

OPEN

Antimalarial Quinoline Drugs Inhibit β -Hematin and Increase Free Hemin Catalyzing Peroxidative Reactions and Inhibition of Cysteine Proteases

Tomás Herraiz ^{1*}, Hugo Guillén¹, Diana González-Peña¹ & Vicente J. Arán²

Malaria caused by *Plasmodium* affects millions people worldwide. *Plasmodium* consumes hemoglobin during its intraerythrocytic stage leaving toxic heme. Parasite detoxifies free heme through formation of hemozoin (β -hematin) pigment. Proteolysis of hemoglobin and formation of hemozoin are two main targets for antimalarial drugs. Quinoline antimalarial drugs and analogs (β -carbolines or nitroindazoles) were studied as inhibitors of β -hematin formation. The most potent inhibitors were quinacrine, chloroquine, and amodiaquine followed by quinidine, mefloquine and quinine whereas 8-hydroxyquinoline and β -carbolines had no effect. Compounds that inhibited β -hematin increased free hemin that promoted peroxidative reactions as determined with TMB and ABTS substrates. Hemin-catalyzed peroxidative reactions were potentiated in presence of proteins (i.e. globin or BSA) while antioxidants and peroxidase inhibitors decreased peroxidation. Free hemin increased by chloroquine action promoted oxidative reactions resulting in inhibition of proteolysis by three cysteine proteases: papain, ficin and cathepsin B. Glutathione reversed inhibition of proteolysis. These results show that active quinolines inhibit hemozoin and increase free hemin which in presence of H_2O_2 that abounds in parasite digestive vacuole catalyzes peroxidative reactions and inhibition of cysteine proteases. This work suggests a link between the action of quinoline drugs with biochemical processes of peroxidation and inhibition of proteolysis.

Malaria is a parasitic infection caused by *Plasmodium* that affects hundreds millions people worldwide and causes almost half a million deaths each year¹. It remains a major infectious disease due to the lack of an effective vaccine and widespread resistance to available drugs. During infection, *Plasmodium* passes over several stages including an intraerythrocytic stage, in which parasite degrades 60–80% of host hemoglobin that is used as food support for its development and growth. Hemoglobin is oxidized to methemoglobin within parasite digestive vacuole and is hydrolyzed by aspartic proteases into free heme (Fe^{3+}) (ferriprotoporphyrin IX) and denatured globin. Globin is hydrolyzed by cysteine proteases (i.e. falcipains) and exopeptidases into small peptides and amino acids used for protein synthesis^{2–5}. Digestion of hemoglobin releases large quantities of heme (Fe^{3+}) that accumulates and reaches high concentrations (up to 300–500 mM). Those high concentrations are thought to be toxic for *Plasmodium* through membrane disruption, lipid peroxidation, and protein and DNA oxidation^{2,6–11}. Free heme (Fe^{3+}) might also interfere with hemoglobin degradation pathway^{12,13}. *Plasmodium* uses a system to detoxify heme (Fe^{3+}) called biocrystallization based on the formation of hemozoin pigment which appears as a dark black crystalline spot (a dark brown pigment) in red blood cells of infected patients^{14–18}. Hemozoin is chemically and structurally identical to β -hematin, a heme dimer that crystallizes under the acidic conditions of digestive vacuole of *Plasmodium* (pH values of 4.8–5.0)^{18–20}. It contains two heme (Fe^{3+}) monomers reciprocally linked through coordination complexes between the carboxyl group of a propionate side chain of one monomer and the iron (Fe^{3+}) atom in the porphyrin ring of another monomer^{19,21}. β -Hematin is stored in crystalline form

¹Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN). Spanish National Research Council (CSIC), Juan de la Cierva 3, 28006, Madrid, Spain. ²Instituto de Química Médica (IQM-CSIC), Juan de la Cierva 3, 28006, Madrid, Spain. *email: tomas.herraiz@csic.es

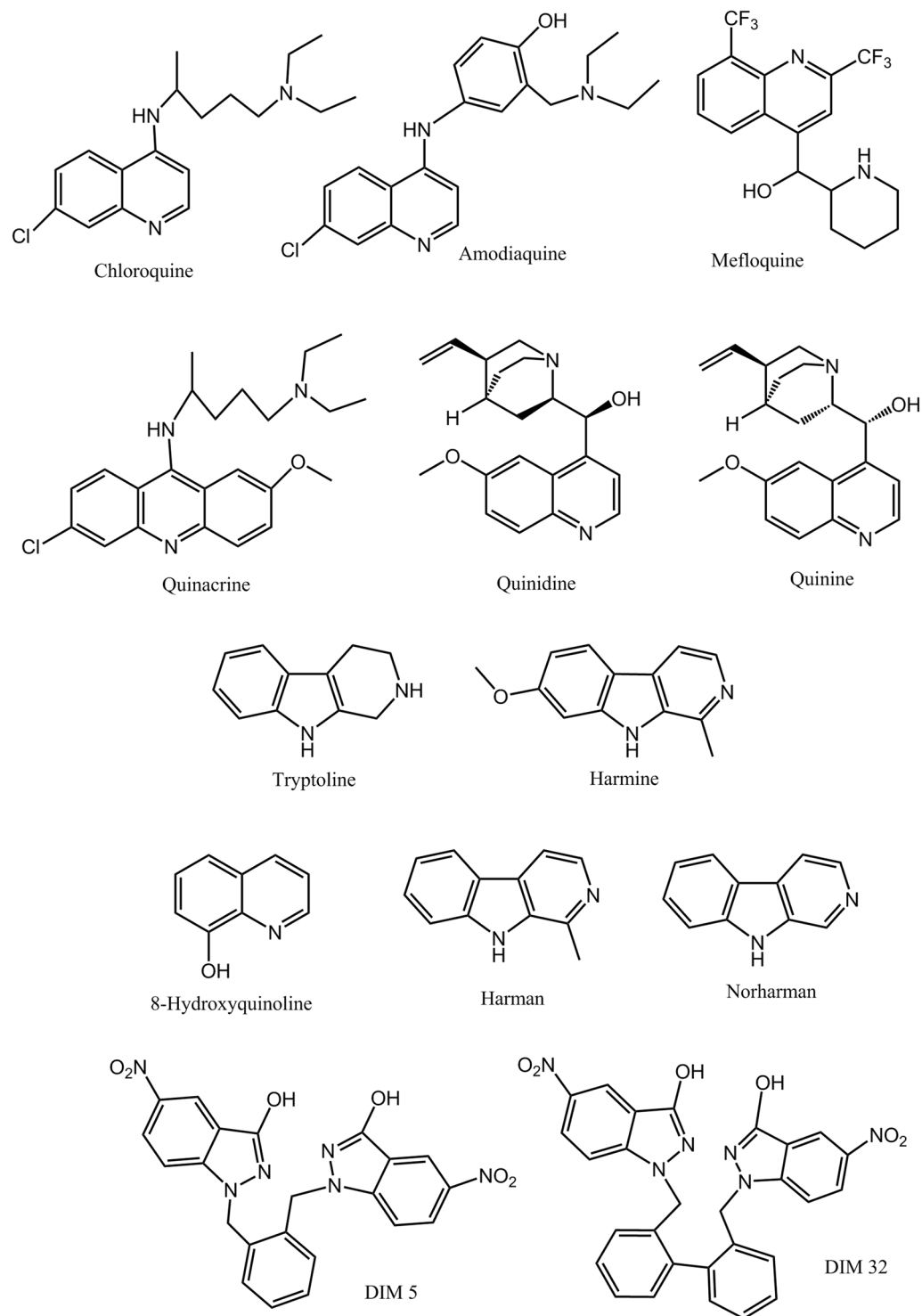


Figure 1. Quinoline drugs, β -carboline alkaloids, and nitroindazole compounds studied as inhibitors of hemozoin (β -hematin).

in the digestive vacuole where it is apparently nontoxic for *Plasmodium*²². The process of detoxification of heme is not unique of *Plasmodium* and occurs in other organisms that use hemoglobin such as *Schistosoma mansoni*, and *Rhodnius prolixus*²³. The mechanisms underlying the formation of β -hematin from hemoglobin are still poorly understood despite this process is vital for the survival of parasite²⁴. It appears to be catalyzed by lipids and/or heme detoxification proteins^{24–29} but it also occurs in absence of biological materials³⁰.

The synthesis of hemozoin constitutes a unique system of *Plasmodium* to detoxify heme; therefore its inhibition is a useful target for antimalarial drugs action^{2,18,31,32}. Quinoline drugs (e.g. chloroquine) (Fig. 1) have been successfully used for years to treat malaria. Currently, these drugs are less effective as parasites are

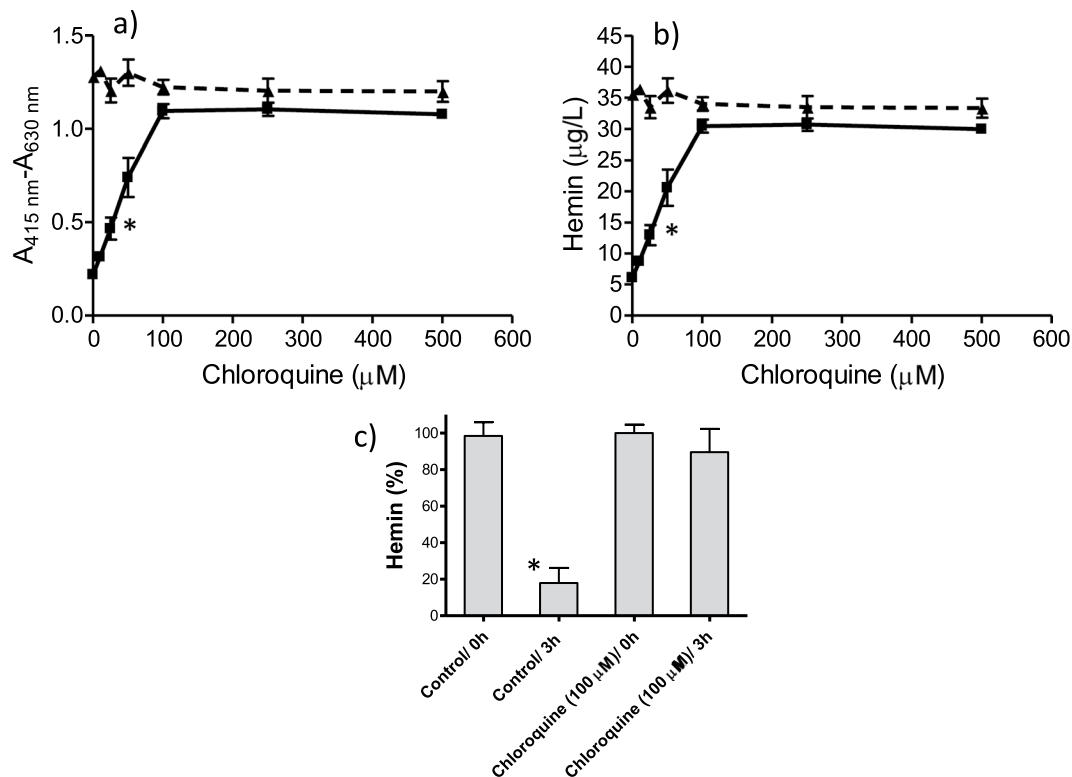


Figure 2. Increase of free hemin (inhibition of β -hematin) in presence of chloroquine. Absorbance ($A_{415\text{ nm}} - A_{630\text{ nm}}$) (a) and concentration of free hemin (b) (initial \blacktriangle , and after incubation 3 h, \blacksquare); and hemin (%) in control and chloroquine (100 μM) (0 and 3 h) (c). Hemin was incubated with chloroquine (0–500 μM) at pH 4.8 in presence of tween20. Data are average from seventeen assays. (*) Data are significantly different ($p < 0.01$) from control without chloroquine for this one and higher concentrations of chloroquine (a,b), and from control (0h) (c).

