 workstation were used for the molecular modeling experiments. The structure of hemin was obtained from the Cambridge Crystallographic Data Bank and uploaded into Spartan 5.0. Hematin μ-oxo dimer was built with constraints of 168° for the Fe−O−Fe angle and 1.76 Å for the Fe−O distance, data derived from the X-ray structure of ferritetraphenylporphyrin μ-oxo dimer.[63] The minimum energy conformer of hematin μ-oxo dimer was established by rotating one of the hematin moieties along the Fe−O−Fe axis through 360° in 5° steps and calculating the relative energies using the Merck molecular force field (MMFF94).[64] The minimum energy conformer was then subject to PM3 calculation to determine its electrostatic potential surface.[65,66]

The energy-minimized structures of CQ and CQ analogues **1**, **6**, **7**, and **11** were subject to a conformational search at the AM1 single-point level to obtain the low-energy conformers. Using Gaussian 94, the lowest and most abundant (population density > 75%) conformers were optimized at the ab initio quantum chemical level with the 3-21G* split-valence basis set. Molecular electrostatic potentials and three-dimensional electronic profiles were calculated on the 3-21G*-optimized geometries for the five structures. A similar sequence was followed for the energy minimization of **1** and 6-chloro-4-aminoquinoline followed by optimization at the ab initio quantum chemical level with the 6-31G** basis set. From the energy-minimized structure of hematin μ-oxo dimer and CQ, a model of a 1:2 CQ−hematin μ-oxo dimer complex[3f,7] was built in which the electron-deficient electrostatic potential surfaces of the hematin μ-oxo dimer interact favorably with the electron-rich electrostatic potential surface of the quinoline heterocycle of CQ.

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References

(1) Bray, P. G.; Ward, S. A. A Comparison of the Phenomenology and Genetics of Multidrug Resistance in Cancer Cells and Quinoline Resistance in *Plasmodium falciparum*. *Pharmacol. Ther.* **1998**, *77*, 1−28.

(2) (a) Ridley, R. G. Haemoglobin Degradation and Haem Polymerization as Antimalarial Drug Targets. *J. Pharm. Pharmacol.* **1997**, *49S*, 43−48. (b) Ridley, R. G.; Dorn, A.; Vippagunta, S. R.; Vennerstrom, J. L. Haematin (haem) Polymerization and its Inhibition by Quinoline Antimalarials. *Ann. Trop. Med. Parasitol.* **1997**, *91*, 559−566.

(3) (a) Chou, A. C.; Chevli, R.; Fitch, C. D. Ferriprotoporphyrin IX Fulfills the Criteria for Identification as the Chloroquine Receptor of Malaria Parasites. *Biochemistry* **1980**, *19*, 1543−1549. (b) Egan, T. J.; Mavuso, W. W.; Ross, D. C.; Marques, H. M. Thermodynamic Factors Controlling the Interaction of Quinoline Antimalarial Drugs With Ferriprotoporphyrin IX. *J. Inorg. Biochem.* **1997**, *68*, 137−145. (c) Sullivan, D. J., Jr.; Gluzman, I. Y.; Russell, D. G.; Goldberg, D. E. On the Molecular Mechanism of Chloroquine's Antimalarial Action. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11865−11870. (d) Sullivan, D. J., Jr.; Matile, H.; Ridley, R. G.; Goldberg, D. E. A Common Mechanism for Blockade of Heme Polymerization by Antimalarial Quinolines. *J. Biol. Chem.* **1998**, *273*, 31103−31107. (e) Bray, P. G.; Mungthin, M.; Ridley, R. G.; Ward, S. A. Access to Hematin: The Basis of Chloroquine Resistance. *Mol. Pharmacol.* **1998**, *54*, 170−179. (f) Dorn, A.; Vippagunta, S. R.; Matile, H.; Jaquet, C.; Vennerstrom, J. L.; Ridley, R. G. An Assessment of Drug-Hematin Binding as a Mechanism for Inhibition of Hematin Polymerisation by Quinoline Antimalarials. *Biochem. Pharmacol.* **1998**, *55*, 727−736.

