Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials

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The malaria parasite feeds by degrading haemoglobin in an acidic food vacuole, producing free haem moieties as a by-product. The haem in oxyhaemoglobin is oxidized from the Fe(II) state to the Fe(III) state with the consequent production of an equimolar concentration of H_2O_2. We have analysed the fate of haem molecules in *Plasmodium falciparum*-infected erythrocytes and have found that only about one third of the haem is polymerized to form haemozoin. The remainder appears to be degraded by a non-enzymic process which leads to an accumulation of iron in the parasite. A possible route for degradation of the haem is by reacting with H_2O_2, and we show that, under conditions designed to resemble those found in the food vacuole, i.e., at pH 5.2 in the presence of protein, free haem undergoes rapid peroxidative decomposition. Chloroquine and quinacrine are shown to be efficient inhibitors of the peroxidative destruction of haem, while epiquinine, a quinoline compound with very low antimalarial activity, has little inhibitory effect. We also show that chloroquine enhances the association of haem with membranes, while epiquinine inhibits this association, and that treatment of parasitized erythrocytes with chloroquine leads to a build-up of membrane-associated haem in the parasite. We suggest that chloroquine exerts its antimalarial activity by causing a build-up of toxic membrane-associated haem molecules that eventually destroy the integrity of the malaria parasite. We have further shown that resistance-modulating compounds, such as chlorpromazine, interact with haem and efficiently inhibit its degradation. This may explain the weak antimalarial activities of these compounds.

Key words: chlorpromazine, drug resistance, malaria, *Plasmodium falciparum*.

INTRODUCTION

Chloroquine has been used to treat malaria for nearly 50 years, however, resistance to this drug has now increased to the point where it is virtually useless in many malarious regions (see [1] for review). It is essential that the mode of action of chloroquine is understood so that novel antimalarial drugs can be developed. The precise details of the mechanism of action of chloroquine are the subject of controversy, however, it has been assumed for some time that the quinoline antimalarials act by interfering with parasite feeding. The parasite feeds by degrading haemoglobin to amino acids in an acidic food vacuole, producing free haem, ferriprotoporphyrin IX, as a toxic by-product. The mature human erythrocyte contains 310–350 mg/ml haemoglobin, which equates to a haem concentration of about 20 mM. If the majority of the haemoglobin is digested during intraerythrocytic growth, and the released haem is allowed to accumulate within the food vacuole, which occupies 3–5% of the volume of the infected erythrocyte [2], then the vacuolar haem level could reach approx. 0.5 M. Haem inhibits enzymes and destabilizes membranes (see [1] for a discussion of the deleterious effects of haem) so that the parasite is faced with, what is essentially, a massive toxic waste problem. The parasite lacks haem oxygenase [3] and it has been proposed that it disposes of the haem, at least in part, by polymerizing it into long insoluble polymers of haemozoin [4]. The polymerization process is autocatalytic [5] and has been shown to be relatively inefficient *in vitro* [6]. This suggests that the parasite has an alternative means of disposing of toxic haem moieties.

A second toxic insult derives from the fact that when oxyhaemoglobin is released into the food vacuole of the parasite at pH 5.2, it is rapidly converted to methaemoglobin [7], with the concomitant reaction of the released electron with oxygen to produce a superoxide anion [8]. At pH 5, the superoxide anion will spontaneously dismutate to H_2O_2 (Scheme 1A). The parasite possesses the oxidant defence enzymes, superoxide dismutase and glutathione peroxidase [9,10], but these are probably located in the parasite cytoplasm. The food vacuole environment will be partly protected from oxidative insult by enzymes adopted from the erythrocyte when the parasite engulfs its meal of haemoglobin, however, these host-derived enzymes may be rapidly degraded by vacuolar proteases, leaving the proteins and lipids of the food vacuole susceptible to oxidative damage. Thus the degradation of haemoglobin produces a heavy oxidative burden which leaves the parasite very susceptible to further oxidative stress [11].

It has been known for some time that haem can react with H_2O_2, displaying both catalase-like and peroxidase-like activities that regenerate the haem molecule. In addition, haem can undergo peroxidative reactions that destroy the porphyrin ring [12,13]. The enzyme-like activities of haem have been extensively studied as models for haem-containing enzymes [14,15]. We have re-examined the catalytic activities of haem, under conditions designed to resemble those found in the food vacuole, and have found that they could contribute significantly to the degradation of haem and to the breakdown of reactive oxygen species. We show that chloroquine and other quinoline antimalarials are very efficient inhibitors of the degradation of both haem and

Abbreviations used: 8AC6, *N*,*N*′-bis-(4-{[4-(diethylamino)-1-methylbutyl]amino}quinolin-8-yl)adipamide; OPD, *o*-phenylenediamine.