becoming resistant. Quinoline drugs exert their antimalarial action by interfering with heme detoxification^{33–35}. Nevertheless, their ultimate mechanism of action remains unknown and more insights are needed to understand how these drugs may produce toxic effects to parasite in order to increase their pharmacological efficacy and lower resistances^{2,36–41}. Previous studies suggested that quinolines could inhibit peroxidative degradation of heme contributing to build-up of toxic membrane-associated heme that would destroy membranes⁴² whereas others have reported a minor role of quinolines in the degradation of heme⁴³. The current work determines the actions of quinoline drugs and related compounds (e.g. β -carbolines and nitroindazoles) having antimalarial activity^{44,45} on the synthesis of hemozoin and free hemin build-up. We have developed an assay to determinate the conversion of hemin into hemozoin, and subsequently it is used to investigate the activity of quinoline drugs. Results show that active quinolines (e.g. chloroquine) increase free hemin that is involved in peroxidative reactions and inhibition of proteolysis. Then, this work highlights a link between free hemin build-up by quinoline drugs with peroxidative effects (oxidative effects) and inhibition of cysteine proteases (inhibition of proteolysis). These events may underlay the basics for toxicity of antimalarial quinoline drugs in blood feeding organisms including *Plasmodium* and could be useful for the development of new antimalarial agents.

Results

Formation of β -hematin and inhibition by quinoline drugs. Hemin incubated at 37 °C and pH 4.8 (pH of digestive vacuole) in presence of tween 20 crystallized and precipitated as a dark (black) powder that was isolated and had IR spectra exhibiting bands at 1210, 1663 and 1712 cm^{-1} (Supplementary Figure 1) corresponding to β -hematin or hemozoin, the pigment of *Plasmodium*^{18,24,46}. β -Hematin has UV-VIS spectrum with lower relative absorbance at 415 nm and higher at 630 nm than free hemin, so that the concentration and relative contribution of free hemin can be assessed from $A_{415\text{ nm}} - A_{630\text{ nm}}$ ^{47,48}. A high proportion of initial hemin (>80%) is converted to β -hematin during incubation time (Fig. 2). Drugs that inhibit β -hematin proportionally increase free hemin measured as $A_{415\text{ nm}} - A_{630\text{ nm}}$ (Fig. 2a,b). Thus, chloroquine inhibited β -hematin and increased hemin that reached more than 80% of the initial hemin at 100 μM and higher concentrations of drug (Fig. 2b,c). This effect can be seen in photographic image (Supplementary Fig. 2). In presence of chloroquine the hemin remained in solution for hours but it was removed and converted into β -hematin in absence of drug (Supplementary Fig. 3a). The ability of quinolines to inhibit β -hematin, and consequently to increase hemin was subsequently studied (Fig. 3). Table 1 lists the concentration of drug needed to reach a 50% inhibition of β -hematin (i.e. allowing a 50% increase of free hemin) (IC_{50}). The most active compounds were quinacrine, chloroquine and amodiaquine whereas quinidine, mefloquine, and quinine had lower activity. The highest inhibition was also produced by

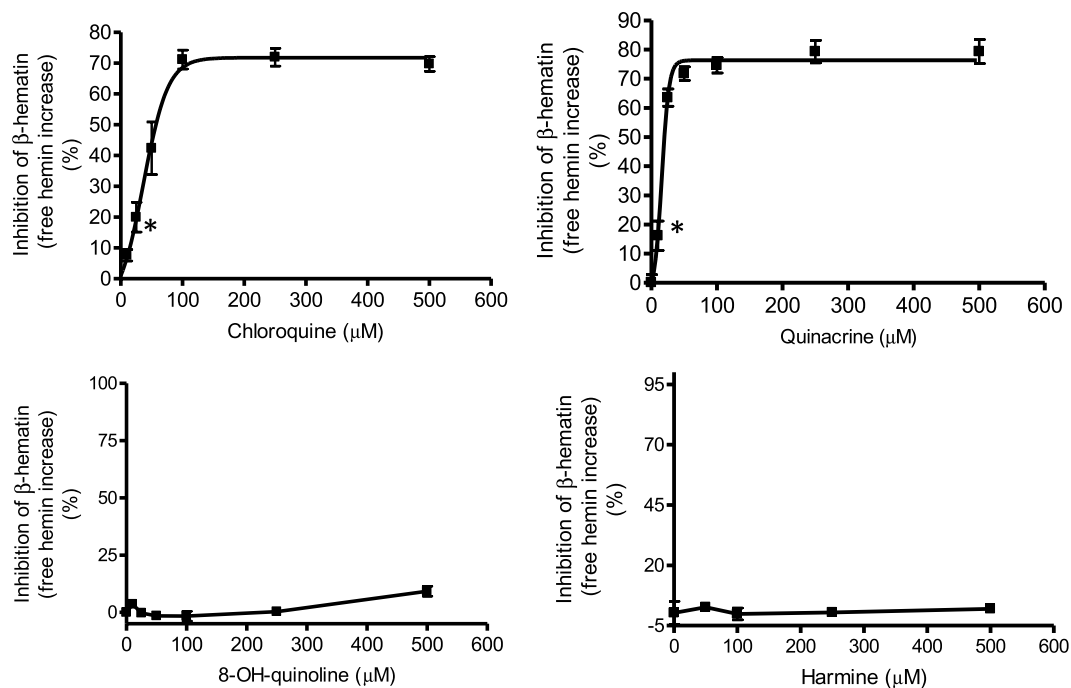


Figure 3. Inhibition (%) of β -hematin (or increase of free hemin) by chloroquine, quinacrine, 8-OH-quinoline and harmine. Data are average \pm SEM from seventeen assays for chloroquine, eight for quinacrine, and three for 8-OH-quinoline and harmine, respectively. Basal values in absence of drug are subtracted, and chloroquine and quinacrine are adjusted to non-linear regression curves. (*) Data are significantly different from control without drug for this one and higher concentrations of drug ($p < 0.01$).

| Compound | IC ₅₀ (μ M) | Max. inhibition (max. free hemin) (%) ^a | EC ₅₀ (μ M) ^b | Max. oxidation of TMB (%) ^c |
|----------------------|-----------------------------|----------------------------------------------------|------------------------------------------|----------------------------------------|
| Chloroquine | 56.9 | 89.0 \pm 3.1 | 28.1 | 96.9 \pm 3.5 |
| Quinacrine | 20.7 | 96.1 \pm 4.1 | 7.7 | 94.7 \pm 8.5 |
| Amodiaquine | 50.1 | 83.3 \pm 2.3 | 36.6 | 59.8 \pm 3.6 |
| Mefloquine | 201 | 70.2 \pm 5.1 | 40.3 | 68.9 \pm 9.3 |
| Quinine | 211 | 65.9 \pm 18 | 40.2 | 63.1 \pm 7.2 |
| Quinidine | 159 | 82.3 \pm 12 | 47.4 | 63.8 \pm 3.6 |
| 8-Hydroxyquinoline | — | 21.4 \pm 3.7 | — | — |
| Harmine | — | 24.5 \pm 2.1 | — | 28 |
| Harman | — | 13.0 \pm 5.5 | — | 35 |
| Norharman | — | 53.8 \pm 9.3 | — | 25 |
| Tryptoline | — | 18.4 \pm 4.8 | — | 26 |
| Nitroindazole DIM 5 | 17.4 | 70.7 \pm 2.3 | — | 36.6 |
| Nitroindazole DIM 32 | 17.0 | 73.4 \pm 1 | — | 23 |

Table 1. Inhibition of β -hematin (hemozoin) (i.e. free hemin increase) by quinoline drugs, β -carbolines and nitroindazoles and oxidation of TMB. ^aMaximum (%) of inhibition of β -hematin or maximum (%) of free hemin reached with drug. Controls without drugs contained a 17.86 \pm 1.98% of free hemin remaining from the initial hemin (100%) and the rest converted into β -hematin. ^bEC₅₀ is the concentration of drug allowing to reach a 50% oxidation of TMB. ^cMaximum oxidation (%) achieved with drug taking the basal oxidation in presence of drug without incubation as 100%. The compounds were tested in a range 0–500 μ M except for indazoles that was 0–100 μ M.

quinacrine and chloroquine. 8-Hydroxyquinoline and β -carbolines (norharman, harman, harmine and tryptoline) did not inhibit β -hematin, and consequently did not increase hemin (Fig. 3 and Table 1). Indazole compounds (DIM 5 and DIM 32) gave IC₅₀ values comparable to chloroquine in agreement with former results⁴⁵. However, these drugs behaved differently to quinolines as they precipitated hemin which redissolved during assay of absorbance contributing to hemin.