(4) (a) Slater, A. F. G.; Cerami, A. Inhibition By Chloroquine of a Novel Haem Polymerase Enzyme Activity in Malaria Trophozoites. *Nature* **1992**, *355*, 167−169. (b) Orjih, A. U.; Fitch, C. D. Hemozoin Production by *Plasmodium falciparum*: Variation With Strain and Exposure to Chloroquine. *Biochim. Biophys.*

Acta **1993**, *1157*, 270−274. (c) Egan, T. J.; Ross, D. C.; Adams, P. A. Quinoline Anti-Malarial Drugs Inhibit Spontaneous Formation of β-Hematin (Malaria Pigment). *FEBS Lett.* **1994**, *352*, 54−57. (d) Dorn, A.; Stoffel, R.; Matile, H.; Bubendorf, A.; Ridley, R. G. Malarial Haemozoin/β-Hematin Supports Haem Polymerization in the Absence of Protein. *Nature* **1995**, *374*, 269−271.

(5) (a) Chou, A. C.; Fitch, C. D. Hemolysis of Mouse Erythrocytes by Ferriprotoporphyrin IX and Chloroquine. *J. Clin. Invest.* **1980**, *66*, 856−858. (b) Orjih, A. U.; Banyal, H. S.; Chevli, R.; Fitch, C. D. Hemin Lyses Malaria Parasites. *Science* **1981**, *214*, 667−669. (c) Ginsburg, H.; Demel, R. A. The Effect of Ferriprotoporphyrin IX and Chloroquine on Phospholipid Monolayers and the Possible Implications to Antimalarial Activity. *Biochim. Biophys. Acta* **1983**, *732*, 316−319. (d) Hebbel, R. P.; Eaton, J. W. Pathobiology of Heme Interaction with the Erythrocyte Membrane. *Semin. Hematol.* **1989**, *26*, 136−149. (e) Ginsburg, H.; Famin, O.; Zhang, J.; Krugliak, M. Inhibition of Glutathione-Dependent Degradation of Heme by Chloroquine and Amodiaquine as a Possible Basis for Their Antimalarial Mode of Action. *Biochem. Pharmacol.* **1998**, *56*, 1305−1313. (f) Sugioka, Y.; Suzuki, M. The Chemical Basis for the Ferriprotoporphyrin IX-Chloroquine Complex Induced Lipid Peroxidation. *Biochim. Biophys. Acta* **1991**, *1074*, 19−24.

(6) (a) Fitch, C. D.; Kanjananggulpan, P. The State of Ferriprotoporphyrin IX in Malaria Pigment. *J. Biol. Chem.* **1987**, *262*, 15552−15555. (b) Slater, A. F. G.; Swiggard, W. J.; Orton, B. R.; Flitter, W. D.; Goldberg, D. E.; Cerami, A.; Henderson, G. B. An Iron-Carboxylate Bond Links the Heme Units of Malaria Pigment. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 325−329. (c) Bohle, S. D.; Dinnebier, R. E.; Madsen, S.; Stephens, P. W. Characterization of the Products of the Heme Detoxification Pathway in Malarial Late Trophozoites by X-ray Diffraction. *J. Biol. Chem.* **1997**, *272*, 713−716.

(7) Moreau, S.; Perly, B.; Biguet, J. Interactions de la Chloroquine Avec la Ferriprotoporphyrine IX. Étude par Résonance Magnétique Nucléaire. *Biochimie* **1982**, *64*, 1015−1025.

(8) Adams, P. A.; Berman, P. A. M.; Egan, T. J.; Marsh, P. J.; Silver, J. The Iron Environment in Heme and Heme-Antimalarial Complexes of Pharmacological Interest. *J. Inorg. Biochem.* **1996**, *63*, 69−77.

(9) (a) Cohen, S. N.; Phifer, K. O.; Yielding, K. L. Complex Formation Between Chloroquine and Ferrihaemic Acid In Vitro, and its Effect on the Antimalarial Action of Chloroquine. *Nature* **1964**, *202*, 805−806. (b) Blauer, G.; Ginsburg, H. Complexes of Antimalarial Drugs with Ferriprotoporphyrin IX, *Biochem. Int.* **1982**, *5*, 519−523. (c) Jearnpipatkul, A.; Govitrapong, P.; Yuthavong, Y.; Wilairat, P.; Panijpan, B. Binding of Antimalarial Drugs to Hemozoin from *Plasmodium berghei*. *Experientia* **1980**, *36*, 1063−1064. (d) Jearnpipatkul, A.; Panijpan, B. Molecular complexes of quinoline antimalarials with iron-porphyrin components of protease-digested methemoglobin. *Chem. Biol. Interact.* **1980**, *33*, 83−90.