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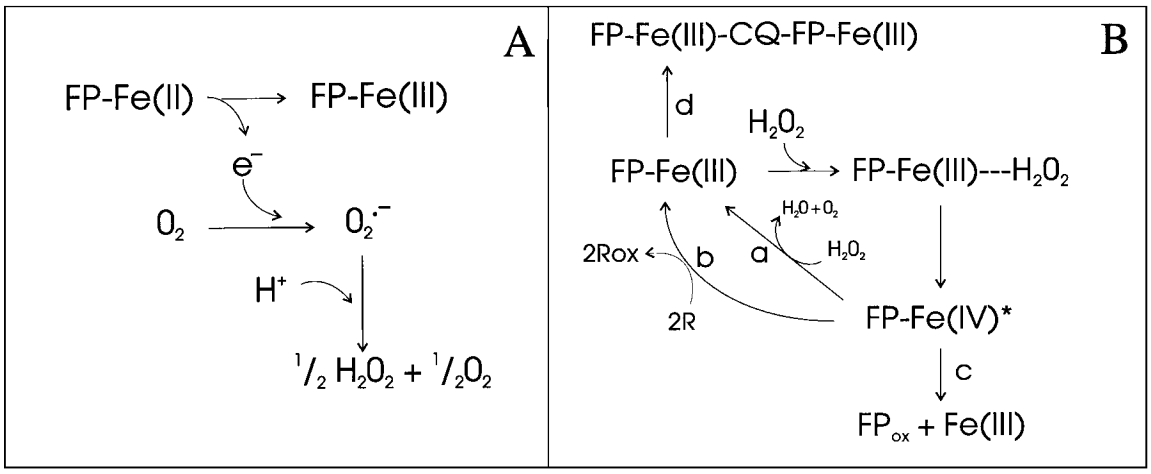

Scheme 1 (A) Generation of H_2O_2 in the food vacuole and (B) putative mechanism for the catalase- and peroxidase-like activities of haem

(**A**) In the acid conditions of the food vacuole, haem, ferriprotoporphyrin IX (FP), is rapidly oxidized to the Fe(III) state with the transfer of an electron to oxygen to generate a superoxide radical. The superoxide is spontaneously converted to H_2O_2. (**B**) H_2O_2 oxidizes the porphyrin ring of haem to produce an activated intermediate [FP-Fe(IV)*] that can participate in a catalase-like reaction (reaction a) or peroxidase-like reactions (reaction b). The substrate 'R' may be glutathione, protein or lipid. In addition, peroxidative cleavage reactions can destroy the porphyrin ring (reaction c). Chloroquine forms a tight complex with haem (reaction d) which interferes with these reactions. See reference [15] for the proposed molecular identity of the ferryl intermediate, FP-Fe(IV)*.

H_2O_2 and that chloroquine enhances the association of haem with membranes both in model systems and in malaria-infected erythrocytes. We suggest that these phenomena underlie the mechanism of action of chloroquine.

MATERIALS AND METHODS

Materials

Chloroquine, chlorpromazine, verapamil and desipramine were obtained from Sigma, U.S.A. Mefloquine was kindly donated by Hoffmann-La Roche, Switzerland. Epiquinine was supplied by B. Sharpless, Scripps Research Institute, La Jolla, CA, U.S.A. *N*,*N*′-Bis-(4-{[4-(diethylamino)-1-methylbutyl]amino}quinolin-8-yl)adipamide (8AC6) was synthesized as described previously [16].

Analysis of the haemoglobin content and the total haem and iron contents of *Plasmodium falciparum*-infected and uninfected erythrocytes

A chloroquine-sensitive strain of *P. falciparum* (3D7) was continuously cultured, as described previously [17], under an atmosphere of 1% O_2, 5% CO_2, 94% N_2. The level of oxygenation of the haemoglobin in erythrocytes maintained under this low oxygen tension was determined spectrophotometrically as described by Riggs [18]. Mature parasite-infected erythrocytes were isolated from synchronous cultures using differential centrifugation through a sorbitol/Percoll gradient, as described by Aley et al. [19]. The parasitized erythrocytes were harvested and gradually readjusted to isotonicity. To measure total haem content, aliquots (250 μl) containing equal numbers of either parasitized erythrocytes or uninfected erythrocytes (approx. 10^7 cells) were mixed with 250 μl of 10% (w/v) SDS and 500 μl of 1 M NaOH, and then sonicated for 10 min to denature proteins and to dissolve all forms of haem. Following an incubation for 2 h at room temperature, the concentration of haem was determined spectrophotometrically at 404 nm, assuming a molar absorption coefficient of 9.08×10^4 $M^{-1} \cdot cm^{-1}$ [20]. The level of iron was determined using a modification of the procedure of Carter [21]. Parasite samples were solubilized in 5% (w/v) SDS, then mixed with 100 μl of trichloroacetic acid and 200 μl of H_2O_2, and sonicated for 30 min. The soluble material was incubated for 5 min with ascorbic acid (1 ml, 0.02% in 0.1 M HCl), then mixed with ammonium acetate [0.8 ml, 10% (w/v)] and ferrozine [200 μl containing ferrozine (0.6 mg) and neocuproine (0.6 mg) in water], and assayed spectrophotometrically at 562 nm, assuming a molar absorption coefficient of 2.79×10^4 $M^{-1} \cdot cm^{-1}$. For assays of haemoglobin levels, aliquots (25 μl) containing equal numbers of either parasitized erythrocytes or uninfected erythrocytes (approx. 10^7 cells) were extensively vortexed in 500 μl of 5 mM sodium phosphate, pH 8, and centrifuged at 27000 ***g*** for 15 min. The level of haemoglobin in the supernatant was determined as the cyanomethaemoglobin form [18].