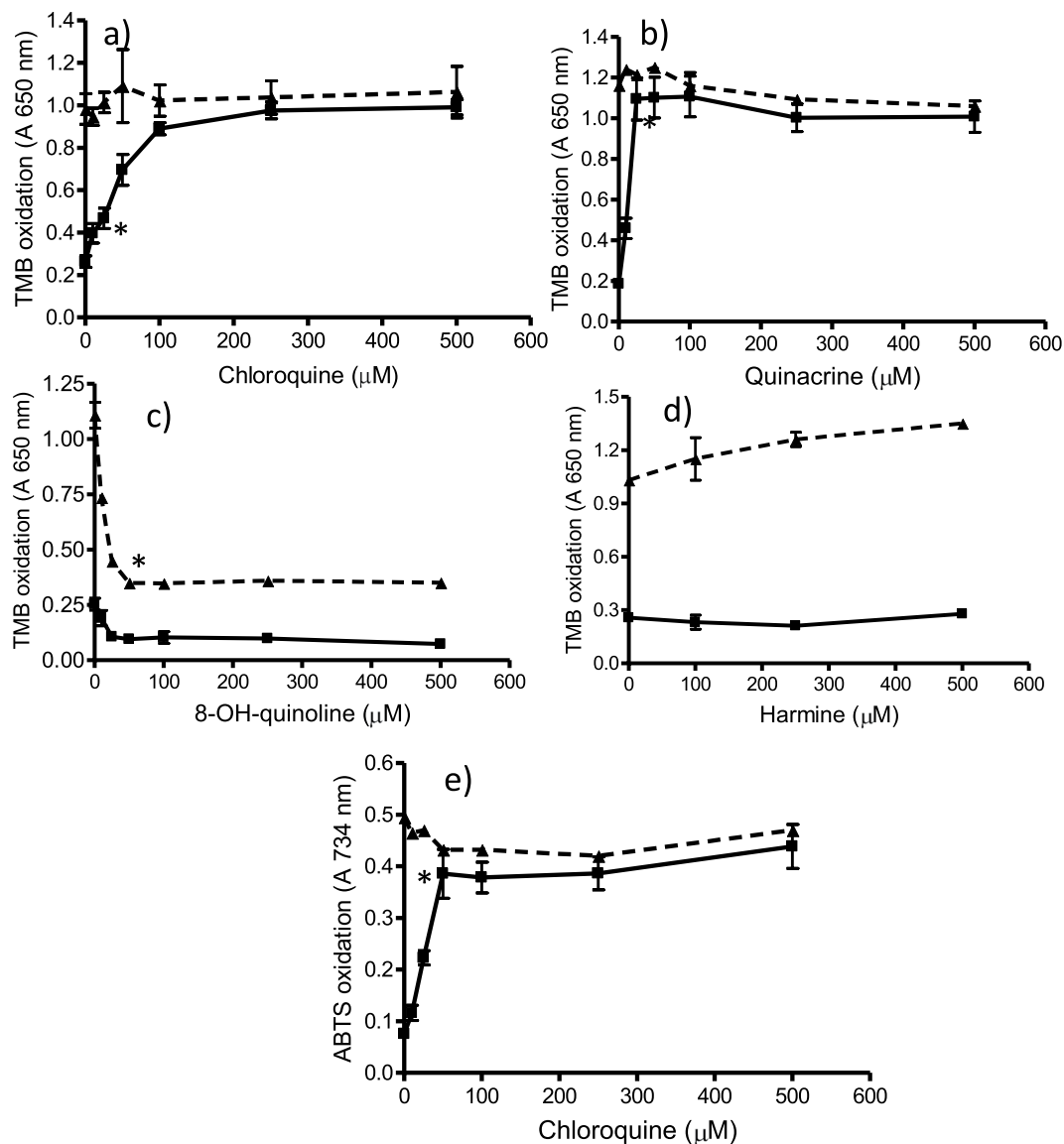


Figure 4. Oxidation of TMB to $\text{TMB}^{+\bullet}$ (absorbance at 650 nm) after incubation of hemin with chloroquine, quinacrine, 8-OH-quinoline and harmine for 3 h (■) (a–d), and oxidation of ABTS to radical $\text{ABTS}^{+\bullet}$ (absorbance at 734 nm) after incubation of hemin with chloroquine for 3 h (■) (e). Oxidation in absence of incubation time ($t=0$) (▲). Oxidation assays were carried out in presence of H_2O_2 (1 mM) as indicated in experimental and data averaged seventeen assays for chloroquine, eight for quinacrine, and three for 8-OH-quinoline and harmine. Oxidation of TMB was determined by absorbance at 650 nm (charge-transfer complex or cation radical, $\text{TMB}^{+\bullet}$) but similar trend was obtained at 450 nm followed acidification (TMB diimine). (*) Data are significantly different ($p < 0.01$) from control without drug for this one and higher concentrations of drug.

Quinolines that inhibit β -hematin increase peroxidation due to hemin build-up. As shown above, quinolines inhibited β -hematin and increased free hemin. It was studied whether this hemin was able to catalyze peroxidative reactions. For this purpose two peroxidase substrates were used (TMB and ABTS). Active quinolines (e.g. chloroquine and quinacrine) increased oxidation of TMB in contrast to inactive compounds (Fig. 4). Moreover, in presence of chloroquine the oxidation of TMB remained high during hours but in absence of drug it decreased simultaneously with hemin (Supplementary Fig. 3b). Peroxidation in presence of those active quinolines was due to free hemin build-up by the action of drugs (Fig. 3 and Fig. 4). Indeed, solutions of hemin standard catalyzed the oxidation of TMB (measured at 650 nm or at 450 nm after acidification) or ABTS (734 nm) in presence of H_2O_2 under pH conditions of digestive vacuole (pH 4.8), and oxidation increased with hemin and H_2O_2 concentrations (Supplementary Fig. 4). The oxidation in presence of drugs was calculated and EC_{50} values were determined as the amount of drug needed to reach a 50% of initial oxidation (Table 1). The most active compounds were quinacrine (EC_{50} of 7.7 μM) and chloroquine (EC_{50} of 28.1 μM) which also produced the highest levels of oxidation (96.9 and 94.7%, respectively). Quinidine, quinine, amodiaquine and mefloquine gave

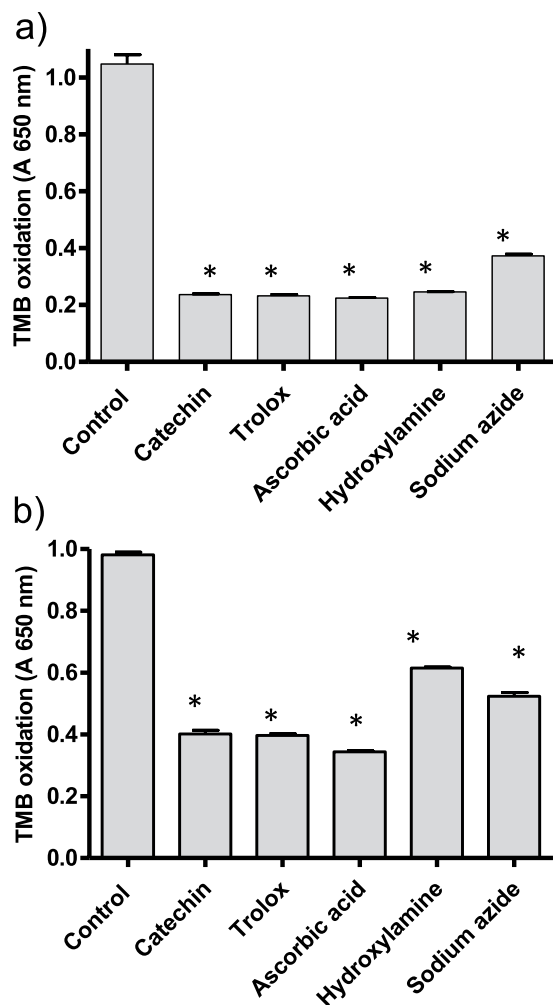


Figure 5. Inhibition of the oxidation of TMB by antioxidants and peroxidase inhibitors. Oxidation of TMB was determined in incubation media (3 h) containing hemin plus chloroquine (100 μ M) in presence of H₂O₂ (1 mM) and the corresponding antioxidant (1 mM) (a); or H₂O₂ (100 μ M), globin (10 μ M) and antioxidant (1 mM) (b). Controls had no antioxidant added. Oxidation of TMB to TMB^{•+} cation radical was measured at 650 nm following incubation at 37 °C, 20 min. (*) Data are significantly different from control ($p < 0.01$).

levels of oxidation lower than 70%. β -Carbolines (norharman, harman, harmine and tryptoline) and nitroindazoles did not increase oxidation whereas 8-hydroxyquinoline decreased basal oxidation owing to antioxidant effects (Fig. 4c). Peroxidation produced by hemin increase in presence of chloroquine was qualitatively similar with ABTS (Fig. 4e) where an EC₅₀ of 32.6 μ M was measured. Therefore, inhibition of crystallization of hemin (β -hematin) resulted in higher levels of free soluble hemin that catalyzed oxidation of peroxidase substrates in presence of H₂O₂. A significant positive correlation was found among free hemin increase ($A_{415\text{ nm}} - A_{630\text{ nm}}$) (Fig. 3) and oxidation of TMB (Fig. 4) for chloroquine (0–500 μ M) (correlation coefficient $r = 0.99$, $p < 0.001$), quina-crine (0–500 μ M) ($r = 0.96$, $p < 0.001$), amodiaquine (0–500 μ M) ($r = 0.98$, $p < 0.001$), mefloquine (0–500 μ M) ($r = 0.81$, $p < 0.05$), quinine (0–500 μ M) ($r = 0.84$, $p < 0.05$) and quinidine (0–250 μ M) ($r = 0.87$, $p < 0.05$). A significant positive correlation was also found among free hemin and oxidation of ABTS for chloroquine (correlation coefficient $r = 0.99$, $p < 0.001$). In contrast, no correlation was found for nitroindazoles despite these compounds reduced hemin crystallization. Neither for 8-hydroxyquinoline or β -carbolines that did not decrease crystallization of hemin nor promote oxidation. Peroxidation produced by free hemin in presence of chloroquine (100 μ M) was diminished by antioxidants and peroxidase inhibitors such as trolox, ascorbic acid, catechin, hydroxylamine and sodium azide (Fig. 5a). Therefore, antioxidants protected against peroxidation catalyzed by free hemin allowed by quinoline drugs.