(10) de Almeida Ribeiro, M. C.; Augusto, O.; da Costa Ferreira, A. M. Inhibitory Effect of Chloroquine on the Peroxidase Activity of Ferriprotoporphyrin IX. *J. Chem. Soc., Dalton Trans.* **1995**, 3759−3766.

(11) O'Neill, P. M.; Bray, P. G.; Hawley, S. R.; Ward, S. A.; Park, B. K. 4-Aminoquinolines-Past, Present, and Future: A Chemical Perspective. *Pharmacol. Ther.* **1998**, *77*, 29−58.

(12) Price, C. C.; Roberts, R. M. The Synthesis of 4-Hydroxyquinolines. I. Through Ethoxymethylenemalonic Ester. *J. Am. Chem. Soc.* **1946**, *68*, 1204−1208.

(13) De, D.; Krogstad, F. M.; Cogswell, F. B.; Krogstad, D. J. Aminoquinolines that Circumvent Resistance in *Plasmodium falciparum* In Vitro. *Am. J. Trop. Med. Hyg.* **1996**, *55*, 579−583.

(14) Surrey, A. R.; Hammer, H. F. Some 7-Substituted 4-Aminoquinoline Derivatives. *J. Am. Chem. Soc.* **1946**, *68*, 113−116.

(15) (a) Yayon, A.; Cabantchik, Z. I.; Ginsburg, H. Identification of the Acidic Compartment of *Plasmodium falciparum*-Infected Erythrocytes as the Target of the Antimalarial Drug Chloroquine. *EMBO J.* **1984**, *3*, 2695−2700. (b) Krogstad, D. J.; Schlesinger, P. H.; Gluzman, I. Y. Antimalarials Increase Vesicle pH in *Plasmodium falciparum*. *J. Cell. Biol.* **1985**, *101*, 2302−2309.

(16) (a) Brown, S. B.; Dean, T. C.; Jones, P. Aggregation of Ferrihaems. Dimerization and Protolytic Equilibria of Protoferrihaem and Deuterioferrihaem in Aqueous Solution. *Biochem. J.* **1970**, *117*, 733−739. (b) Silver, J.; Lukas, B. Mössbauer Studies on Protoporphyrin IX Iron(III) Solutions. *Inorg. Chim. Acta* **1983**, *78*, 219−224. (c) Bednarski, T. M.; Jordan, J. Electron-Transfer Characteristics of the Prosthetic Group of Hemoproteins. *J. Am. Chem. Soc.* **1967**, *89*, 1552−1558. (d) Buchler, J. W. Hemoglobin-An Inspiration for Research in Coordination Chemistry. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 407−423. At low concentrations of free hematin monomer and at low pH, conditions likely to prevail in the parasite food vacuole, the hematin monomer:μ-oxo dimer equilibrium shifts toward the monomer, although the K_{obs} estimated at $2.8 \times 10^5\ M^{-1}$ at pH 4.8 (ref 16a) still strongly favors the μ-oxo dimer.

(17) White, W. I. Aggregation of Porphyrins and Metalloporphyrins. In *The Porphyrins, Vol. V*; Dolphin, D., Ed.; Physical Chemistry, Part C; Academic Press: New York, 1978; pp 303−339.

(18) Diederich, F.; Smithrud, D. B.; Sanford, E. M.; Wyman, T. B.; Ferguson, S. B.; Carcanague, D. R.; Chao, I.; Houk, K. N. Solvent Effects in Molecular Recognition. *Acta Chem. Scand.* **1992**, *46*, 205−215.

(19) Lemieux, R. U. How Water Provides the Impetus for Molecular Recognition in Aqueous Solution. *Acc. Chem. Res.* **1996**, *29*, 373−380.

(20) Lumry, R.; Rajender, S. Enthalpy−Entropy Compensation Phenomena in Water Solutions of Proteins and Small Molecules: A Ubiquitous Property of Water. *Biopolymers* **1970**, *9*, 1125−1227.

