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An Assessment of Drug-Haematin Binding as a Mechanism for Inhibition of Haematin Polymerisation by Quinoline Antimalarials

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ABSTRACT. Chloroquine is thought to exert its antimalarial activity by preventing the polymerisation of toxic haematin released during proteolysis of haemoglobin in the *Plasmodium* digestive vacuole. However, the molecular mechanisms by which this inhibition occurs and the universality of this mechanism for other quinoline antimalarials remain to be established. We demonstrate here a correlation for eight antimalarial quinolines between inhibition of haematin polymerisation *in vitro* and inhibition of *P. falciparum* growth in culture, confirming haematin polymerisation as the likely target of quinoline blood schizonticides. Furthermore, using isothermal titration microcalorimetry, a correlation was observed between the haematin binding constant of these compounds and their ability to inhibit haematin polymerisation, suggesting that these compounds mediate their activity through binding to haematin. It was also observed that the compounds bind primarily to the μ-oxo dimer form of haematin rather than the monomeric form. It is postulated that this binding inhibits haematin polymerisation by shifting the haematin dimerisation equilibrium to the μ-oxo dimer, thus reducing the availability of monomeric haematin for incorporation into haemozoin. These data reconcile the haematin polymerisation theory with the Fitch hypothesis, which states that chloroquine mediates its activity through binding to haematin. BIOCHEM PHARMACOL **55**;6:727–736, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. malaria; haematin polymerisation; quinoline antimalarials; drug-haematin binding; μ-oxo dimer; haemozoin

Haem (iron II-protoporphyrin IX) serves as the prosthetic group for many different haemoproteins, including haemoglobin. During their growth in erythrocytes, malarial parasites degrade large quantities of haemoglobin in an acidic lysosomal food vacuole, and toxic, monomeric haem is released [1, 2]. In higher eukaryotes, haem is oxidatively degraded by a haem oxygenase which breaks the porphyrin ring to release carbon monoxide, ferric ion and biliverdine [3]. Malarial parasites, however, employ a different mechanism to detoxify haem. An insoluble polymer, termed malaria pigment or haemozoin and based upon haematin (iron III-protoporphyrin IX), is formed. The advantage of this process, compared with the more ubiquitous haem oxygenase pathway, might be that toxicity problems caused by the release of the large quantities of iron and carbon monoxide are avoided.

It is now widely accepted that haemozoin is essentially identical to β-haematin [4, 5]. By the use of resonance Raman microspectrometry, electron paramagnetic resonance spectrometry, and magnetic susceptibility measurements, different research groups have found that haemozoin contains a five coordinate iron (III) complex in a high spin state [6, 7]. Four of the iron (III) bonds in each haematin subunit link to the planar porphyrin ring, while the fifth is believed to link to a propionic acid side-chain of the adjacent haematin unit [5, 7].

Practical implications of these observations arose when it was discovered that a trophozoite lysate could support the polymerisation of haematin *in vitro* [8]. It was postulated that chloroquine and quinine inhibited haematin polymerisation in the parasite and that failure to detoxify the haem resulted in parasite death. It was initially believed that this reaction was catalysed by a haem polymerase enzyme and that chloroquine inhibited this enzyme activity. However, chloroquine also inhibited synthetic β-haematin formation, synthesized at nonphysiological temperature in the absence of trophozoite lysate [9]. In addition, the polymerisation reaction promoted by trophozoite lysate was found to be protein-independent and was solely dependent on the presence of preformed haemozoin [10]. Thus, haematin polymerisation, once initiated, is a physico-chemical process and is not enzyme-mediated. However, protein(s) and/or lipids may play a role in initiating the process in the parasite [11–13]. These data led us to conclude that chloroquine inhibited haemozoin elongation by binding to