Peroxidation catalyzed by free hemin build-up with drugs proceeds in presence of H₂O₂ at the pH of digestive vacuole. As proteins abound in digestive vacuole, peroxidation was evaluated under these conditions. Oxidation of TMB or ABTS by hemin solutions increased in presence of globin (Supplementary Fig. 5). In presence of globin, the oxidation of TMB or ABTS by free hemin accumulated during incubation was produced at much lower concentration of H₂O₂ (for TMB, 100 μ M vs 1000 μ M in absence of protein) (Fig. 6). Oxidation depended on the presence of both hemin and H₂O₂. A significant positive correlation was found among free hemin build-up

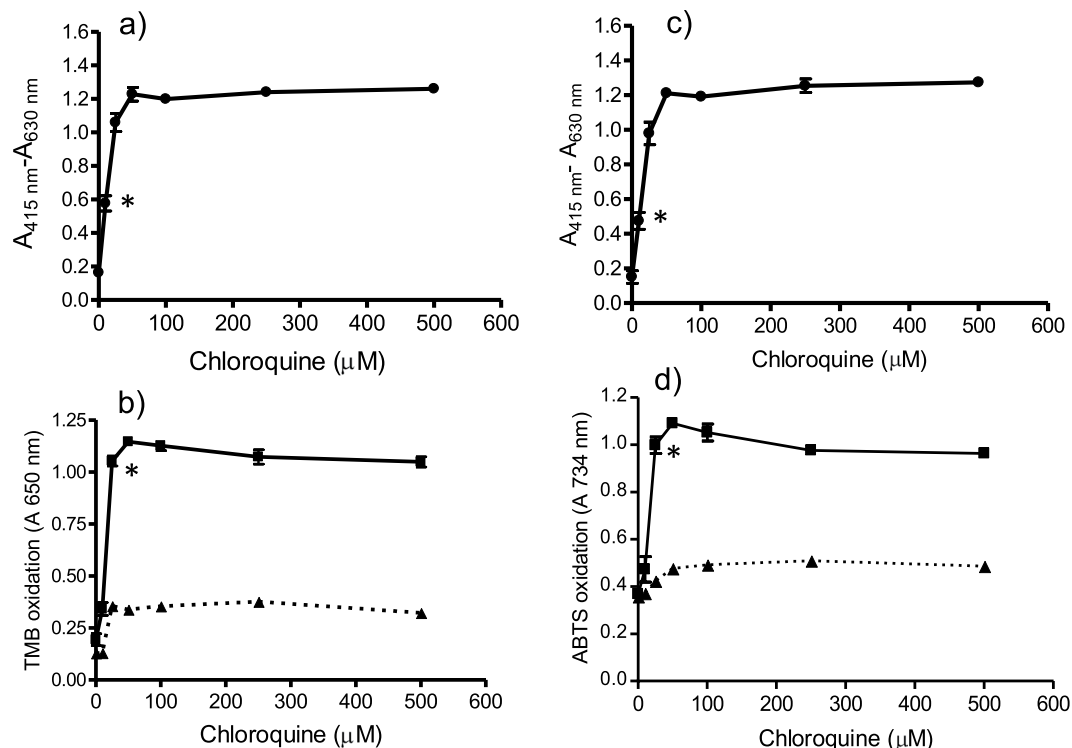


Figure 6. Hemin incubated with chloroquine (a,c) (●), and corresponding oxidation of TMB (b) or ABTS (d) in presence of globin (■). Incubation of hemin with chloroquine was carried out for 3 h and free hemin determined by absorbance ($A_{415\text{ nm}} - A_{630\text{ nm}}$). Oxidation of TMB was determined in presence of globin (10 μM) and H_2O_2 (100 μM) (b) and oxidation of ABTS in presence of globin (10 μM) and H_2O_2 250 μM (d). Controls in absence of H_2O_2 (-▲-). Assays were in triplicate and data are significantly different from control without drug for this one and higher concentrations of drug ($p < 0.01$) (*).

($A_{415\text{ nm}} - A_{630\text{ nm}}$) produced by chloroquine and the oxidation of TMB ($r = 0.97$, $p < 0.01$) or ABTS ($r = 0.92$, $p < 0.01$). The same qualitative trend of oxidation by hemin incubated with chloroquine was obtained in presence of BSA instead of globin (Supplementary Fig. 6). Therefore, free hemin build-up in the media by the action of quinoline drugs catalyzes peroxidative reactions that are enhanced in presence of proteins such as globin or BSA. Antioxidants and peroxidase inhibitors also decreased oxidation in presence of globin (Fig. 5b).

Chloroquine inhibits cysteine proteases due to free hemin build-up. Cysteine proteases metabolize hemoglobin in a process that is essential for growth and survival of parasite. The proteolytic activity of various cysteine proteases with incubation media of hemin with chloroquine was evaluated using Z-Phe-Arg-AMC (Supplementary Fig. 7). Proteolytic activity of papain, a model of cysteine protease that is similar to parasite's falcipains, was inhibited up to a 50% by incubation media of hemin with increasing levels of chloroquine in assays containing H_2O_2 (Fig. 7a). Proteolytic activity was also inhibited in presence of globin at lower concentrations of H_2O_2 (Fig. 7b). The inhibition of papain correlated well with free hemin build-up by the action of chloroquine while chloroquine itself did not inhibit proteolysis. Other cysteine proteases such as ficin and cathepsin B were also inhibited in presence of increased concentrations of chloroquine (Fig. 8a,b) owing to enhanced levels of free hemin. Moreover, the same qualitative trend was obtained in presence of protein BSA instead of globin (Supplementary Fig. 8). These results show that free hemin build-up resulting from inhibition of β -hemin by quinoline drugs inhibits cysteine proteases in presence of H_2O_2 . When glutathione was added no inhibition of proteolysis occurred (Fig. 9), suggesting that this antioxidant was able to reverse inhibition of proteolysis caused by hemin and H_2O_2 .

Discussion

Heme crystallization system in the digestive vacuole of *Plasmodium* has been a useful target for antimalarial drugs^{16,49,50}. Chloroquine and other quinolines (Fig. 1) exert antimalarial actions by interfering with this system. These drugs accumulate into the acidic digestive vacuole reaching up to millimolar concentrations, and prevent heme sequestration resulting in toxicity⁵¹. The biochemical mechanisms underlying these processes are still poorly understood despite their importance for the design of novel and more efficient drugs against resistant parasites⁵². *Plasmodium* detoxifies heme through its conversion to insoluble crystalline ferriprotoporphyrin IX dimer called hemozoin (β -hemin). This process may occur by self-assembly (autocatalytic) near lipid/water surfaces^{30,37,53,54}, or be catalyzed by specific heme detoxification proteins^{24,55}. Drugs targeting this process have been screened on the basis of differential solubilization of β -hemin and hemin^{27,56}. These assays are often troubled

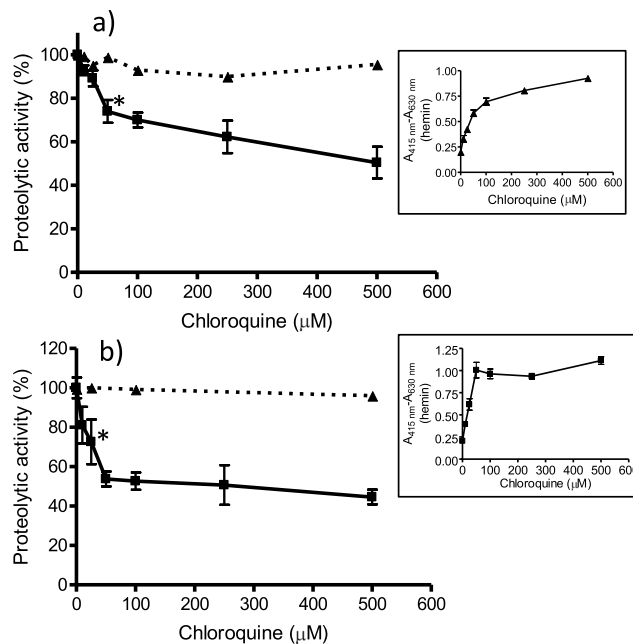


Figure 7. Inhibition of papain proteolytic activity by hemin incubated with chloroquine (3 h) (■). Proteolysis was measured as AMC released from Z-Phe-Arg-AMC from incubation media containing hemin plus chloroquine in presence of 1 mM H₂O₂ (a), or 75 μM H₂O₂ and globin (40 μM) (b). Controls (t = 0) without hemin (▲). Inserts correspond to hemin pattern during incubation (3 h). Basal proteolysis in absence of chloroquine was 21.22 ± 0.42 μM (a) or 46.41 ± 2.41 μM of AMC (b). Assays were in quadruplicate. (*) Data are significantly different (p < 0.01) from control without drug for this one and higher concentrations of drug.

by the formation of aggregates distinct from β-hematin. A spectrophotometric assay was used here to assess the contribution of free hemin and β-hematin^{27,47,57}. In this assay, active quinolines inhibited β-hematin formation and proportionally increased free hemin. Chloroquine, quinacrine and amodiaquine were the most active drugs whereas quinidine, quinine and mefloquine had lower potency. Two nitroindazoles had activity comparable to chloroquine and quinacrine whereas 8-hydroxyquinoline and β-carbolines were inactive. It is generally assumed that active quinoline drugs (Fig. 1) interact with free hemin and block hemozoin synthesis. The incorporation of quinoline-heme complexes into the growing crystal of hemozoin helps to terminate the process of crystallization of hemin^{35,58}. Results obtained herein and elsewhere suggest that drugs with protonated nitrogen and an aliphatic chain with a tertiary nitrogen have higher activity whereas the pyridine nitrogen has less effect^{33,37}. The electron rich planar area of quinoline interacts with hemin whereas basic nitrogen interacts with anionic sites^{33,59}. These quinoline-heme interactions show molecular diversity and quinolines may act differentially with various forms of heme (monomer vs dimer) and perturb equilibrium between them playing a role in the inhibition of β-hematin^{60,61}. β-Carbolines are quinoline analogs with reported antimalarial activity^{44,62}. Inhibition of hemozoin and intercalation of DNA have been proposed as possible mechanisms of action for β-carbolines. Results obtained here show that these substances do not inhibit β-hematin suggesting they may work under other mechanisms⁶³.

Active quinolines increased free hemin that catalyzed peroxidation reactions under conditions occurring in digestive vacuole. A significant correlation was found between the free hemin increased due to the action of antimalarial drugs and peroxidation (oxidative effects). Then, active quinolines may ultimately increase oxidative effects in the parasite through increased free hemin. The most active pro-oxidant drugs were by this order: chloroquine, quinacrine, amodiaquine, quinine, quinidine and mefloquine. 8-Hydroxyquinoline and β-carbolines were unable to increase oxidation as they lacked inhibitory effects against β-hematin and consequently did not increase free hemin. The occurrence of phenolic substituents in the quinoline structure (i.e. 8-hydroxyquinoline) decreased oxidation catalyzed by hemin likely due to antioxidant properties (Fig. 4). Nitroindazoles (DIM 5 and DIM 32) did not promote oxidation because they precipitated hemin that was not available for peroxidative reactions. However, this hemin contributed to absorbance as it redissolved during assay. Thus, differences between assays of hemin/β-hematin by absorbance and oxidation could detect possible aggregates of hemin different from β-hematin as occurred here with nitroindazoles.