Analysis of membrane-associated haem levels in erythrocyte ghosts and in *P. falciparum*-infected erythrocytes

Haemoglobin-free erythrocyte membranes were prepared by hypotonic lysis. White ghosts suspended in 20 mM sodium phosphate, pH 7, were mixed with haem (100 μM) and incubated at 37 °C for 30 min. The membranes were collected by centrifugation at 100000 ***g*** and the level of haem in the pellet was determined spectrophotometrically after solubilization in SDS or using the ferrozine method [21] to determine the level of iron. Levels of membrane-associated haem in parasitized erythrocytes were determined using a modification of the procedure of Chou and Fitch [22]. Synchronized cultures (30 ml, 15–20% trophozoite stages, 4% haematocrit) of the 3D7 strain of *P. falciparum* were incubated for 4 h in the presence of increasing concentrations of chloroquine or chlorpromazine. Mature parasite-infected erythrocytes were collected on a sorbitol/Percoll gradient and aliquots (50 μl) containing equal numbers of either parasitized erythrocytes or uninfected erythrocytes (approximately 10^8 cells) were mixed with 200 μl of 5 mM sodium phosphate, pH 7, 4 °C, and sonicated for 10 min to disrupt the membranes and release any haemoglobin. The membranes were pelleted at 27000 ***g***. The washing procedure was repeated and membrane

pellets were resuspended in 5 % (w/v) SDS. Following sonication for 10 min, the insoluble haemozoin was pelleted and the concentration of SDS-soluble haem was determined spectrophotometrically.

Preparation of β-haematin

β-Haematin was prepared as described by Slater et al. [23], then washed four times with 1 % (w/v) SDS and a further two times with distilled water. Fourier-transform IR spectroscopy was used to confirm the formation of the β-haematin polymer. For all experiments, β-haematin was sonicated in the appropriate buffer to form a fine suspension.

Assays of enzyme-like activities of haem

Stock solutions of ferriprotoporphyrin IX were prepared daily in NaOH. The peroxidative decomposition of haem was monitored by measuring the decrease in absorption at the Soret band (360 nm). Aliquots (0.2 ml or 1 ml) of 200 mM sodium acetate, pH 5.2, 1 mg/ml BSA and 15 μM haem were equilibrated at 20 °C. The reaction was initiated by the addition of H_2O_2 and followed spectrophotometrically. For studies of sonicated β-haematin, the sample was dissolved in 50 mM NaOH prior to spectrophotometric analysis. The catalase-like activity of haem was measured by monitoring oxygen evolution using a Clark oxygen electrode. The electrode chamber was filled with 4 ml of haem [or sonicated β-haematin or bovine liver catalase (Sigma)] in 200 mM sodium acetate, pH 5.2, with or without 1 mg/ml BSA, and equilibrated at 37 °C. The reaction was started by the addition of H_2O_2 to a final concentration of 5 mM. The peroxidase-like activity of haem was monitored by following the oxidation of *o*-phenylenediamine (OPD, Sigma). Aliquots (200 μl) of 5.5 mM OPD, 25 mM sodium citrate, pH 5.2, 100 mM NaCl, 5 μM haem with or without 1 mg/ml BSA were added to the wells of a 96-well plate. The reaction was started by the addition of H_2O_2 (2 mM). Plates were incubated at 20 °C or 37 °C for 1 h and the oxidation of OPD was measured at 490 nm after the addition of 50 μl of 3 M HCl. Alternatively, reactions were carried out on a larger scale with 1 ml aliquots of 5 μM haem or the equivalent concentration of sonicated β-haematin. Samples were centrifuged before assessment of OPD oxidation. For inhibition studies, chloroquine, epiquinine, quinacrine, chlorpromazine, verapamil and desipramine were added from stocks in water. 8AC6 and mefloquine were added from stocks in 10 % (v/v) DMSO in water. Mixtures of drugs and haem were made prior to addition to the haem activity assays. The interaction of chlorpromazine with haem was examined by monitoring the change in the absorption profile of haem upon the addition of chlorpromazine (chlorpromazine/haem, molar ratio of 2:1).

RESULTS AND DISCUSSION

The malaria parasite feeds by degrading haemoglobin with the production of free haem and reactive oxygen species as toxic by-products. To estimate the extent of haem production during intraerythrocytic growth, we have determined the level of haemoglobin in control and schizont-infected erythrocytes. We find that mature-stage-parasitized erythrocytes contain only 25 % of the haemoglobin levels of control erythrocytes (Table 1), indicating that most of the haemoglobin is degraded, presumably with the release of the haem moieties into the food vacuole. It has been assumed that the released haem is detoxified by polymerization into polymers of haemozoin [4], however, when a haem balance sheet, accounting for the fate of haem moieties liberated during haemoglobin catabolism in *Plasmodium berghei* was prepared [24], it was found that only 20–30 % of the haem was sequestered into haemozoin. A similar balance sheet has not previously been prepared for *P. falciparum*, however, haem polymerization has been shown to be a relatively inefficient process *in vitro* [6]. We therefore have sought to determine the efficiency of conversion of haem into its polymeric form *in vivo*. To do this, we have made an analysis of the total haem content of equal numbers of uninfected erythrocytes and erythrocytes infected with mature stages of *P. falciparum*. The haem present as either haemoglobin, haemozoin or free or membrane-bound haem was released by disruption of the cells in the presence of SDS and NaOH. If all of the haem released during haemoglobin digestion is converted into haemozoin, then the total haem content should remain unchanged. However, in three separate experiments, we found that schizont-infected erythrocytes contain only about half as much haem as uninfected erythrocytes (Table 1). These data indicate that of the 75 % of the haemoglobin molecules that are digested, only about one third of the released haem molecules are polymerized to form haemozoin, while the remainder are disposed of by an alternative mechanism.

Table 1 Fate of haem in malaria-infected erythrocytes

Equal numbers of isolated schizont-infected and uninfected erythrocytes were assayed for haemoglobin levels or solubilized in SDS/NaOH and assayed spectrophotometrically for total haem and total iron. Membrane-associated haem is defined as the SDS-soluble haem that remains associated with cells after lysis to remove haemoglobin and any water-soluble haem moieties. Values are the mean ± S.D. from three separate experiments.