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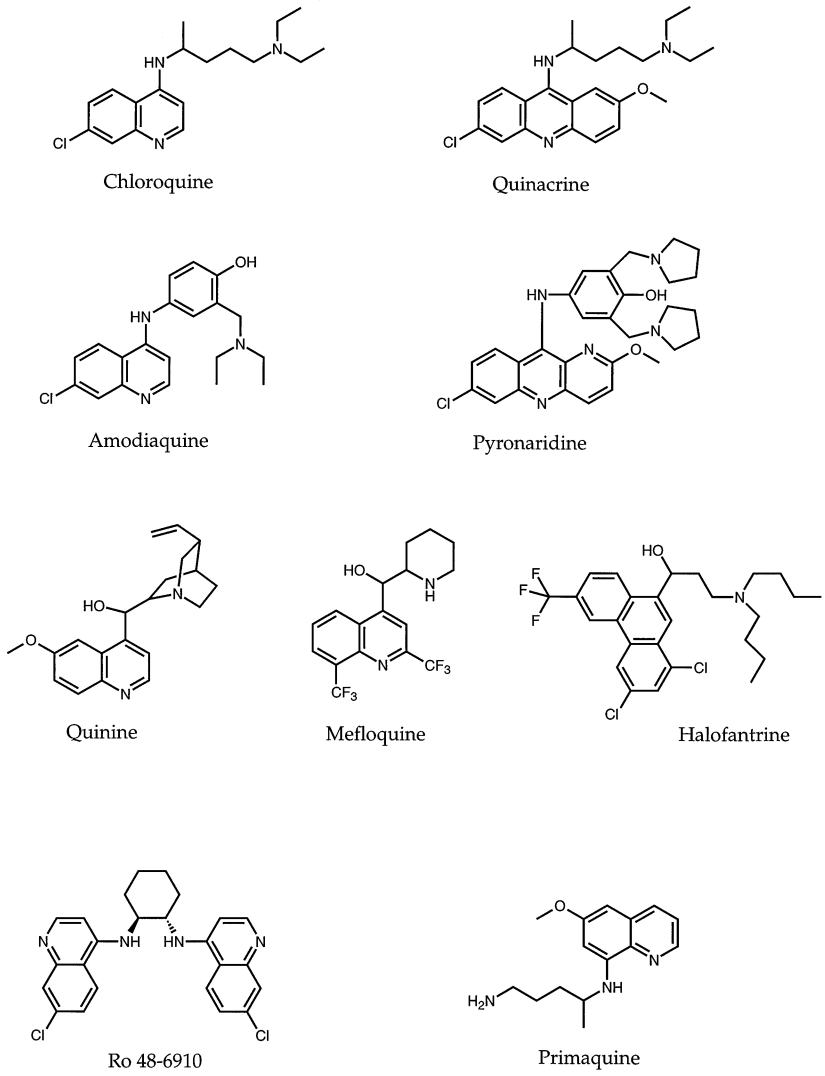

FIG. 1. Structures of quinoline antimalarials used in this study.

monomeric haematin (α-haematin), thus preventing its incorporation into the β-haematin polymer [10]. This supported an original hypothesis by Fitch and colleagues, who had postulated that haematin (or ferriprotoporphyrin IX) was the receptor for chloroquine in malaria parasites [14].

In this paper, we further test this hypothesis and compare the ability of several types of quinoline antimalarials to inhibit haematin polymerisation and inhibit parasite growth in culture. We then use isothermal titration microcalorimetry (ITC)§ to measure the binding affinity with which these compounds interact with haematin and compare this with their ability to inhibit haematin polymerisation. We discuss their antimalarial activity in light of these results. Throughout this manuscript, we use haematin to refer to iron III-protoporphyrin IX rather than the generic term haem used in many previous articles on this topic. The term haem is reserved for iron II-protoporphyrin IX.

§ *Abbreviations:* ITC, isothermal titration microcalorimetry; K_a, association constant; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Compounds

All compounds for this study were obtained from the Hoffmann–La Roche inventory. They included the blood schizonticides chloroquine, quinacrine, amodiaquine, pyronaridine, quinine, mefloquine, halofantrine, and a bisquinoline, Ro 48-6910, *trans*-N^1,N^2-bis(7-chloro-quinolin-4-yl)cyclohexane-1,2-diamine [15, 16]. Primaquine [17], an 8-aminoquinoline which is inactive against blood stage parasites and hence unlikely to interfere with haemozoin formation, but which is active against liver stage parasites, was included as a negative control. The structures of these compounds are given in Fig. 1.

Parasitological Methods

Plasmodium falciparum strain NF54 was cultured according to the method of Trager and Jensen [18] with minor variations as described [10]. Compound inhibition of parasite growth was determined by a semiautomated microdilu-

tion assay using asynchronous stock cultures [19] as described previously [20].

Haematin Polymerisation Assay

Reactions were carried out essentially as described previously [10], using purified haemozoin from the malarial parasite *P. falciparum* [21] to initiate the reaction. Briefly, aliquots of haemozoin containing 10–20 μmol haematin were mixed with 140 μM [^{14}C]-haemin (University of Leeds Innovations Industrial Services Ltd; specific activity 0.16 μCi/μmol) in either 500 mM sodium acetate, pH 4.8, or 250 mM sodium phosphate, pH 6.5, in a final volume of 100 μL on a Greiner microtiter plate, and incubated overnight at 37°. Unincorporated haematin was removed from the insoluble haemozoin by filtrating through Multiscreen HV plates (0.45 μm hydrophobic low protein binding durapore membrane, MHVB N45, Millipore AG). The microtiter plates were washed with 1 mL 2% SDS in 0.1 M sodium bicarbonate buffer, pH 9.1, with 1 mL of 0.1 M sodium bicarbonate, pH 9.1, with 1 mL of 50 mM Tris-HCl, pH 7.5, and with 1 mL of distilled water. The membrane-bound insoluble haemozoin was determined by scintillation counting using the TopCount™ Microplate Scintillation System (Canberra Packard S.A.).

Inhibition of Haematin Polymerisation

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 haemozoin formation in a drug-free control. The values of triplicate assays were plotted semi-logarithmically (DeltaGraph Pro 3.5 and CA-Cricket Graph III 1.5.2) and the IC_{50} values [μM] calculated graphically ± SD.