Increased peroxidation by quinoline drugs was evidenced in presence of H₂O₂ and depended on hemin. Reactive oxygen species (ROS) could participate in the mechanism of action of quinoline drugs (i.e. chloroquine). Indeed, *Plasmodium* breaks down high levels of hemoglobin to peptides and amino acids. During this process free heme (Fe²⁺) converts into hemin (Fe³⁺) with subsequent production of superoxide anion (O₂^{•-}) that under acidic conditions dismutates to H₂O₂⁶⁴. It is estimated that up to 15 mM H₂O₂ could be produced within digestive vacuole, exposing parasites to high fluxes of ROS^{42,65}. The activity of chloroquine against *P. falciparum* is enhanced in presence of H₂O₂^{33,66,67} whereas hemin increases *in vivo* after treatment with chloroquine⁶⁸. As shown here, those high levels of H₂O₂ could be involved in peroxidase reactions catalyzed by the increased levels

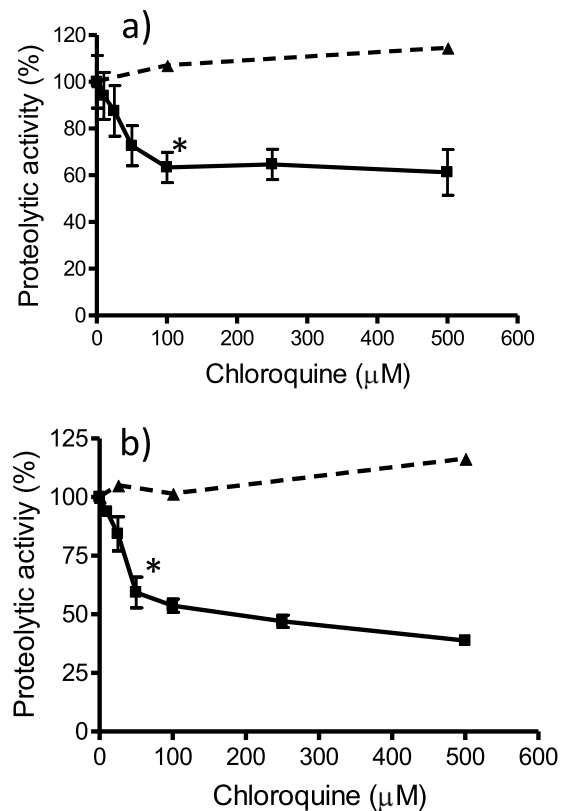


Figure 8. Inhibition of ficin (a) or cathepsin B (b) proteolytic activity by hemin incubated with chloroquine (3 h) (■). Proteolysis was measured as AMC released from Z-Phe-Arg-AMC in presence of globin (40 μM) and H₂O₂ (75 μM). Basal proteolysis in absence of drug was 24.0 ± 2.69 μM (a) and 28.9 ± 0.5 μM (b) of AMC. Assays with ficin and cathepsin were in quintuplicate and duplicate, respectively. Controls (t = 0) without hemin (▲) (*) Data are significantly different (p < 0.01) from controls without drug for this and higher concentrations of drug.

of free hemin allowed by quinoline drugs. Interestingly, proteins abundant in the digestive vacuole and this work has shown that peroxidation catalyzed by free hemin highly increased in presence of globin resulting in lower concentrations of H₂O₂ needed for oxidation. Therefore, the digestive vacuole contains enough H₂O₂ with high levels of proteins such as globin, that could result in increased oxidative effects (oxidative stress) triggered by soluble free hemin allowed by antimalarial drugs (chloroquine). Previously, it has been suggested that chloroquine could act through oxidative stress because it may host highly reactive radicals⁶⁹. Results in this work, however, suggest that active quinolines contribute to oxidative stress thorough an increase of free hemin but they have no pro-oxidant effect by themselves. Thus, peroxidative actions depended on the presence of soluble free hemin in incubation media although this hemin might be in different forms and as hemin-quinoline complexes⁶¹ that keep peroxidative actions. Quinoline drugs which accumulate into the parasite digestive vacuole increase soluble free hemin which may trigger peroxidation and oxidative stress supporting the assumption that accumulation of free hemin may result in oxidative damage to lipid membranes, proteins, and DNA with subsequent toxicity for the parasite^{6,42,70}. Resistance to quinolines may arise from a decrease in drug level at the site of action and/or mechanisms of defense against heme^{16,33,41,71}. Antioxidants and peroxidase ligands (trolox, catechin, ascorbic acid, sodium azide and hydroxylamine) inhibited peroxidative actions of hemin. The antioxidant system of parasite could reduce peroxidase actions of hemin and decrease efficacy of quinolines. In this regard, drugs that increase hemin accumulation while decreasing antioxidant effects might be most useful to increase antimalarial activity.

Inhibition of proteolysis is a current target for new antimalarial drugs^{4,15,72}. A number of protease inhibitors are active against *Plasmodium*^{5,73}. Numerous cysteine proteases have been identified in the genome of *Plasmodium*. They are involved in essential processes for parasite survival and growth such hemoglobin digestion, erythrocyte rupture and host cell invasion^{5,73}. *Plasmodium* consumes large quantities of hemoglobin from host cell. Once inside the parasite, hemoglobin is shuttled to digestive vacuole where it is digested by successive action of aspartate and cysteine proteases such as papain-like falcipains⁵. This process results in high levels of globin, peptides and amino acids that are transported into cytoplasm and used for cell growth¹⁵. Antimalarial drugs could interfere with hemoglobin digestion during blood stages of malarial life-cycle^{33,68,74}. Chloroquine inhibits lysosomal functions³⁴ and protein degradation in trophozoite-infected erythrocytes⁷⁵. The inhibition of lysosomal function may lead to parasite starvation^{76,77} and vacuole abnormalities¹⁵. Results in this work have shown that free hemin enhanced by the action of chloroquine was able to inhibit cysteine proteases. This was evidenced for three distinct enzymes: papain, ficin and cathepsin B. Papain is similar to falcipain in *Plasmodium*

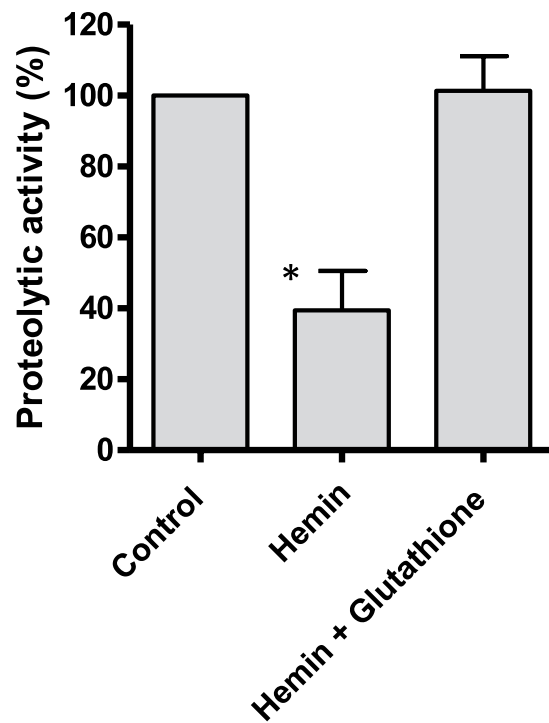


Figure 9. Inhibition of papain proteolytic activity by hemin incubated with chloroquine (100 μM). Proteolysis of Z-Phe-Arg-AMC was carried out as mentioned in experimental in presence of globin (40 μM) and H_2O_2 (75 μM), and with or without glutathione (500 μM). (*) Data are significantly different ($p < 0.05$) from control and from hemin plus glutathione.

whereas cathepsin B participates in the degradation of hemoglobin in *S. mansoni*^{78,79}. Inhibition was due to free hemin (or hemin complexes) allowed by chloroquine but not to chloroquine itself. It occurred in presence of H_2O_2 suggesting a likely involvement of peroxidative reactions that increased in presence of proteins such as globin. Cysteine proteases contain an essential sulfhydryl group sensitive to oxidation and inactivation due to the formation of mixed disulfides^{80,81}, or sulfenic acid (R-SOH) that are reversible in presence of glutathione⁸². Here, the inhibition of proteolysis by cysteine proteases due to free hemin allowed by chloroquine was reversed with glutathione. Previously, chloroquine response to *Plasmodium berguei*, a rodent malaria species, was affected by glutathione levels⁶⁷. Then, inactivation of cysteine proteases by quinoline drugs might occur under conditions of oxidative stress in a deficit of glutathione. Then, the antioxidant system of parasite could contribute to reduce inhibition of proteolysis triggered by chloroquine.

Beyond *Plasmodium*, heme aggregation is an efficient mechanism of detoxification in other organisms that use hemoglobin such as *S. mansoni* or *R. prolixus*^{23,64,83,84}. Chloroquine increases free hemin in the midgut of *R. prolixus* resulting in lipid peroxidation⁸⁵. Blood feeding organisms including *Plasmodium* contain proteases that break down hemoglobin affording heme and also globin, peptides and amino acids that are used for growth and survival (Fig. 10). Heme is oxidized to hemin (Fe^{3+}) releasing H_2O_2 . The former organisms detoxify hemin by its conversion into non-toxic hemozoin. However, in presence of active quinolines (e.g. chloroquine or quinacrine), hemin may remain free in the vacuole to interact with globin or other proteins, and can be oxidized by H_2O_2 to higher oxidative iron forms (oxoferryl iron ($\text{Fe}(\text{IV})=\text{O}$) or oxoferryl porphyrin π -cation radicals [$(\text{P}^{+})\text{Fe}^{\text{IV}}=\text{O}$]) which are highly oxidant species resembling compound I of heme peroxidases^{86–88}. These species can oxidize small molecules and metabolites, proteins, lipid membranes, and DNA resulting in parasite damage. Under these conditions free hemin may also inactivate enzymes such as papain-like cysteine proteases that are essential for parasite survival and growth.

Taken together results obtained in this study shed light on the mechanisms of toxicity of antimalarial quinoline drugs in blood feeding organisms. However, these results and evidences should be tested further and confirmed *in vivo* in the own malaria parasite where complexity increases substantially and the actual conditions could differ (e.g. H_2O_2 concentrations in digestive vacuole, specific cysteine proteases involved, antioxidant mechanisms, among many other factors), and these changes might affect quinoline-heme interactions as well as peroxidative actions and inhibition of proteolysis.

In conclusion, results show that active quinoline drugs inhibit β -hematin resulting in increased levels of free hemin. Under conditions expected in the parasite digestive vacuole this free hemin catalyzes oxidative reactions and inhibition of cysteine proteases. Peroxidation and inhibition of proteolysis due to free hemin was enhanced in presence of globin. Results evidenced a positive correlation between quinoline drugs, free hemin increase, and enhanced peroxidation and inhibition of proteolysis. These mechanisms suggest how these drugs may work against malaria parasite. Thus, anti-parasitic drugs that increase free hemin could trigger oxidative stress through

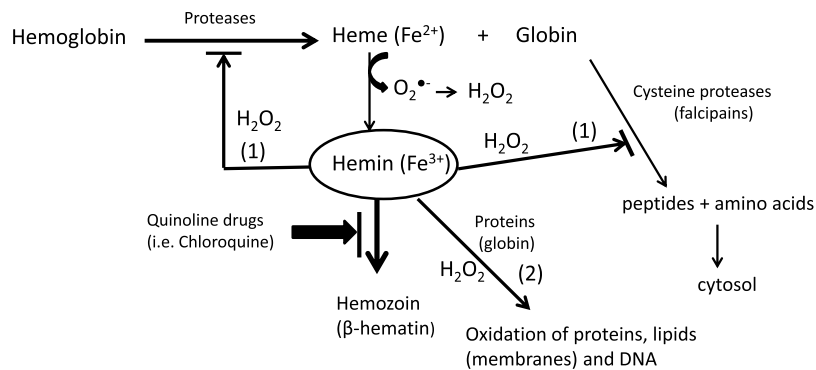


Figure 10. Mechanism of action of quinoline drugs inhibiting hemozoin in the digestive vacuole of *Plasmodium* inside erythrocytes or in other organisms that use hemoglobin as food. This inhibition enhances the levels of free hemin which promote peroxidase reactions with H_2O_2 arising from heme (Fe^{2+}) oxidation. Hemin and peroxidase reactions inhibit cysteine proteases needed for degradation of proteins used for parasite growth (1), and may also oxidize proteins (enzymes), lipids and DNA resulting in parasite damage (2).

peroxidase reactions resulting in inhibition of proteolysis and damage to macromolecules and membranes causing toxicity and parasite death. Oxidation promoted by chloroquine was inhibited by antioxidants and peroxidase inhibitors and the inhibition of proteolysis blocked by glutathione. Therefore, the antioxidant system of parasite could contribute to quinoline drug resistance. These results could be useful for the design of more powerful anti-malarial drugs.