	Uninfected erythrocytes (nmol/10^7 cells)	Schizont-infected erythrocytes (nmol/10^7 cells)
Haemoglobin	20 ± 2	5 ± 1
Total haem	21 ± 1	9 ± 2
Total iron	20.0 ± 0.5	19.3 ± 1.5
Membrane-associated haem	0.04 ± 0.01	0.40 ± 0.05

The pathway for removal of the haem molecules could involve simple diffusion out of the infected erythrocyte or alternatively could involve some degradation process. To investigate the fate of the 'missing' haem molecules, we have analysed total iron levels in uninfected and schizont-infected erythrocytes. If haem is lost by diffusion into the surrounding medium, the total iron content should also decrease. By contrast, in three separate experiments performed in triplicate we found equal levels of iron in uninfected erythrocytes and schizont-infected erythrocytes (Table 1). This indicates that the porphyrin ring of the haem molecules is destroyed at a site within the parasite (presumably within the food vacuole) with the consequent release of the iron moieties.

Haem undergoes peroxidative decomposition

The malaria-infected erythrocyte appears to lack the enzyme, haem oxygenase, that is used by other organisms for the enzymic destruction of haem [3]. We therefore explored the possibility that the haem molecules might be degraded by a non-enzymic route. An interesting observation from the early work of Brown et al. [25] was that haem can react with H_2O_2 to bring about its own peroxidative decomposition (Scheme 1B, reaction c). It is possible that peroxidative destruction of haem occurs under the conditions of the food vacuole as H_2O_2 is produced during haemoglobin degradation (Scheme 1A). We have exam-

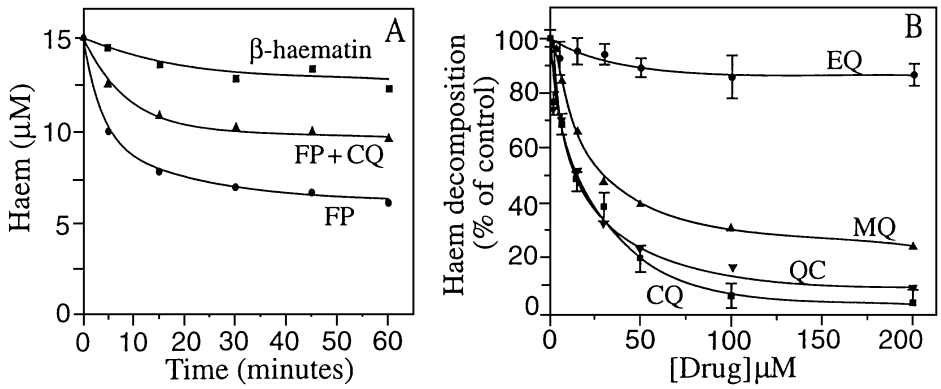

Figure 1 Peroxidative decomposition of haem

(**A**) Samples of haem (FP; 15 μM), sonicated β-haematin (15 μM) or a haem/chloroquine mixture (15 μM haem/30 μM chloroquine) were incubated at 20 °C, in the presence of 200 mM sodium acetate, pH 5.2, 1 mg/ml BSA. The reaction was initiated by the addition of H_2O_2 (2 mM) and followed spectrophotometrically at 360 nm. (**B**) Inhibition of the peroxidative decomposition of haem was monitored for a sample containing haem (15 μM) in the presence of increasing concentrations of the inhibitory drug. Values are the mean $\pm$ S.D. from triplicate measurements in a typical experiment. CQ, chloroquine; EQ, epiquinine; MQ, mefloquine; QC, quinacrine.

ined the destruction of haem under conditions designed to resemble those of the food vacuole. The milieu of the food vacuole is likely to be rich in intact and partially degraded proteins as well as the haem-binding proteins, histidine-rich proteins I and II [26]. In an effort to mimic these conditions, all experiments examining the peroxidative decomposition of haem were carried out in the presence of BSA, which binds haem via hydrophobic interaction sites [27]. Under the conditions of our experiments, we found that haem is rapidly decomposed, as monitored by a decrease in the absorbance of the Soret band (Figure 1A). The rate of destruction of haem was slightly lower at pH 5.2 than at pH 7.4 (results not shown), however, the decomposition rate was still quite rapid under the presumed conditions of the food vacuole (50 % decomposition in 20 min). In agreement with a previous report [25], we found that the rate of peroxidative destruction of haem decreased in an almost linear fashion with decreasing concentrations of H_2O_2 and increased with increasing haem concentrations. For example, at an equimolar ratio of H_2O_2 and haem (100 μM each), 20 % of the haem was decomposed in a 30 min time period (results not shown). The rate of destruction decreased slightly with increasing ionic strength over the range examined (50–500 mM sodium acetate; results not shown). In preliminary experiments, we have shown that, in the presence of recombinant histidine-rich protein II, haem degradation occurs at a rate similar to that observed in the presence of BSA (G. Kocak, P. Loria, and L. Tilley, unpublished work), again suggesting that the decomposition of haem observed in these *in vitro* studies could occur under the conditions of the food vacuole. These data suggest that peroxidative degradation of haem may account for the fate of a proportion of the haem that is 'lost' from parasitized erythrocytes.