Isothermal Titration Microcalorimetry (ITC)

ITC experiments were carried out on an OMEGA titration calorimeter from Microcal Inc. [22, 23] at 37°. An aliquot of a 5 mM stock solution of haematin in 0.01 N NaOH was diluted up to the required concentrations (0.05–0.5 mM) in 250 mM phosphate buffer, pH 6.5, or in 50 mM phosphate buffer, pH 6.9 containing 150 mM KCl. The haematin was titrated with a 1.0–10.0 mM solution of drugs dissolved in the same medium using a 100 μL syringe rotating at 400 rpm. Titration experiments consisted of 20–30 injections of 2–5 μL each. Three to four experiments were performed for each drug.

Addition of a small amount of 200 mM acetate buffer, pH 4.0 was required to dissolve amodiaquine in the pH 6.9 buffer and quinine in both the pH 6.5 and 6.9 buffers. Due to the very limited solubility of both mefloquine and halofantrine in these buffers, it was necessary to use water and 80% aqueous EtOH, respectively, to dissolve these two drugs.

Heats of dilution and mixing were obtained by 20 to 30 injections of each drug into the two buffer solutions. After having first subtracted heats of dilution and mixing from each injection heat pulse, association constants (K_a values), enthalpy change ($\Delta H°$) and stoichiometry (n) were obtained by construction of binding isotherms by means of nonlinear least squares fitting of the titration data using Origin® software.

Determination of Soluble Haematin Concentration at pH 4.8

Saturated haematin solutions in 500 mM sodium acetate, pH 4.8, were prepared from a known quantity of haematin ($n = 3$) and stirred for 1 hr. The insoluble material was then collected by filtration on 0.2 μM Millipore® Type Eg Cellotate (cellulose acetate) membranes. Both this material and the filter papers were then stirred in 0.01 M NaOH to dissolve the solid haematin. After appropriate dilution in 0.01 M NaOH, haematin concentrations were determined at 380 nM using a standard curve. The concentration of soluble haematin in the pH 4.8 buffer was then calculated via mass balance.

RESULTS

Correlation between Inhibition of Haematin Polymerisation and Inhibition of Parasite Growth in Culture

We tested compounds against a strain highly susceptible to quinoline antimalarials, namely *P. falciparum* NF54. We compared these values with the IC_{50} values for compound inhibition of haematin polymerisation at pH 4.8, which approximates to the pH of the food vacuole in the parasite. The results, shown in Fig. 2, demonstrate that there is a good correlation between hematin polymerisation inhibition and parasite growth inhibition for quinoline-containing blood schizonticides ($r = 0.91$, $P = 0.002$). All the antimalarial blood schizonticides inhibited haematin polymerisation with an IC_{50} between 10 and 200 μM and inhibited parasite growth in culture against the NF54 strain with IC_{50} value between 2 and 25 nM. Primaquine, the 8-aminoquinoline used as a negative control because of its inactivity against blood stage parasite growth, was inactive in the haematin polymerisation assay ($IC_{50} > 2500$ μM).

Establishment of Conditions for Allowing a Direct Comparison of Drug-Haematin Binding and Inhibition of Haematin Polymerisation

The ideal situation would be to carry out both drug-haematin binding and haematin polymerisation experiments at pH 4.8 to 5.4, the pH of the parasite food vacuole [24, 25]. However, binding data at pH 6.0 and below could not be obtained due to the poor solubility of haematin. For example, the concentration of soluble haematin in saturated haematin solutions in 500 mM acetate buffer, pH 4.8 was 1.4 ± 0.2 μM, which was too low to obtain the

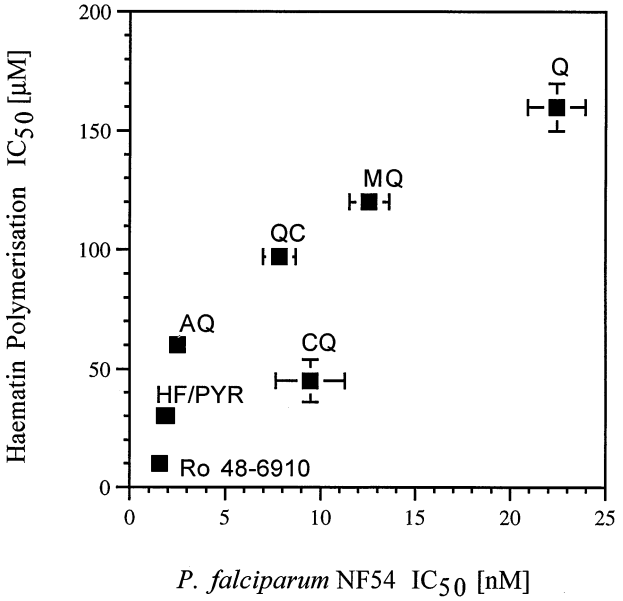

FIG. 2. Correlation of drug inhibition of haematin polymerisation induced by haemozoin at pH 4.8 and inhibition of parasite growth using the chloroquine-sensitive *P. falciparum* strain NF54. Values are given for halofantrine (HF), pyronaridine (PYR), amodiaquine (AQ), chloroquine (CQ), quinine (Q), quinacrine (QC), mefloquine (MQ) and the bisquinoline Ro 48-6910 and show the mean values (±SD) obtained from a minimum of three experiments. Where error bars are not visible, these fell within the boundaries of the square symbol. Halofantrine and pyronaridine occupy the same space in the curve.