Material and Methods

Hemin chloride, chloroquine, quinacrine, amodiaquine, mefloquine, 8-hydroxyquinoline, quinine, quinidine and the β -carbolines norharman, tryptoline, harman, and harmine were obtained from Sigma-Aldrich. Indazole compounds 1,1'-[2,2'-biphenyldiyl]bismethylene]bis(5-nitro-1H-indazol-3-ol) (DIM-32) and 1,1'-(*o*-xylylene) bis(5-nitro-1H-indazol-3-ol) (DIM-5) were previously synthesized⁴⁵. 3,3',5,5'-Tetramethylbenzidine (TMB), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and tween 20 were obtained from Sigma and cysteine from Merck. DMSO and hydrogen peroxide (H_2O_2) were from Scharlau and Panreac, respectively. Globin was obtained from bovine hemoglobin (Sigma) by precipitation in acetone-0.1% HCl at low temperature^{89,90}, and lab-stored crystallized and lyophilized bovine serum albumin (BSA) was from Sigma. Cysteine proteases: papain from *Carica papaya*, ficin from fig tree latex and cathepsin B from bovine spleen were obtained from Sigma. The peptide Z-Phe-Arg-AMC was purchased from Bachem, and 7-amino-4-methylcoumarin (AMC) from Sigma.

Crystallization of hemin to β -hematin and inhibition by quinoline drugs. Eppendorf tubes (1 mL, final volume) containing by order: hemin chloride dissolved in DMSO (8 μ L to reach 0.036 mg/mL, final conc.), quinoline or β -carboline drug dissolved in water (0–500 μ M, final conc.), or nitroindazole dissolved in DMSO (0–100 μ M, final conc.), freshly prepared detergent tween 20 dissolved in water (15 μ L to reach 0.015 mg/mL, final conc.), and 100 mM acetate buffer (pH 4.8) to reach 1 mL, were vortexed, and incubated in the dark at 37 °C for 2 h at 700 rpm using a thermo shaker (Biosan TS100). Then, the tubes were slightly moved and kept in the dark for 1 h at room temperature to continue with precipitation of crystallized hemin (β -hematin). During the assay free hemin disappears from the media as it is converted to β -hematin that precipitates. Control assays without incubation were also carried out. After the incubation period, an aliquot (400 μ L) of supernatant was used for studying peroxidation as mentioned below. With the rest (600 μ L) a colorimetric method was used to determine the effect of drugs on hemin crystallization. Colorimetric methods afford comparable results to those of purification of β -hematin^{47,91,92}. For this, the 600 μ L were pipetted three times, and absorbance measured first at 630 nm and then at 415 nm against a blank of 100 mM acetate buffer (pH 4.8). Any drug that inhibits the synthesis of β -hematin will increase free hemin, so that its activity as inhibitor of hemin crystallization can be determined. The concentration of hemin was calculated from a calibration curve of absorbance ($A_{415\text{ nm}} - A_{630\text{ nm}}$) vs hemin standard. To $A_{415\text{ nm}} - A_{630\text{ nm}}$ values from incubation media containing the drug were subtracted those without drug, and divided by $A_{415\text{ nm}} - A_{630\text{ nm}}$ with drug without incubation (control at $t = 0$). It affords a measure of remaining free hemin (%) that correlates with inhibition of β -hematin. IC_{50} values (concentration of drug that inhibits a 50% the formation of β -hematin or that increases free hemin a 50%) and the highest percentage of inhibition of hemin crystallization (or maximum % of free hemin achieved) were determined. The IC_{50} values were calculated from at least five different incubation experiments for each compound (0–500 μ M concentration range) using GraphPad Prism 4.0. Controls with drugs without the incubation period were also prepared. DMSO used as solvent for indazole compounds had no effect up to 15%. To study the fate of hemin during assay, UV-VIS spectra of solutions of hemin and hemin plus chloroquine (100 μ M) were monitored during incubation time (37 °C, 700 rpm).

Isolation and characterization of β -hematin. Forty eppendorf tubes (1 mL, final volume) containing hemin dissolved in DMSO (0.036 mg/mL, final conc.), freshly prepared tween 20 (final conc., 0.015 mg/mL), and 995 μ L 100 mM acetate buffer (pH 4.8) were vortexed and incubated in the dark at 37 °C for 2 h while shaking

at 700 rpm using a thermo shaker. The tubes were kept at room temperature in the dark for 1 hour and then centrifuged at 10000 rpm for 10 min. The precipitate was washed with buffer, centrifuged again and washed two additional times with water. The precipitate of β -hematin, a black powder, was dried and characterized by IR spectrometry.

Peroxidation activity of hemin and activity of quinoline drugs. Incubations of hemin with quinoline drugs were carried out as mentioned above and used to study peroxidation as follows:

Oxidation of peroxidase substrates TMB and ABTS. Incubation media containing hemin, quinoline drugs and tween 20 in acetate buffer (pH 4.8) were prepared as above and incubated for 2 h at 37 °C (700 rpm) and kept 1 h at room temperature. Controls without incubation period were also prepared. An aliquot (400 μ L) of supernatant was added to 100 mM acetate buffer (pH 4.8) (106 μ L), 10 mM H₂O₂ (60 μ L) (1 mM, final conc.) and 8.83 mM 3,3',5,5'-tetramethylbenzidine (TMB) dissolved in methanol (34 μ L) (500 μ M, final conc.) to reach 600 μ L (final volume). The tubes were vortexed and incubated in the dark for 20 min at 37 °C in a water bath. An aliquot (500 μ L) was used to determine oxidation of TMB by absorbance measured at 650 nm (blue charge-transfer complex from TMB^{•+} radical cation), and then at 450 nm following addition of 2 N HCl (250 μ L) (yellow TMB diimine) using acetate buffer as a blank^{93,94}. Oxidation of TMB was confirmed by measuring the corresponding UV-VIS absorption spectra. Oxidations with hemin and hemin plus drug were also determined at time zero (absence of incubation). The concentration of drug needed to reach a 50% of the initial oxidation of TMB taken as 100% was calculated as EC₅₀, and values calculated from at least five different incubation experiments. In other parallel experiments, an aliquot (400 μ L) of supernatant from incubations of hemin plus quinoline drugs (2 + 1 h) or from controls without incubation, was added with methanol (110 μ L), 10 mM H₂O₂ solution (60 μ L) (1 mM, final conc.) and 10 mM ABTS solution in water (30 μ L) (500 μ M, final conc.), and tubes incubated at 37 °C for 10 min in the dark. Absorbance at 734 nm was measured to determine oxidation of ABTS (ABTS^{•+} cation radical)⁹⁵. Oxidation of ABTS was confirmed with the corresponding UV-VIS absorption spectra. The concentration of drug needed to reach a 50% of the initial oxidation of ABTS that was taken as 100% was calculated as EC₅₀ and values calculated from at least five different incubation experiments. Control assays were also carried out in order to determine the influence of drugs, hemin and H₂O₂ in the oxidation of TMB and ABTS substrates.

Oxidation of TMB and ABTS in presence of globin or BSA. Eppendorf tubes (1 mL, final volume) containing by order: hemin dissolved in DMSO (0.036 mg/mL), chloroquine (0–500 μ M), freshly prepared tween 20 (0.015 mg/mL) and acetate buffer (pH 4.8) 100 mM, were vortexed and incubated as above at 37 °C for 2 h in the dark in a shaker at 700 rpm and kept at room temperature for 1 h. Controls without incubation period were also prepared. An aliquot of 400 μ L was taken from supernatant and added to globin (10 μ M, final concentration) or BSA (20 μ M, final concentration) solutions, H₂O₂ (100 μ M, final concentration) and 3,3',5,5'-tetramethylbenzidine (TMB) dissolved in methanol (500 μ M, final concentration) to reach 600 μ L (final volume), and vortexed and incubated in the dark for 20 min at 37 °C in a water bath. An aliquot of 500 μ L was used to determine oxidation of TMB by absorbance at 650 nm (charge-transfer complex) and 450 nm after addition of 2 N HCl (250 μ L) (TMB diimine). In other parallel experiments, an aliquot of supernatant (400 μ L) taken from samples containing hemin and drug after incubation (2 + 1 h) or from controls without incubation was added to globin solution (10 μ M, final conc.), H₂O₂ solution (250 μ M, final conc.) and ABTS solution in water (500 μ M, final concentration), and incubated at 37 °C for 10 min in the dark. Then, absorbance at 734 nm was measured to determine oxidation products of ABTS (ABTS^{•+} cation radical)⁹⁵. Controls in absence of H₂O₂ or hemin were also measured.

To determine the effects of antioxidants and peroxidase inhibitors on the oxidation of TMB, incubation media of hemin with chloroquine (100 μ M) were carried out as above (2 + 1 h), and oxidation of TMB was carried out in presence of catechin (1 mM), trolox (1 mM), ascorbic acid (1 mM), sodium azide (1 mM) or hydroxylamine (1 mM) in absence or in presence of proteins as mentioned above.