The concentrations of haem and H_2O_2 in the food vacuole are difficult to estimate, however, when the contents of an endocytic vesicle are released into the food vacuole at pH 5.2, any oxyhaemoglobin will immediately be converted to methaemoglobin with the production of an equimolar level of H_2O_2 [7,8] (Scheme 1A). We found that even under the reduced oxygen conditions used for the culture of malaria parasites, more than 90 % of the haemoglobin was maintained in the oxygenated state (results not shown). Thus, upon oxidation of the oxyhaemoglobin, the local concentration of H_2O_2 in the food vacuole could reach millimolar levels. The haem moieties will be released more slowly as the globin is degraded by proteases [28], however, haem levels could also gradually build up to millimolar levels or higher. We have measured membrane-associated haem in uninfected and schizont-infected erythrocytes (Table 1). Free haem in the food vacuole is likely to equilibrate between lipid bilayers and haem-binding proteins [26,29], so an estimate of membrane-associated haem gives some indication of the level of free haem in the cell. Trophozoite-infected erythrocytes contain 0.40 ± 0.05 nmol of haem per 10^7 cells. The location of this haem is not known, but as very little haem appears to escape the parasite (as judged by the total iron levels), much of the haem may be associated with membranes within the parasite. If even half of this amount of haem in located in the food vacuole, which represents less than 5 % of the total volume of the infected erythrocytes [30], a local haem concentration of a few millimolar could be reached. A concentration of haem in the millimolar range is sufficient to cause substantial membrane lysis and peroxidative damage, although the presence of specialized haem-binding proteins in the food vacuole environment may protect the food vacuole membrane from the lytic effects of the haem [31]. None the less, the data suggest that the parasite is living on a 'knife edge' whereby its mechanisms for detoxifying haem may only be just sufficient to prevent the toxic effects of this metabolic waste-product.

The peroxidative degradation of haem is thought to involve a reaction with H_2O_2 to form a ferryl intermediate [14,25] (Scheme 1B, reaction c). Transfer of electrons within the Fe(IV) intermediate of haem results in the opening of the porphyrin ring (Scheme 1B, reaction c). This pathway is similar to that catalysed by haem oxygenase except that the billirubin intermediate is rapidly converted to colourless dipyrrolic fragments [25]. Chelation of the haem moiety has been shown to prevent the formation of the ferryl intermediate [12,14] and would be expected to decrease the rate of haem destruction. The polymerization of haem molecules to form β-haematin (the form of haem in haemozoin) involves the formation of a non-covalent co-ordination complex with the ferric iron of each haem moiety chelated onto a carboxy side chain of the adjacent moiety [22,32]. We found that preparations of β-haematin underwent peroxidative decomposition at a much slower rate than unpolymerized haem (Figure 1A). Thus, once the haem is converted to haemozoin, it is probably protected from destruction.

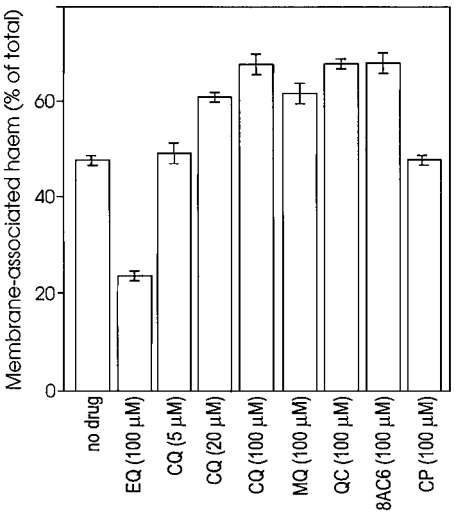

Figure 2 Effect of antimalarial drugs on the level of association of haem with a model membrane system

Erythrocyte ghosts were incubated with 100 μM haem in the presence or absence of antimalarial drugs. The amount of haem associated with the membrane pellet was assayed as iron using the ferrozine method [21]. Values are the mean $\pm$ S.D. from triplicate measurements in a typical experiment. CP, chlorpromazine; other abbreviations are as in the legend to Figure 1.

Table 2 Effect on antimalarial drugs on the level of membrane-associated haem in malaria-infected erythrocytes

Cultures of synchronized trophozoite-infected erythrocytes (3D7 strain) were incubated for 4 h in the presence of increasing concentrations of drug prior to isolation of the parasitized erythrocytes and determination of the level of membrane-associated haem. Values are the mean $\pm$ S.D. from two separate experiments.

Treatment	Membrane-associated haem (nmol/10^7 cells)
No drug	0.40 ± 0.05
Chloroquine (1 μM)	0.46 ± 0.02
Chloroquine (10 μM)	0.69 ± 0.03
Chlorpromazine (100 μM)	0.43 ± 0.02

Antimalarial drugs inhibit the peroxidative decomposition of haem and enhance the binding of haem to membranes

Chloroquine forms a complex with haem with a 1 : 2 stoichiometry [33,34] (Scheme 1B, reaction d) and an estimated affinity ranging from 10^{-6} M to 10^{-9} M depending on the conditions of analysis [33,35]. The interaction of chloroquine with haem has previously been shown to inhibit haem polymerization [4–6]. We have therefore examined the ability of chloroquine to inhibit the peroxidative decomposition of haem under conditions resembling those of the food vacuole. At a concentration of about 15 μM, chloroquine caused approx. 50 % inhibition of the peroxidative destruction of haem (Figure 1B). This concentration is well within the millimolar concentrations of chloroquine that are thought to be present in the food vacuole [36]. It is therefore likely that chloroquine would substantially inhibit the destruction of haem *in vivo*. We suggest that this underlies the molecular basis of action of chloroquine.

We examined the abilities of some other antimalarial drugs to inhibit the peroxidative destruction of haem. The 9-aminoacridine, quinacrine, was an effective inhibitor suggesting that this drug acts via a similar mechanism of action to chloroquine. This drug has previously been shown to interact with haem [35] and to act as an efficient inhibitor of haem polymerization [22]. The quinolinemethanol antimalarial drug, mefloquine, also showed some inhibition of the destruction of haem, but was less effective than chloroquine (Figure 1B). Epiquinine, which has very little antimalarial activity [37], is ineffective as an inhibitor of haem degradation.