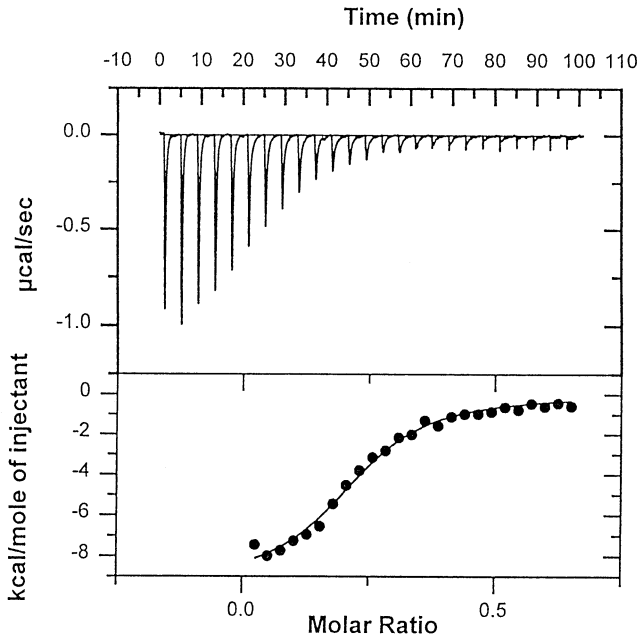

FIG. 4. Heats of binding and binding isotherm for the calorimetric titration of 0.05 M haematin with 2 μL injections of 1 mM chloroquine in 250 mM phosphate buffer, pH 6.5 at 37°.

requisite enthalpy data in ITC drug-haematin binding experiments. In a pH profile of haematin polymerisation (Fig. 3), activity was detected across a broad pH range, with maximal activity up to pH 6.0. Thereafter, activity fell to zero at pH 7.0, but 50% activity was still observed at pH 6.5. As haematin was fully soluble at pH 6.5, the comparative studies were carried out at this pH.

Drug-Haematin Binding

A representative titration curve and corresponding binding isotherm from a single ITC experiment carried out at pH 6.5 is shown in Fig. 4. The ITC data obtained for the series of quinoline-containing drugs at pH 6.5 are shown in Table 1. The results revealed that the bisquinoline Ro 46-6910

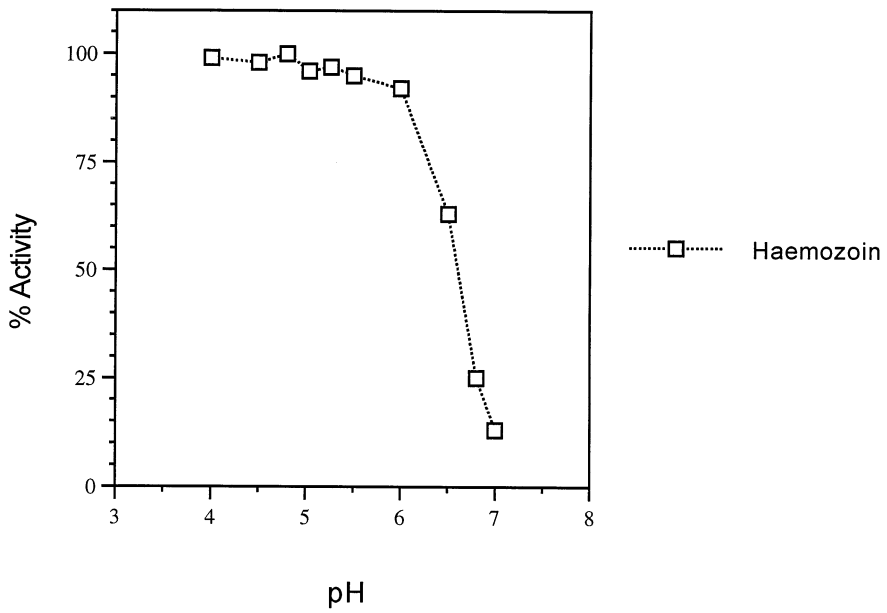

FIG. 3. The pH profile of haematin polymerisation. Assays up to pH 5 were carried out using 500 mM sodium acetate buffer. Assays above pH 5 were performed using 250 mM phosphate buffer.