(21) Blokzijl, W.; Engberts, J. B. F. N. Hydrophobe Effekte−Ansichten und Tatsachen. *Angew. Chem.* **1993**, *105*, 1610−1648.

(22) Muller, N. Search for a Realistic View of Hydrophobic Effects. *Acc. Chem. Res.* **1990**, *23*, 24−28.

(23) Gilli, P.; Ferretti, V.; Gilli, G.; Borea, P. A. Enthalpy−Entropy Compensation in Drug-Receptor Binding. *J. Phys. Chem.* **1994**, *98*, 1515−1518.

(24) (a) Hunter, C. A.; Sanders, J. K. M. The Nature of $\pi-\pi$ Interactions. *J. Am. Chem. Soc.* **1990**, *112*, 5525−5534. (b) Hunter, C. A. Arene-Arene Interactions: Electrostatic or Charge Transfer? *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1584−1586.

(25) (a) Ridley, R. G.; Hofheinz, W.; Matile, H.; Jaquet, C.; Dorn, A.; Masciadri, R.; Jolidon, S.; Richter, W. F.; Guenzi, A.; Girometta, M. A.; Urwyler, H.; Huber, W.; Thaithong, C.; Peters, W. 4-Aminoquinoline Analogues of Chloroquine With Shortened Side Chains Retain Acitivity Against Chloroquine-Resistant *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* **1996**, *40*, 1846−1854. (b) De, D.; Krogstad, F. M.; Byers, L. D.; Krogstad, D. J. Structure−Activity Relationships for Antiplasmodial Activity among 7-Substituted 4-Aminoquinolines. *J. Med. Chem.* **1998**, *41*, 4918−4926.

(26) (a) Geary, T. G.; Divo, A. A.; Jensen, J. B. Activity of Quinoline Containing Antimalarials Against Chloroquine-Sensitive and Resistant Strains of *Plasmodium falciparum* In Vitro. *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 499−503. (b) Aderounmu, A. F. In Vitro Assessment of the Antimalarial Activity of Chloroquine and Its Major Metabolites. *Ann. Trop. Med. Parasitol.* **1984**, *78*, 581−585.

(27) Veignei, E.; Moreau, S. The Mode of Action of Chloroquine. Non-Weak Base Properties of 4-Aminoquinolines and Antimalarial Effects on Strains of *Plasmodium*. *Ann. Trop. Med. Parasitol.* **1991**, *85*, 229−237.

(28) Bray, P. G.; Hawley, S. R.; Mungthin, M.; Ward, S. A. Physicochemical Properties Correlated with Drug Resistance and the Reversal of Drug Resistance in *Plasmodium falciparum*. *Mol. Pharmacol.* **1996**, *50*, 1559−1566.

(29) Hawley, S. R.; Bray, P. G.; Mungthin, M.; Atkinson, J. D.; O'Neill, P. M.; Ward, S. A. Relationship Between Antimalarial Drug Activity, Accumulation, and Inhibition of Heme Polymerization in *Plasmodium falciparum* In Vitro. *Antimicrob. Agents Chemother.* **1998**, *42*, 682−686.

(30) O'Neill, P. M.; Willock, D. J.; Hawley, S. R.; Bray, P. G.; Storr, R. C.; Ward, S. A.; Park, B. K. Synthesis, Antimalarial Activity, and Molecular Modeling of Tebuquine Analogues. *J. Med. Chem.* **1997**, *40*, 437−448.

(31) Vippagunta, S. R.; Dorn, A.; Ridley, R. G.; Vennerstrom, J. L. Unpublished results.

(32) Koh, H. L.; Go, M. L.; Ngiam, T. L.; Mark, J. W. Conformational and Structural Features Determining In Vitro Antimalarial Activity in Some Indolo[3,2-c]Quinolines, Anilinoquinolines and Tetrahydroindolo[3,2-d]Benzazepines. *Eur. J. Med. Chem.* **1994**, *29*, 107−113.

(33) The $N_{quin}-N_{diethyl}$ distances are 8.615 Å (3-21G*) and 8.508 Å from X-ray data (Karle, J. M.; Karle, I. L. Redetermination of the Molecular Structure of the Antimalarial Chloroquine Bis-(dihydrogenphosphate) Dihydrate. *Acta Crystallogr.* **1988**, *C44*, 1605−1608).