It has been shown previously that chloroquine enhances the association of haem with phospholipid monolayers [38] and promotes haem-catalysed lipid peroxidation [39]. We have therefore examined the ability of chloroquine and other drugs to affect the level of binding of haem to lipid bilayers, using the erythrocyte membrane as a convenient model system. Chloroquine, quinacrine, the novel bisquinoline, 8AC6, and to some extent mefloquine increased the level of membrane-associated haem, while epiquinine caused a marked decrease in membrane-associated haem (Figure 2).

The above demonstrations of the ability of chloroquine to inhibit haem destruction and to promote the association of haem with membranes would suggest that treatment of *P. falciparum*-infected erythrocytes with chloroquine would lead to a build-up of membrane-associated haem. The level of membrane-associated haem in *P. berghei*-infected erythrocytes has previously been reported to increase upon chloroquine treatment [22]. We have undertaken similar experiments to determine levels of membrane-bound haem in control and chloroquine-treated *P. falciparum*-infected erythrocytes. We found that exposure of trophozoite-infected erythrocytes to 1–10 μM chloroquine for 4 h, caused a substantial increase in the level of membrane-associated haem (Table 2). Assuming that half of this amount of haem is present in the food vacuole, this represents an increase in the estimated haem concentration within the food vacuole from about 4 to 7 mM. This may be sufficient to overwhelm any mechanism for the protection of parasite membranes from the toxic effects of haem, resulting in disruption of the membranes and death of the parasite. These data strongly support the proposal that chloroquine functions at least in part by increasing the toxicity of haem.

Haem also displays catalase-like and peroxidase-like activities which are inhibited by quinoline antimalarial drugs

The level of peroxidative haem destruction *in vivo* will depend on the availability of H_2O_2. While H_2O_2 is constantly produced by the conversion of oxyhaemoglobin to methaemoglobin, a number of processes occur in the food vacuole to decompose H_2O_2. Host-derived catalase may be important in this process as this enzyme remains active at pH 5, however, host glutathione peroxidase would have very little activity under the conditions of the food vacuole [40,41]. Another possible route for H_2O_2 breakdown comes from the observation that haem itself can decompose H_2O_2 via a catalase-like activity [14,15] (Scheme 1B, reaction a). We have re-examined the catalase-like activity of haem under food vacuole conditions (Figure 3A). In the absence of haem, no O_2 was produced over the time-course of the experiment. In the presence of haem (at 55 μM) H_2O_2 is rapidly converted to O_2 (Figure 3A). The rate of degradation of H_2O_2 is similar to that with 0.01 μM bovine catalase (Figure 3A), indicating that the catalase enzyme is approx. 5000 times more efficient as a catalyst than free haem. Catalase protomers are present in the erythrocyte

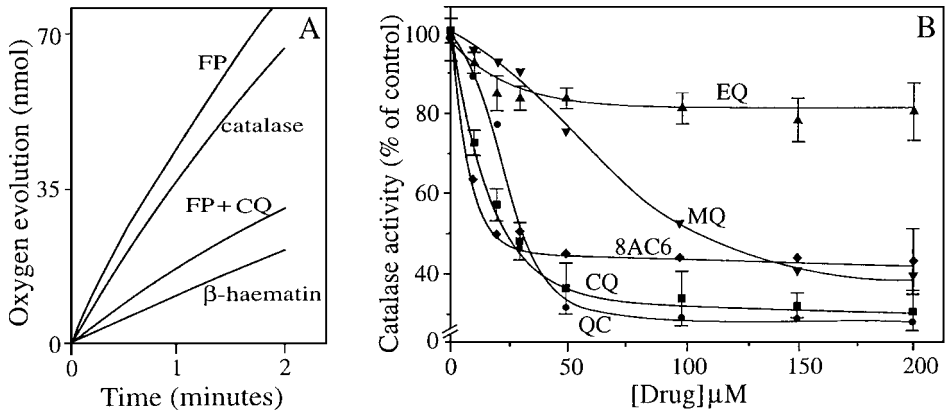

Figure 3 Catalase-like activity of haem

(**A**) Samples of haem (55 μM), sonicated β-haematin (55 μM), catalase (0.5 μg/ml, 0.01 μM monomers) or a haem/chloroquine mixture (55 μM haem/110 μM chloroquine) were incubated at 37 °C, in 200 mM sodium acetate, pH 5.2, in the chamber of a Clark oxygen electrode. The reaction was initiated by the addition of H_2O_2 (5 mM). The data are the traces of continuous recordings from a Clark oxygen electrode. (**B**) Inhibition of the catalase-like activity of haem was monitored for a sample containing haem (55 μM) in the presence of increasing concentrations of the modulating drug. Values are the mean ± S.D. from triplicate measurements in a typical experiment. Abbreviations are defined in the legend to Figure 1.