TABLE 1. Stoichiometry and thermodynamics of drug-haematin binding at pH 6.5[d]

Drug[a]	n	$1/n$	K_a (10^5 M^{-1})	ΔG (kcal/mole)	ΔH (kcal/mole)	TΔS (kcal/mole)
Chloroquine	0.24 ± 0.03	4.2	4.0 ± 1.7	−7.87 ± 0.37	−10.1 ± 1.0	−2.23 ± 1.20
Quinacrine	0.23 ± 0.03	4.4	5.0 ± 0.5	−8.07 ± 0.06	−9.33 ± 0.20	−1.26 ± 0.24
Amodiaquine	0.26 ± 0.03	3.9	0.93 ± 0.30	−7.04 ± 0.16	−9.40 ± 0.71	−2.36 ± 0.87
Pyronaridine	0.15 ± 0.01	6.7	3.0 ± 0.24	−7.76 ± 0.05	−10.9 ± 0.4	−3.14 ± 0.40
Quinine	0.20 ± 0.02	5	0.21 ± 0.02	−6.12 ± 0.06	−13.4 ± 1.5	−7.28 ± 1.60
Mefloquine[b]	0.34 ± 0.07	2.9	0.12 ± 0.04	−5.77 ± 0.21	−4.50 ± 1.01	1.27 ± 1.21
Halofantrine[c]	0.86 ± 0.07	1.2	0.46 ± 0.07	−6.61 ± 0.10	−5.74 ± 0.20	0.873 ± 0.127
Primaquine	0.14 ± 0.01	7.1	0.16 ± 0.07	−5.90 ± 0.27	−9.00 ± 1.40	−3.10 ± 1.62
Ro 48-6910	0.054 ± 0.011	19	6.2 ± 1.9	−8.18 ± 0.21	−5.80 ± 0.32	2.83 ± 0.54

[a] Chloroquine, quinacrine, pyronaridine, amodiaquine, quinine, primaquine, Ro 48-6910 and haematin were dissolved in 250 mM phosphate buffer, pH 6.5. ITC experiments were conducted at 37°.

[b] Mefloquine and haematin were dissolved in distilled water and the pH was adjusted to 6.5.

[c] Halofantrine and haematin were dissolved in 80% aqueous ethanol.

[d] ITC data (not shown) were also obtained at pH 6.9, the cytosolic pH of both normal and malaria-infected red cells [26]. The thermodynamics of drug-haematin binding at the two pH values were very similar.

and chloroquine and its analogs, amodiaquine, quinacrine and pyronaridine, had drug:haematin association constants (K_a values) of 0.93 to 6.2 × 10^5 M^{-1}. In contrast, the two quinolinemethanols, quinine and mefloquine, and the structurally related halofantrine, bound significantly more weakly to haematin with association constants of 0.21, 0.12, and 0.46 × 10^5 M^{-1} respectively. Primaquine, which failed to inhibit haematin polymerisation, bound to haematin (K_a = 0.16 × 10^5 M^{-1}) in the same range as the quinolinemethanols.

Stoichiometry

Under our experimental conditions of haematin concentration and pH, haematin exists largely in the form of its μ-oxo dimer [26–28]. For chloroquine, quinacrine and amodiaquine, a $1/n$ value very close to 4 was obtained, which indicated a 1:2 drug:haematin μ-oxo dimer binding stoichiometry, consistent with a model proposed by Moreau *et al.* [29]. For pyronaridine and primaquine, a value of $1/n$ close to 6 indicates a 1:3 drug:haematin μ-oxo dimer binding stoichiometry. The uniquely low stoichiometry of the strongly binding bisquinoline, Ro 48-6910, (0.05; $1/n \approx 20$), suggests that a 1:10 drug:haematin μ-oxo dimer complex is formed. Interestingly, the two quinolinemethanols, quinine ($1/n \approx 5$) and mefloquine ($1/n \approx 3$), appear to bind an odd number of haematin molecules, indicating that they may bind to haematin monomers as well as μ-oxo dimers. However, one cannot easily compare the mefloquine and halofantrine stoichiometry data, as these results were obtained in water and 80% aqueous ethanol, respectively, and a significantly lower extent of haematin dimerization occurs in these solvents compared to buffered salt-containing media [30, 31].

Enthalpy and Entropy Contributions

Drug-haematin μ-oxo dimer binding was enthalpy-driven for all compounds. We attribute this binding enthalpy largely to π–π interactions (*vide infra*) between the drugs and haematin. For mefloquine, halofantrine, and Ro 48-6910, drug-haematin μ-oxo dimer binding was also entropy-driven. However, for chloroquine, quinacrine, amodiaquine, pyronaridine, quinine and primaquine, a loss of entropy was observed (Table 1). Binding entropies, whether favored or disfavored, are in part a function of changes in the hydrogen-bonded network of water [32] in the hydrophobic hydration shell of the drug-haematin μ-oxo dimer complexes vs. free drug and free haematin μ-oxo dimer. The entropic penalty (TΔS°) for quinacrine-haematin μ-oxo dimer binding was lowest among the 4-aminoquinolines. We hypothesize that this may have resulted from a greater number of favorable drug-haematin binding geometries accommodated by the larger acridine vs. quinoline heterocycle in quinacrine vs. chloroquine. We suggest that an analogous result was not obtained for pyronaridine vs. amodiaquine because of the offsetting steric inhibition to binding in the form of a second pyrrolidinomethyl substituent in pyronaridine. Even though the enthalpy (ΔH°) of drug-haematin binding for quinine was substantially greater than that of chloroquine, the entropic penalty (TΔS°) for this binding was sufficiently high to afford a lower free energy (ΔG°) of binding, a manifestation of enthalpy-entropy compensation [33].