(34) Constantinides, I.; Satterlee, J. D. UV−Visible and Carbon NMR Studies of Chloroquine Binding to Urohemin I Chloride and Uroporphyrin I in Aqueous Solutions. *J. Am. Chem. Soc.* **1988**, *110*, 4391−4395.

(35) Caughey, W. S.; Barlow, C. H.; O'Keeffe, D. H.; O'Toole, M. C. Spectroscopic Studies of Cis and Trans Effects in Hemes and Hemins. *Ann. N.Y. Acad. Sci.* **1973**, *206*, 296−309.

(36) Heaton, N. J.; Bello, P.; Herradón, B.; del Campo, A.; Jiménez-Barbero, J. NMR Study of Intramolecular Interactions between Aromatic Groups: van der Waals, Charge-Transfer, or Quadrupolar Interactions? *J. Am. Chem. Soc.* **1998**, *120*, 12371−12384.

(37) Schneider, H.; Wang, M. Ligand-Porphyrin Complexes: Quantitative Evaluation of Stacking and Ionic Contributions. *J. Org. Chem.* **1994**, *59*, 7464−7472.

(38) Ferrari, V.; Cutler, D. J. Temperature Dependence of the Acid Dissociation Constants of Chloroquine. *J. Pharm. Sci*. **1987**, *76*, 554−556.
(39) Macomber, D. B.; Sprinz, H.; Tousimis, A. J. Morphological Effects of Chloroquine on *Plasmodium berghei* in Mice. *Nature* **1967**, *214*, 937.
(40) Fitch, C. D.; Chevli, R.; Gonzalez, Y. Chloroquine-Resistant *Plasmodium falciparum*: Effect of Substrate on Chloroquine and Amodiaquine Accumulation. *Antimicrob. Agents Chemother.* **1974**, *6*, 757−762.
(41) Hawley, S. R.; Bray, P. G.; O'Neill, P. M.; Kevin Park, B.; Ward, S. A. The Role of Drug Accumulation in 4-Aminoquinoline Antimalarial Potency. *Biochem. Pharmacol.* **1996**, *52*, 723−733.
(42) (a) Vippagunta, S. R.; Dorn, A.; Bubendorf, A.; Ridley, R. G.; Vennerstrom, J. L. Deferoxamine: Stimulation of Hematin Polymerization and Antagonism of its Inhibition by Chloroquine. *Biochem. Pharmacol*. **1999**, *58*, 817−824. (b) Blauer, G.; Ginsburg, H. Complexes of Antimalarial Drugs With Ferriprotoporphyrin IX. *Biochem. Int.* **1982**, *5*, 519−523. (c) Grinberg, L. N.; Soprunov, F. F. Metabolism of Erythrocyte Infected with Malaria Parasite and the Action of Antimalarial Drugs. *Biomed. Biochim. Acta* **1983**, *11/12*, S317−S321.
(43) Saliba, K. J.; Folb, P. I.; Smith, P. J. Role for the *Plasmodium falciparum* Digestive Vacuole in Chloroquine Resistance. *Biochem. Pharmacol.* **1998**, *56*, 313−320.
(44) Bass, G. E.; Hudson, D. R.; Parker, J. E.; Purcell, W. P. Mechanism of Antimalarial Activity of Chloroquine Analogues From Quantitative Structure Activity Studies. Free Energy Related Model. *J. Med. Chem*. **1971**, *14*, 275−282.
(45) Thompson, P. E.; Werbel, L. M. 4-Aminoquinolines. *Antimalarial Agents: Chemistry and Pharmacology*; Academic Press: New York, 1972; pp 150−193.
(46) Coatney, G. R.; Cooper, W. C.; Eddy, N. B.; Greenberg, J. *Survey of Antimalarial Agents*; Public Health Monograph No. 9; U.S. Government Printing Office: Washington, DC, 1953.
(47) Su, X.; Kirkman, L A.; Fujioka, H.; Wellems, T. E. Complex Polymorphisms in an ∼330 Kda Protein are Linked to Chloroquine-Resistant *P. falciparum* in Southeast Asia and Africa. *Cell* **1997**, *91*, 593−603.
(48) Wünsch, S.; Sanchez, C. P.; Gekle, M.; Grobe-Wortmann, L.; Wiesner, J. Differential Stimulation of the Na±/H± Exchanger Determines Chloroquine Uptake in *Plasmodium falciparum*. *J. Cell. Biol*. **1998**, *140*, 335−345.