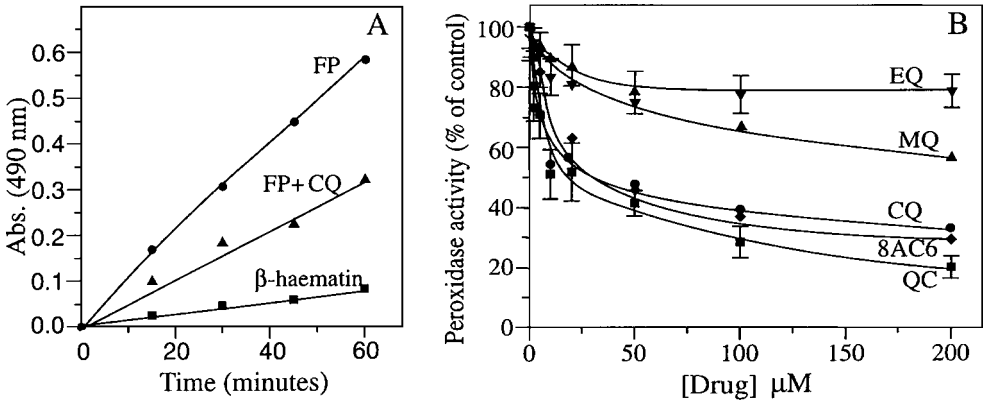

Figure 4 Peroxidase-like activity of haem

(**A**) Samples of haem (FP; 5 μM), sonicated β-haematin (5 μM) or a haem/chloroquine mixture (5 μM haem/1 μM chloroquine) were incubated at 20 °C, in the presence of 25 mM sodium citrate, pH 5.2, 100 mM NaCl. Oxidation of OPD was initiated by the addition of H_2O_2 (2 mM) and monitored by following the absorbance at 490 nm. (**B**) Inhibition of the peroxidase activity of haem was monitored for a sample containing haem (5 μM) in the presence of increasing concentrations of the inhibitory drug. Values are the mean ± S.D. from triplicate measurements in a typical experiment. Abbreviations are defined in the legend to Figure 1.

cytosol at a concentration of 4–8 μM [42], i.e. at about a 4000-fold lower concentration than the molar concentration of haemoglobin protomers (20 mM). This suggests that host-derived catalase and free haem could make similar contributions to the catalytic degradation of H_2O_2 in the parasite food vacuole. However, as proteolytic enzymes in the digestive vacuole degrade the host catalase, its contribution may be substantially decreased. Indeed, available estimates indicate that host-derived catalase makes only a limited contribution to H_2O_2 breakdown in malaria-infected erythrocytes [43]. The catalase-like activity of haem was greatly decreased upon polymerization of the haem to β-haematin (Figure 3A), presumably due to prevention of the formation of the ferryl intermediate (Scheme 1B).

Quinoline antimalarial drugs have previously been shown to inhibit the catalase-like activity of haem [15]. We have re-examined the abilities of a number of antimalarial drugs to inhibit haem-catalysed destruction of H_2O_2 under the conditions of our experiments. Chloroquine, quinacrine and the novel bis-4-aminoquinoline, 8AC6 [6,16], were all effective inhibitors of the catalase-like activity of haem while mefloquine showed a lower level of inhibition and epiquinine had very little effect (Figure 3B). Chloroquine had no effect on the activity of catalase (results not shown). These data suggest that the accumulation of chloroquine in the food vacuole could cause a build-up of H_2O_2 which could be harmful to the parasite.

Free haem has also been reported to display peroxidase-like activity against oxidizable substrates [14,44] (Scheme 1B, reaction b). We found that haem is an efficient catalyst of the oxidation of OPD under conditions which mimic those of the food vacuole (Figure 4A). These experiments were performed at either 20 °C or 37 °C; the oxidation rate was found to be approximately 20 % higher at 37 °C (results not shown). The peroxidase-like activity of haem may also contribute to the breakdown of reactive oxygen species in the food vacuole at the expense of substrates such as glutathione. As was the case for the catalase-like activity of haem, polymerized haem displayed a much lower level of

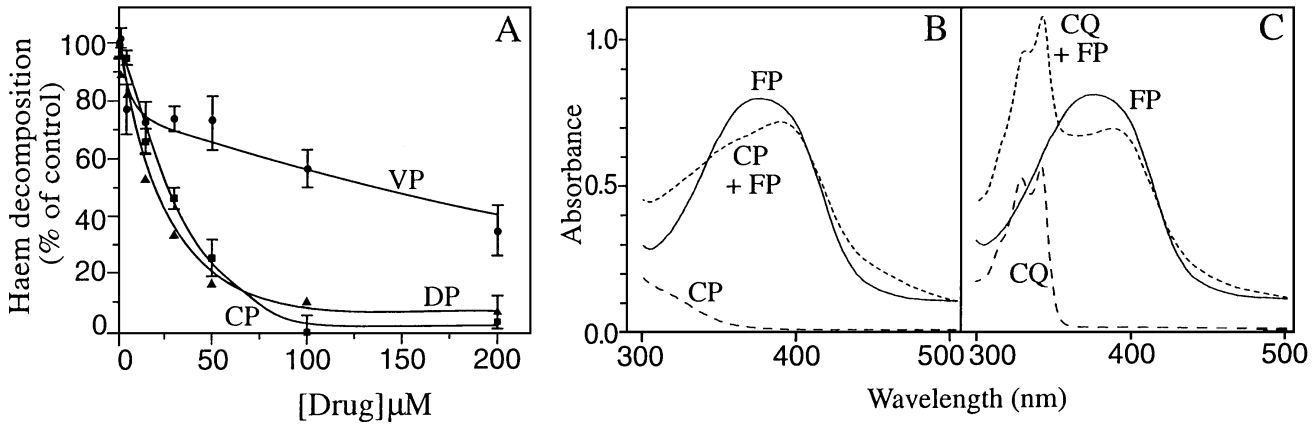

Figure 5 Effect of resistance-reversing agents on haem activities

(**A**) Inhibition of the peroxidative decomposition of haem was monitored for a sample containing haem (15 μM) in the presence of increasing concentrations of the inhibitory drug. Values are the mean $\pm$ S.D. from triplicate measurements in a typical experiment. CP, chlorpromazine; DP, desipramine; VP, verapamil. (**B** and **C**) Interaction of haem with chloroquine and chlorpromazine. Samples of haem (FP; 15 μM), chloroquine (CQ; 30 μM), chlorpromazine (CP; 30 μM), a haem/chloroquine mixture (15 μM haem/30 μM chloroquine) and a haem/chlorpromazine mixture (15 μM haem/30 μM chlorpromazine) were prepared in 200 mM sodium acetate, pH 5.2, 1 mg/ml BSA and visible absorption spectra were collected using a Cary 1E spectrophotometer.