Inhibition of Haematin Polymerisation at pH 4.8 vs. pH 6.5

A comparison of drug-mediated inhibition of haematin polymerisation at pH 4.8 and pH 6.5 is given in Table 2. All compounds, with the exception of the 8-aminoquinoline, primaquine, inhibited haematin polymerisation at both pH values. Interestingly, all compounds thought to bind the μ-oxo dimer (Table 1), with the exception of amodiaquine, exhibited a lower IC_{50} at the higher pH. This is internally consistent with the concept that these compounds inhibit haematin polymerisation by binding to haematin μ-oxo dimer, as at the higher pH the dimerisa-

TABLE 2. Comparison of drug inhibition of haematin polymerisation at pH 4.8 and pH 6.5

	Sodium acetate pH 4.8	Sodium phosphate pH 6.5
Compound	Haemozoin-initiated [μM]	
Chloroquine	45 ± 9	11 ± 1
Amodiaquine	60 ± 1	60 ± 1
Quinine	160 ± 10	25 ± 1
Mefloquine	120 ± 1	60 ± 1
Halofantrine	30 ± 2	50 ± 1
Pyronaridine	30 ± 2	15 ± 1
Quinacrine	97 ± 5	10 ± 0.6
Ro 48-6910	10 ± 1	9 ± 1.5
Primaquine	>2500	>2500

tion constant is increased 30-fold [26]. The lack of a reduction in the IC_{50} for amodiaquine may relate to a variation in its ionisation state between the two pH values ($pK_a1 = 7.1$) [34]. Only halofantrine demonstrated an increased IC_{50} at the higher pH value, and it is interesting to note that this was the only compound among those tested which stoichiometrically appears to bind to haematin monomer under the conditions tested (Table 1).

Correlation between Drug-Haematin Binding and Drug Inhibition of Haematin Polymerisation

A correlation between the IC_{50} for drug-mediated inhibition of haematin polymerisation and the drug-haematin association constant was found ($r = -0.82$, $P = 0.01$) at pH 6.5 (Fig. 5). This indicates that inhibition of polymerisation is mediated by the binding of the drug to haematin, probably as the μ-oxo dimer.

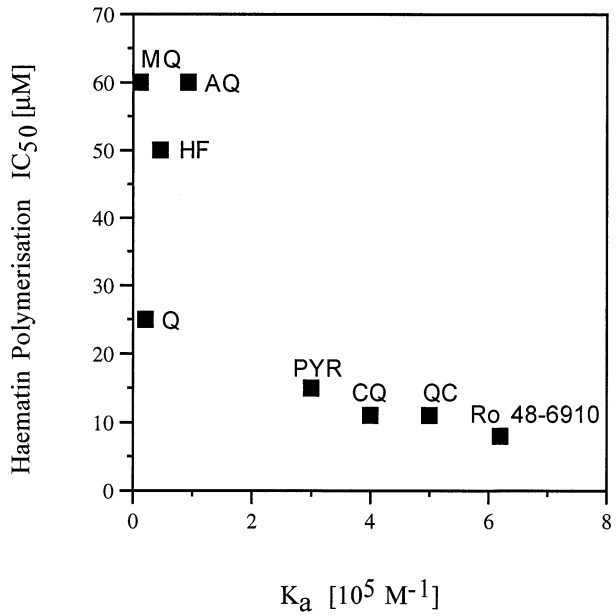

FIG. 5. Correlation between inhibition of haematin polymerisation induced by haemozoin at pH 6.5 and the drug-haematin binding constant determined at pH 6.5 for the same eight drugs listed in Fig. 1. Note that the error bars fall within the boundaries of the square symbols.

DISCUSSION

The results presented here build on our earlier hypothesis that haematin polymerisation is not enzyme-dependent and that quinoline-containing antimalarials most likely exert their activity through direct interaction with haematin [10], as previously proposed by Fitch [14].

Correlation between Haematin Binding, Inhibition of Haematin Polymerisation, and Inhibition of Parasite Growth in Culture

The strong correlation observed between the ability of quinoline blood schizonticides to inhibit haematin polymerisation and to inhibit malarial parasite growth in culture supports the concept that these compounds exert their antimalarial activity through inhibition of haematin polymerisation. The fact that primaquine, a quinoline inactive against blood stage malaria parasites, failed to inhibit haematin polymerisation served as an important negative control to support this hypothesis.