(49) Ridley, R. G.; Hudson, A. T. Quinoline Antimalarials. *Exp. Opin. Ther. Patents* **1998**, *8*, 121−135.
(50) Carmack, M.; Bullitt, Jr., O. H.; Handrick, G. R.; Kissinger, L. W.; Von, I. The Synthesis of 4-(4-Amino-1-methylbutylamino)-7-chloroquinoline and Some 4-(4-Monoalkylamino-1-methylbutylamino)-7-chloroquinolines. *J. Am. Chem. Soc.* **1946**, *68*, 1220−1225.
(51) Drake, N. L.; Creech, H. J.; Garman, J. A.; Haywood, S. T.; Peck, R. M.; VanHook, J. O.; Walton, E. Synthetic Antimalarials. The Preparation of Certain 4-Aminoquinolines. *J. Am. Chem. Soc.* **1946**, *68*, 1208−1213.
(52) Price, C. C.; Leonard, N. J.; Reitsema, R. H. Synthesis of 4-Hydroxyquinolines. V. A Direct Synthesis from β-Anilinoacrylates. *J. Am. Chem. Soc.* **1946**, *68*, 1256−1259.
(53) Steck, E. A.; Hallock, L. L.; Holland, A. J.; Fletcher, L. T. Quinolines. V. Some Polysubstituted 4-(4′-Diethylamino-1′-methylbutylamino)quinolines. *J. Am. Chem. Soc.* **1948**, *70*, 1012−1015.
(54) Steck, E. A.; Hallock, L. L.; Holland, A. J. Quinolines. III. The Synthesis of 5- and 7-Chloro and Bromo-3-methyl-4-dialkylaminoalkylaminoquinolines. *J. Am. Chem. Soc.* **1946**, *68*, 380−383.
(55) Hofheinz, W.; Jaquet, C.; Jolidon, S. Aminochinolin-Derivate mit einer Wirksamkeit gegen Malariaerreger. European Patent Application 94116281.0, June 1995.
(56) Baker, R. H.; Dodson, R. M.; Riegel, B. The Dealkylation of 4-(4-Diethylamino-1-methylbutylamino)-7-chloroquinoline, SN 7618. *J. Am. Chem. Soc.* **1947**, *69*, 704.
(57) Ellis, J.; Gellert, E.; Robson, J. Synthesis of Some New Iodoquinolines. *Aust. J. Chem*. **1973**, *26*, 907−911.
(58) Baker, R. H.; Lappin, G. R.; Albisetti, C. J., Jr.; Riegel, B. The Decarboxylation of Nitro Substituted 3-Carboxy-4-quinolinols by Pyrolysis of Their Silver Salts. *J. Am. Chem. Soc.* **1946**, *68*, 1267.
(59) Bevington, P. R. *Data Reduction and Error Analysis in the Physical Sciences*; McGraw-Hill: New York, 1969; p 236.
(60) Ashong, J. O.; Blench, I. P.; Warhurst, D. C. The Composition of Haemozoin from *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **1989**, *83*, 167−172.
(61) Trager, W.; Jensen, J. B. Human Malaria Parasites in Continuous Culture. *Science* **1976**, *193*, 673−675.
(62) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. Quantitative Assessment of Antimalarial Activity In Vitro by a Semiautomated Microdilution Technique. *Antimicrob. Agents Chemother.* **1979**, *16*, 710−718.
(63) Fleischer, E. B.; Srivastava, T. S. The structure and properties of μ-Oxo bis(tetraphenylporphineiron (III)). *J. Am. Chem. Soc.* **1969**, *91*, 2403−2405.
(64) Halgren, T. A. Merck Molecular Force Field. 1. Basis, Form, Scope, Parametrization, and Performance of MMFF94. *J. Comput. Chem*. **1996**, *17*, 490−641.
(65) Politzer, P.; Truhlar, D. G. *Chemical Applications of Atomic and Molecular Electrostatic Potentials*; Plenum: New York, 1981.
(66) Naray-Szabo, G.; Ferenczy, G. G. Molecular Electrostatics. *Chem. Rev*. **1995**, *95*, 829−847.

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