peroxidase-like activity compared with free haem (Figure 4A). Chloroquine, quinacrine and 8AC6 were potent inhibitors of the peroxidase-like activity of haem, while mefloquine was significantly less active and epiquinine showed very little inhibitory activity (Figure 4B). Again, these data suggest that the presence of chloroquine is likely to lead to a build-up of toxic H_2O_2 in the food vacuole. In combination with a build-up of haem, this may lead to peroxidative disruption of the membranes. In this context, it is interesting to note that, in contrast to its effects on the peroxidase-like activities of haem against water-soluble substrates, chloroquine enhances the haem-catalysed peroxidation of lipids ([39]; P. Loria and L. Tilley, unpublished work).

Resistance-modulating agents inhibit the peroxidative destruction of haem

We have also examined the effects of drugs that are known to modulate resistance of *P. falciparum* to chloroquine on the various activities of the haem molecule. Chloroquine resistance is thought to arise, at least in part, from a decreased level of chloroquine uptake, although some studies suggest that an alteration in the drug target may also be involved [30,36,45]. Chloroquine resistance can be reversed *in vitro* and in animal models by co-administration of compounds such as verapamil, chlorpromazine and desipramine [46–48]. These reagents have been shown to enhance chloroquine uptake by chloroquine-resistant parasites, although recent work has shown that the apparent reversal of chloroquine resistance by these drugs cannot entirely be accounted for by an increase in chloroquine accumulation [49,50]. We have made the interesting observation that some of these resistance-modulating agents inhibit the peroxidative decomposition of haem (Figure 5A). At a concentration of 100 μM, chlorpromazine almost completely inhibited the breakdown of haem (Figure 5A). The inhibitory activity of chlorpromazine appears to involve a direct interaction of this drug with haem. The presence of chlorpromazine decreased the intensity of the Soret band in the haem absorption spectrum and shifted that absorption wavelength maximum from 380 nm to 395 nm (Figure 5B). Similar spectral changes were observed for the interaction of chloroquine with haem (Figure 5C). The inhibitory effects of chlorpromazine on haem decomposition may relate to a previous report showing that chlorpromazine inhibits the oxidation of the haem moiety in methaemoglobin by H_2O_2 [51]. Desipramine and to a lesser extent verapamil also inhibited haem breakdown (Figure 5A).

These data suggest that some resistance-modulating drugs might interfere with parasite growth in a manner similar to that for the 4-aminoquinolines. Indeed, drugs such as chlorpromazine and verapamil are known to be toxic to malaria parasites, albeit at relatively high concentrations (i.e., several μM) [46,48,52]. These weakly basic, hydrophobic drugs will be accumulated much less efficiently than chloroquine [36], but at higher external concentrations may reach sufficiently high levels in the food vacuole to exert an effect on haem breakdown. We found, however, that chlorpromazine does not promote haem association with membranes (Figure 2). Moreover, treatment of malaria-infected erythrocytes with this drug did not significantly increase the level of membrane-associated haem (Table 2). The ability of these resistance-reversing drugs to inhibit haem breakdown may underlie their weak antimalarial action and may contribute to their ability to reverse chloroquine resistance. However, the resistance-reversing effects of these drugs presumably also involve additional effects on chloroquine uptake [53,54].

In summary, these studies suggest a scenario in which quinoline antimalarial drugs, such as chloroquine, are efficiently accumulated into the parasite food vacuole, where they (a) inhibit the peroxidative breakdown of haem, (b) interfere with haem polymerization, (c) promote the association of haem with membranes, and (d) inhibit the decomposition of H_2O_2. These combined effects would lead to a build-up of toxic moieties which would irreversibly damage the proteins and lipids of the food vacuole. The involvement of reactive oxygen species in chloroquine action is supported by reports that the activity of chloroquine against *P. falciparum* is enhanced in the presence of H_2O_2 [55] or a glutathione antagonist [56]. The proposal that disruption of the normal function of the food vacuole is the final toxic event in the action of chloroquine is supported by ultrastructural studies which indicate that pharmacologically relevant concentrations of chloroquine lead to swelling of the parasite food vacuole and accumulation of undigested haemoglobin in endocytic vesicles [2,57,58].

The studies presented here confirm the importance of drug-haem interactions as the basis of the antimalarial action of a number of quinoline compounds. This information should help in the design of novel antimalarial drugs. The abilities of different compounds to inhibit the enzyme-like activities of haem appear to correlate well with their abilities to inhibit haem polymerization. For example, we found that antimalarial drugs inhibited the enzyme-like activities of haem in the order: chloroquine $\approx$ quinacrine $\approx$ 8AC6 $>$ mefloquine $\gg$ epiquinine. This correlates very well with the relative effects of these drugs on haem polymerization [6,22,23]. These assays of haem enzyme-like activities may, therefore, be useful as a preliminary screening assay for novel antimalarial compounds.

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