Others have observed a trend linking inhibition of haematin polymerisation and inhibition of parasite growth within specific quinoline subclasses, such as quinine and its stereoisomers [8] and some bisquinolines [35]. In one of the studies [8], no correlation was observed between different quinoline subclasses (e.g., chloroquine vs. quinine). We believe this is due to the fact that in that study trophozoite lysate was used to promote haematin polymerisation, whereas in the present study we have promoted haematin polymerisation using purified haemozoin. In other experiments, we have demonstrated that trophozoite lysate is not an optimal substance for monitoring drug inhibition of haematin polymerisation, due to nonspecific binding of compounds to cytosolic proteins [13].

The hypothesis that pyronaridine exerts its antimalarial activity through inhibition of haematin polymerisation contradicts a previous notion that it acts by inhibition of topoisomerase II [36]. However, this latter theory concerning pyronaridine always had a major weakness, namely that the compound inhibited parasite growth in the low nM range, but only inhibited topoisomerase II in the μM range [36, 37]. It was postulated that a mechanism existed to concentrate the drug into the nucleus of the parasite but no rationale for this was put forward. We believe it is far more likely that pyronaridine, a weak base, concentrates in the food vacuole where it inhibits haematin polymerisation. Recent work in our laboratory demonstrating that pyronaridine is inactive against liver stage parasites *in vitro*, which have active topoisomerase II but do not produce haemozoin, reinforces this conclusion (H. Matile, unpublished results).

The conclusion that quinolines inhibit parasite growth by inhibition of haematin polymerisation requires that they

concentrate in the parasitic food vacuole to levels greater than the micromolar levels required to inhibit haematin polymerisation *in vitro*. It has been demonstrated that accumulation of chloroquine [38] and quinidine [39] occurs in this organelle and mM levels of chloroquine [40] and amodiaquine [34] have been measured in infected erythrocytes, presumably within the food vacuole. It is therefore likely that other quinolines also concentrate in this way, though this has been questioned in the case of mefloquine [41].

Haematin Association Constants Indicate Inhibition of Haematin Polymerisation is Mediated by Binding to Haematin

The measurement of drug:haematin association constants by ITC allowed a more detailed assessment of the molecular mechanisms by which haematin polymerisation might be inhibited. With the exception of a $K_d^{-1} = K_a = 2.9 \times 10^8\ M^{-1}$ (pH 7.4) for a 1:2 chloroquine-haematin complex as measured by equilibrium dialysis [13], these data represent the first direct determination of K_a's for antimalarial drug-haematin binding. Our ITC-derived chloroquine:haematin μ-oxo dimer K_a values of 4.0 and $4.5 \times 10^5\ M^{-1}$ at pH 6.5 and 6.9, respectively, were some 700-fold lower than the K_a (pH 7.4) reported by Chou *et al.* [13]. We attribute this disparity in the chloroquine-haematin binding data to problems inherent in the equilibrium dialysis method such as surface adsorption of haematin and chloroquine. Our direct measurements showing weaker drug-haematin binding affinities for the quinolinemethanols compared to the 4-aminoquinolines are qualitatively in agreement with earlier data obtained *via* indirect methods [14, 42].

The correlation observed between the binding constant and inhibition of haematin polymerisation for the quinoline-containing compounds indicates that the strength of haematin binding is an important parameter in determining the potency of a compound to inhibit haematin polymerisation and parasite growth. However, it is significant that primaquine had no effect on haematin polymerisation, but did bind to haematin μ-oxo dimer with an affinity between that of mefloquine and quinine. Indirect binding data also indicate that primaquine binds to haematin [14, 43]. This suggests that other properties of drug-haematin complexes such as shape, size, or lipophilicity [44], rather than association constants alone, may also play a role in the inhibition of haematin polymerisation.

Stoichiometry of Compound Binding to Haematin Supports Significance of μ-Oxo Dimer

The ITC-derived data clearly demonstrate a 1:4 stoichiometry for an enthalpy-driven drug:haematin binding for chloroquine, quinacrine and amodiaquine. This suggests that these drugs bind to two haematin μ-oxo dimers in a cofacial π–π sandwich-type complex, consistent with a model proposed by Moreau *et al.* [29]. This binding model has also been confirmed in other studies in which NMR [45–48] and Mössbauer spectroscopy [49, 50] were used to ascertain the geometry of binding.

NMR studies show that quinine interacts at the porphyrin periphery, in contrast to chloroquine which binds at the porphyrin center with greater π–π overlap than is possible with quinine [46]. The observed binding stoichiometry of $1/n = 5$ indicates that quinine binds to both one haematin monomer and two haematin μ-oxo dimers. This is consistent with data from NMR experiments [44, 47] suggesting that iron coordination to the 9-hydroxyl group of quinine occurs in addition to π–π stacking. The higher enthalpy and greater loss of entropy evident in quinine:haematin binding, compared to chloroquine, quinacrine and amodiaquine, is also consistent with this proposed iron coordination. In contrast, iron coordination to the three nitrogen atoms of chloroquine was not observed [46]. This is consistent with our binding data for chloroquine, as such iron coordination *trans* to the Fe-O-Fe linkage in haematin μ-oxo dimer would be expected to be extremely weak because the iron is displaced some 0.5 Å out of the porphyrin plane toward the bridging oxygen atom [51].