Proteolytic activity of cysteine proteases and effects of chloroquine. Incubations of hemin with chloroquine were used to investigate possible effects on proteolysis. Proteolysis was studied with three distinct cysteine proteases: papain, ficin and cathepsin-B using Z-Phe-Arg-AMC peptide. Incubation of hemin with or without chloroquine was carried out as above. Eppendorf tubes (1 mL, final volume) containing by order: hemin dissolved in DMSO (0.036 mg/mL), detergent tween 20 (0.015 mg/mL), chloroquine from 0–500 μ M and 100 mM acetate buffer (pH 4.8) were vortexed and incubated for 2 h at 37 °C at 700 rpm and kept for 1 h at room temperature. An aliquot of 600 μ L was taken to study proteolysis as mentioned below. The rest (400 μ L) was mixed by pipetting three times, and absorbance measured at 630 nm and 415 nm to determine hemin as above. Proteolysis was studied as follows: a) An aliquot (600 μ L) from incubation media (2 + 1 h) was added with 100 mM acetate buffer (pH 4.8), papain (100 μ g prot./mL with 3.5 μ M cysteine) and H₂O₂ (1 mM). The tubes were pre-incubated for 5 min at 37 °C and added with Z-Phe-Arg-AMC (100 μ M) (final volume of mixture, 800 μ L). After 20 min at 37 °C, reaction was stopped with 2 N HCl:MeOH (1:1) (80 μ L), and samples analyzed by RP-HPLC to determine proteolysis as AMC released (rate of hydrolysis as μ M AMC released/min). Assays were made in quadruplicate. b) Aliquots (600 μ L) from incubation media (2 + 1 h) were added with 100 mM acetate buffer (pH 4.8), globin (40 μ M), papain (20 μ g prot./mL with 2.5 μ M cysteine), and H₂O₂ (75 μ M). The samples were pre-incubated for 5 min at 37 °C and added with Z-Phe-Arg-AMC (100 μ M) (final volume of mixture, 800 μ L). After 5 min at 37 °C, the reaction was stopped with 2 N HCl:MeOH (1:1) (80 μ L), and samples vortexed and injected into HPLC for analysis of AMC. The assays were made in quadruplicate. c) Aliquots (600 μ L) from incubation media (2 + 1 h) of hemin with chloroquine were added with 100 mM acetate buffer, BSA (60 μ M), papain (20 μ g prot./mL with 2.5 μ M cysteine), and H₂O₂ (75 μ M). The samples were pre-incubated for 5 min at 37 °C and added with Z-Phe-Arg-AMC (100 μ M) (final volume of mixture, 800 μ L). After 5 min at 37 °C, the reaction was stopped with

2 N HCl:MeOH (1:1) (80 μ L), and samples vortexed and injected into HPLC for analysis of AMC. The assays were made in sextuplicate. d) Aliquots (600 μ L) from incubation media of hemin with chloroquine (2 + 1 h) were added with 100 mM acetate buffer, globin (40 μ M), ficin (100 μ g prot./mL with 2.5 μ M cysteine), and H₂O₂ (75 μ M). The samples were pre-incubated for 5 min at 37 °C and added with Z-Phe-Arg-AMC (100 μ M) (final volume of mixture, 800 μ L). After 5 min at 37 °C, the reaction was stopped with 2 N HCl:MeOH (1:1) (80 μ L), and samples injected into HPLC for analysis of AMC. Assays were carried out in quintuplicate. e) Aliquots of 150 μ L of incubation media (2 + 1 h) of hemin with chloroquine were added with globin (40 μ M), cathepsin B (21.75 μ g prot./mL protein with 2.5 μ M cysteine) and H₂O₂ (75 μ M) and pre-incubated 5 min at 37 °C; then added with Z-Phe-Arg-AMC (100 μ M) (final volume, 200 μ L) and incubated 10 min at 37 °C. The reaction was stopped with 2 N HCl:methanol (1:1) (20 μ L), vortexed and injected into the HPLC to determine AMC released into the assays. Assays were carried out in duplicate. The proteolytic activity by papain, ficin or cathepsin B in incubation media of hemin plus chloroquine was calculated as a percentage of initial proteolysis without drug taken as 100%. The effect of glutathione on proteolysis with papain was determined as in protocol b) from incubation media of hemin plus chloroquine (100 μ M) with globin (40 μ M), H₂O₂ (75 μ M) and glutathione (500 μ M).

RP-HPLC analysis of AMC and Z-Phe-Arg-AMC. Samples (20 μ L) of proteolysis assays were injected into a 1050 HPLC equipped with a DAD and a 1046 fluorescence detector. The chromatographic column was a 150 \times 3.8 mm, 4 μ m Novapak-C18 (Waters); Chromatographic conditions were: ammonium phosphate buffer (pH 3) (eluent A), and 20% eluent A in ACN (eluent B); the gradient was at 8 min 32% B and at 18 min 90% B. Detection was carried out with a DAD at 320 and 355 nm and fluorescence at 352 nm excitation and 442 nm emission. AMC released from Z-Phe-Arg-AMC peptide by cysteine proteases was analyzed at 355 nm and concentration obtained by using a calibration curve with AMC standard.

Spectrophotometric (UV-VIS) and infrared (IR) spectra. Absorption spectra from incubation media containing hemin and hemin plus chloroquine as well as from samples of oxidation of TMB and ABTS substrates were measured with a spectrophotometer Beckman Coulter Du 80 at 37 °C. To obtain IR spectra, potassium bromide pellets were prepared from dried samples of β -hematin obtained as mentioned above, and spectra were acquired with an IR Perkin Elmer 681 spectrophotometer.

Data and statistical analysis. The inhibition of hemozoin (%) and oxidation of TMB or ABTS substrates (%) as a function of quinoline drugs incubated with hemin were fit to non-linear regression curves to calculate IC₅₀ or EC₅₀ values using GraphPad Prism, 4.0. Correlation studies and statistical analysis were carried out and significance assumed for $p < 0.05$.

Data availability

Data generated and analyzed during this study are included in the published article (and its supplementary information files). Materials and data are also available from corresponding author on request.

Received: 1 April 2019; Accepted: 3 October 2019;

Published online: 28 October 2019

References

- World Malaria Report 2018. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO.
- Kumar, S., Guha, M., Choubey, V., Maity, P. & Bandyopadhyay, U. Antimalarial drugs inhibiting hemozoin (β -hematin) formation: A mechanistic update. *Life Sci.* **80**, 813–828 (2007).
- Eggleston, K. K., Duffin, K. L. & Goldberg, D. E. Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* **274**, 32411–32417 (1999).
- Roy, K. K. Targeting the active sites of malarial proteases for antimalarial drug discovery: approaches, progress and challenges. *Int. J. Antimicrob. Ag.* **50**, 287–302 (2017).
- Rosenthal, P. J., Falcipains and other cysteine proteases of malaria parasites, in *Cysteine Proteases of Pathogenic Organisms*, in Robinson, M. W. and Dalton, J. P., (Eds), Springer-Verlag Berlin, Berlin, pp. 30–48 (2011).
- Kumar, S. & Bandyopadhyay, U. Free heme toxicity and its detoxification systems in human. *Toxicol. Lett.* **157**, 175–188 (2005).
- Becker, K. *et al.* Oxidative stress in malaria parasite-infected erythrocytes: Host-parasite interactions. *Int. J. Parasitol.* **34**, 163–189 (2004).
- Schmitt, T. H., Frezzatti, W. A. Jr. & Schreier, S. Hemin-induced lipid membrane disorder and increased permeability: A molecular model for the mechanism of cell lysis. *Arch. Biochem. Biophys.* **307**, 96–103 (1993).
- Ryter, S. W. & Tyrrell, R. M. The heme synthesis and degradation pathways: Role in oxidant sensitivity. *Free Radic. Biol. Med.* **28**, 289–309 (2000).
- Tekwani, B. L. & Walker, L. A. Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: Technologies for *in vitro* β -hematin formation assay. *Comb. Chem. High T. Scr.* **8**, 63–79 (2005).
- Orjih, A. U., Banyal, H. S., Chevli, R. & Pitch, C. D. Hemin lyses malaria parasites. *Science* **214**, 667–669 (1981).
- Francis, S. E., Sullivan, D. J. Jr & Goldberg, D. E. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* **51**, 97–123 (1997).
- Pandey, A. V., Tekwani, B. L., Singh, R. L. & Chauhan, V. S. Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. *J. Biol. Chem.* **274**, 19383–19388 (1999).
- Sullivan, D. J. Jr, Gluzman, I. Y. & Goldberg, D. E. Plasmodium hemozoin formation mediated by histidine-rich proteins. *Science* **271**, 219–222 (1996).
- Gupta, P. *et al.* Exploring heme and hemoglobin binding regions of Plasmodium heme detoxification protein for new antimalarial discovery. *J. Med. Chem.* **60**, 8298–8308 (2017).
- Egan, T. J. Physico-chemical aspects of hemozoin (malaria pigment) structure and formation. *J. Inorg. Biochem.* **91**, 19–26 (2002).
- Combrinck, J. M. *et al.* Insights into the role of heme in the mechanism of action of antimalarials. *ACS Chem. Biol.* **8**, 133–137 (2013).