A large variation in binding stoichiometries was observed between different quinoline compounds. Mefloquine, pyronaridine and the bisquinoline Ro 48-6910 all varied significantly from chloroquine and quinine, but each bound to one or more haematin μ-oxo dimers. Further studies, including NMR, will be required to more fully interpret these data.

The Equilibrium between Haematin Monomer and Haematin μ-Oxo Dimer Could Affect Haematin Polymerisation

It is known that the equilibrium between haematin monomer and haematin μ-oxo dimer strongly favors the dimer ($K_{obs} = 4.7 \times 10^7\ M^{-1}$ at pH 7.0), but that this is reduced at lower pH values (K_{obs} estimated at $2.8 \times 10^5\ M^{-1}$ at pH 4.8) [26]. Also, monomeric haematin is required for incorporation into the growing haemozoin chain, as in haematin μ-oxo dimer the two iron atoms are coordinated to the bridging oxygen atom and are unavailable for coordination with the propionate side-chains of the adjacent haematin moiety. The pH profile of haematin polymerisation is consistent with this. Optimal haematin polymerisation occurs at low pH when the equilibrium shifts more to the haematin monomer. The 170-fold increase in the relative concentration of haematin μ-oxo dimer between pH 4.8 and pH 7.0 might explain the cessation of haematin polymerisation at neutral pH.

It is feasible that the binding of the quinoline antimalarials stabilises the μ-oxo dimer relative to the monomer, shifting the dimerisation equilibrium to the right and reducing the amount of haematin monomer available for incorporation into the growing haemozoin. The lower haematin polymerisation IC_{50} values obtained for antima-

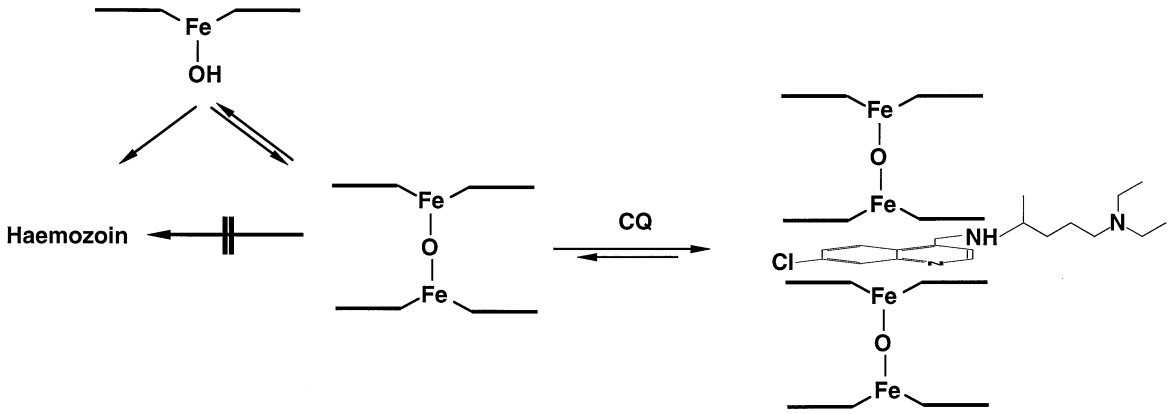

FIG. 6. Proposed model illustrating the mechanism by which chloroquine may inhibit haematin polymerisation by shifting haematin/μ-oxo dimer equilibrium to the right. The chloroquine-μ-oxo dimer complex may 'cap' the growing haemozoin chain, preventing further extension of the haemozoin polymer.

larial quinolines at pH 6.5 compared to pH 4.8 support this concept. Building on a report that chloroquine is integrated into haemozoin in the presence of haematin [39], we postulate that, in addition to shifting the dimerisation equilibrium, chloroquine may inhibit haematin polymerisation by 'capping' the growing β-haematin strand with a stabilised 1:2 chloroquine:haematin μ-oxo dimer complex. A scheme outlining the mechanism by which chloroquine may affect the μ-oxo dimer equilibrium is presented in Fig. 6.

This molecular interpretation effectively integrates the chloroquine-ferriprotoporphyrin IX (hematin) hypothesis [14, 52] with the observed inhibition of haematin polymerisation by chloroquine [8, 10, 53, 54]. However, it is important to realise that formation of 1:2 drug:haematin μ-oxo dimer complexes probably mediate an antimalarial effect by perturbing important parasite biochemistry distinct from haemozoin production, the consequence of which is only indirectly measured in parasite growth inhibition assays [55]. One such example is the positive correlation ($r = 0.938$, $P = 0.062$) (data not shown) between drug:haematin μ-oxo dimer binding constants (K_a's) and IC_{50} values for drug-mediated inhibition of haemoglobin denaturation in the presence of proteinase K for chloroquine, amodiaquine, quinine and mefloquine [56]. In any event, even small perturbations mediated by antimalarial quinolines in the parasite haematin detoxification process may have destructive consequences for the parasite [57–59].

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