18. Coronado, L. M., Nadovich, C. T. & Spadafora, C. Malarial hemozoin: From target to tool. *BBA-Gen Subjects* **1840**, 2032–2041 (2014).
19. Pagola, S., Stephens, P. W., Bohle, D. S., Kosar, A. D. & Madsen, S. K. The structure of malaria pigment β -haematin. *Nature* **404**, 307–310 (2000).
20. Kapishnikov, S. *et al.* Unraveling heme detoxification in the malaria parasite by *in situ* correlative X-ray fluorescence microscopy and soft X-ray tomography. *Sci. Rep.* **7**, 7610, <https://doi.org/10.1038/s41598-017-06650-w> (2017).
21. Slater, A. F. G. Chloroquine: Mechanism of drug action and resistance in Plasmodium falciparum. *Pharmacol. Therapeut.* **57**, 203–235 (1993).
22. Hempelmann, E., Motta, C., Hughes, R., Ward, S. A. & Bray, P. G. Plasmodium falciparum: Sacrificing membrane to grow crystals? *Trends Parasitol.* **19**, 23–26 (2003).
23. Oliveira, M. F. *et al.* Inhibition of heme aggregation by chloroquine reduces Schistosoma mansoni infection. *J. Infect. Dis.* **190**, 843–852 (2004).
24. Chugh, M. *et al.* Protein complex directs hemoglobin-to-hemozoin formation in Plasmodium falciparum. *Proc. Natl. Acad. Sci. USA* **110**, 5392–5397 (2013).
25. Hempelmann, E. & Egan, J. T. Pigment biocrystallization in Plasmodium falciparum. *Trends Parasitol.* **18**, 11 (2002).
26. Bendrat, K., Berger, B. J. & Cerami, A. Haem polymerization in malaria. *Nature* **378**, 138 (1995).
27. Tripathi, A. K., Khan, S. I., Walker, L. A. & Tekwani, B. L. Spectrophotometric determination of de novo hemozoin/beta-hematin formation in an *in vitro* assay. *Anal. Biochem.* **325**, 85–91 (2004).
28. Fitch, C. D., Cai, G. Z., Chen, Y. F. & Shoemaker, J. D. Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. *BBA- Mol. Basis Dis.* **1454**, 31–37 (1999).
29. Tripathi, A. K., Garg, S. K. & Tekwani, B. L. A physicochemical mechanism of hemozoin (beta-hematin) synthesis by malaria parasite. *Biochem. Bioph. Res. Co.* **290**, 595–601 (2002).
30. Egan, T. J. Haemozoin formation as a target for the rational design of new antimalarials. *Drug Design Review* **1**, 93–110 (2004).
31. Huy, N. T. *et al.* High-throughput screening and prediction model building for novel hemozoin inhibitors using physicochemical properties. *Antimicrob. Agents Ch.* **61**, e01607, <https://doi.org/10.1128/AAC.01607-16> (2017).
32. Ketchum, M. A., Lee, A. M., Vekilov, P. G. & Rimer, J. D. Biomimetic assay for hematin crystallization inhibitors: A new platform to screen antimalarial drugs. *Cryst. Growth Des.* **17**, 197–206 (2017).
33. Foley, M. & Tilley, L. Quinoline antimalarials: Mechanisms of action and resistance and prospects for new agents. *Pharmacol. Therapeut.* **79**, 55–87 (1998).
34. Raynes, K. Bisquinoline antimalarials: their role in malaria chemotherapy. *Int. J. Parasit.* **29**, 367–379 (1999).
35. Sullivan, D. J., Gluzman, I. Y., Russell, D. G. & Goldberg, D. E. On the molecular mechanism of chloroquine's antimalarial action. *Proc. Natl. Acad. Sci. USA* **93**, 11865–11870 (1996).
36. Haldar, K., Bhattacharjee, S. & Safeukui, I. Drug resistance in Plasmodium. *Nature. Rev. Microbiol.* **16**, 156–170 (2018).
37. Dorn, A. *et al.* An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. *Biochem. Pharmacol.* **55**, 727–736 (1998).
38. Joshi, M. C. *et al.* *In vitro* antimalarial activity, β -haematin inhibition and structure–activity relationships in a series of quinoline triazoles. *Eur. J. Med. Chem.* **69**, 338–347 (2013).
39. Gunsaru, B. *et al.* Simplified reversed chloroquines to overcome malaria resistance to quinoline-based drugs. *Antimicrob. Agents Ch.* **61**, e01913–16, <https://doi.org/10.1128/aac.01913-16> (2017).
40. Lisewski, A. M. *et al.* Potential role of Plasmodium falciparum exported protein 1 in the chloroquine mode of action. *Int. J. Parasitol.-Drugs* **8**, 31–35 (2018).
41. Reiling, S. J., Krohne, G., Friedrich, O., Geary, T. G. & Rohrbach, P. Chloroquine exposure triggers distinct cellular responses in sensitive versus resistant Plasmodium falciparum parasites. *Sci. Rep.* **8**, 11137, <https://doi.org/10.1038/s41598-018-29422-6> (2018).
42. Loria, P., Miller, S., Foley, M. & Tilley, L. Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochem. J.* **339**, 363–370 (1999).
43. Egan, T. J. *et al.* Fate of haem iron in the malaria parasite Plasmodium falciparum. *Biochem. J.* **365**, 343–347 (2002).
44. Takasu, K. *et al.* pi-delocalized beta-carbolinium cations as potential antimalarials. *Bioorg. Med. Chem. Lett.* **14**, 1689–1692 (2004).
45. Martins Alho, M. *et al.* Synthesis and evaluation of 1,1'-hydrocarbylenebis(indazol-3-ols) as potential antimalarial drugs. *ChemMed Chem* **4**, 78–87 (2009).
46. Egan, T. J., Hempelmann, E. & Mavuso, W. W. Characterisation of synthetic beta-haematin and effects of the antimalarial drugs quindine, halofantrine, desbutylhalofantrine and mefloquine on its formation. *J. Inorg. Biochem.* **73**, 101–107 (1999).
47. Huy, N. T. *et al.* Simple colorimetric inhibition assay of heme crystallization for high-throughput screening of antimalarial compounds. *Antimicrob. Agents Ch.* **51**, 350–353 (2007).
48. Orjih, A. U. On the mechanism of hemozoin production in malaria parasites: activated erythrocyte membranes promote beta-hematin synthesis. *Exp. Biol. Med.* **226**, 746–752 (2001).
49. Perez-Guaita, D. *et al.* Multispectral atomic force microscopy-infrared nano-imaging of malaria infected red blood cells. *Anal. Chem.* **90**, 3140–3148 (2018).
50. Mosaddeque, F. *et al.* Prediction model for antimalarial activities of hemozoin inhibitors by using physicochemical properties. *Antimicrob. Agents Ch.* **62**, e02424–17, <https://doi.org/10.1128/aac.02424-17> (2018).
51. Kuhn, Y., Rohrbach, P. & Lanzer, M. Quantitative pH measurements in Plasmodium falciparum-infected erythrocytes using pHluorin. *Cell. Microbiol.* **9**, 1004–1013 (2007).
52. Bakouh, N. *et al.* Iron is a substrate of the Plasmodium falciparum chloroquine resistance transporter PfCRT in Xenopus oocytes. *J. Biol. Chem.* **292**, 16109–16121 (2017).
53. Egan, T. J. *et al.* Haemozoin (beta-haematin) biomineralization occurs by self-assembly near the lipid/water interface. *FEBS Lett.* **580**, 5105–5110 (2006).
54. Egan, T. J. Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation. *J. Inorg. Biochem.* **102**, 1288–1299 (2008).
55. Jani, D. *et al.* HDP - A novel heme detoxification protein from the malaria parasite. *Plos Pathogens* **4**, e1000053, <https://doi.org/10.1371/journal.ppat.1000053> (2008).
56. Deharo, E. *et al.* A non-radiolabelled ferriprotoporphyrin IX biomineralisation inhibition test for the high throughput screening of antimalarial compounds. *Exp. Parasitol.* **100**, 252–256 (2002).
57. Sandlin, R. D. *et al.* Identification of beta-hematin inhibitors in a high-throughput screening effort reveals scaffolds with *in vitro* antimalarial activity. *Int. J. Parasitol.-Drugs* **4**, 316–325 (2014).
58. Fitzroy, S. M. *et al.* The effects of quinoline and non-quinoline inhibitors on the kinetics of lipid-mediated beta-hematin crystallization. *Langmuir* **33**, 7529–7537 (2017).
59. Ismail, F. M. D., Dascombe, M. J., Carr, P. & North, S. E. An exploration of the structure-activity relationships of 4-aminoquinolines: Novel antimalarials with activity *in-vivo*. *J. Pharm. Pharmacol.* **48**, 841–850 (1996).
60. Gorka, A. P., de Dios, A. & Roepe, P. D. Quinoline drug-heme interactions and implications for antimalarial cytostatic versus cytotoxic activities. *J. Med. Chem.* **56**, 5231–5246 (2013).
61. Casabianca, L. B. *et al.* Quinine and chloroquine differentially perturb heme monomer-dimer equilibrium. *Inorg. Chem.* **47**, 6077–6081 (2008).

62. Ancolio, C. *et al.* Antimalarial activity of extracts and alkaloids isolated from six plants used in traditional medicine in Mali and Sao Tome. *Phytother. Res.* **16**, 646–649 (2002).
63. Herraiz, T. N-methyltetrahydropyridines and pyridinium cations as toxins and comparison with naturally-occurring alkaloids. *Food Chem. Toxicol.* **97**, 23–39 (2016).
64. Egan, T. J. Haemozoin formation. *Mol. Biochem. Parasitol.* **157**, 127–136 (2008).
65. Hunt, N. H. & Stocker, R. Oxidative stress and the redox status of malaria-infected erythrocytes. *Blood Cells* **16**, 499–526 (1990).
66. Malhotra, K., Salmon, D., Lebras, J. & Vilde, J. L. Potentiation of chloroquine activity against *Plasmodium falciparum* by the peroxidase-hydrogen peroxide system. *Antimicrob. Agents Ch.* **34**, 1981–1985 (1990).
67. Lehane, A. M., McDevitt, C. A., Kirk, K. & Fidock, D. A. Degrees of chloroquine resistance in *Plasmodium* - Is the redox system involved? *Int. J. Parasitol.-Drugs* **2**, 47–57 (2012).
68. Combrinck, J. M. *et al.* Optimization of a multi-well colorimetric assay to determine haem species in *Plasmodium falciparum* in the presence of anti-malarials. *Malaria J.* **14**, 253, <https://doi.org/10.1186/s12936-015-0729-9> (2015).
69. Monti, D. *et al.* Does chloroquine really act through oxidative stress? *FEBS Lett.* **522**, 3–5 (2002).
70. Sugioka, Y. & Suzuki, M. The chemical basis for the ferriprotoporphyrin-IX-chloroquine complex induced lipid-peroxidation. *BBA* **1074**, 19–24 (1991).
71. Lee, A. H. *et al.* Evidence for regulation of hemoglobin metabolism and intracellular ionic flux by the *Plasmodium falciparum* chloroquine resistance transporter. *Sci. Rep.* **8**, 13578, <https://doi.org/10.1038/s41598-018-31715-9> (2018).
72. Himangini, Pathak, D. P., Sharma, V. & Kumar, S. Designing novel inhibitors against falcipain-2 of *Plasmodium falciparum*. *Bioorg. Med. Chem. Lett.* **28**, 1566–1569 (2018).
73. Lisk, G. *et al.* Changes in the plasmodial surface anion channel reduce leupeptin uptake and can confer drug resistance in *Plasmodium falciparum*-infected erythrocytes. *Antimicrob. Agents Ch.* **52**, 2346–2354 (2008).
74. McKerrow, J. H., Caffrey, C., Kelly, B., Loke, P., & Sajid, M. Proteases in parasitic diseases, in Annual Review of Pathology: Mechanisms of disease, Annual Reviews, Palo Alto, pp. 497–536 (2006).
75. Zarchin, S., Krugliak, M. & Ginsburg, H. Digestion of the host erythrocyte by malaria parasites is the primary target for quinolin-containing antimalarials. *Biochem. Pharmacol.* **35**, 2435–2442 (1986).
76. Homewood, C. A., Warhurst, D. C., Baggaley, V. C. & Peters, W. Lysosomes, pH and anti-malarial action of chloroquine. *Nature* **235**, 50–52 (1972).
77. Goldberg, D. E., Slater, A. F. G., Cerami, A. & Henderson, G. B. Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. *Proc. Natl. Acad. Sci. USA* **87**, 2931–2935 (1990).
78. Rosenthal, P. J. Cysteine proteases of malaria parasites. *Int. J. Parasit.* **34**, 1489–1499 (2004).
79. Lipps, G., Fullkrug, R. & Beck, E. Cathepsin B of *Schistosoma mansoni* - Purification and activation of the recombinant proenzyme secreted by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 1717–1725 (1996).
80. Xian, M., Chen, X., Liu, Z., Wang, K. & Wang, P. G. Inhibition of papain by S-nitrosothiols: Formation of mixed disulfides. *J. Biol. Chem.* **275**, 20467–20473 (2000).
81. Hampton, M. B., Fadeel, B. & Orrenius, S. Redox regulation of the caspases during apoptosis. *Ann N.Y. Acad. Sci.* **854**, 328–335 (1998).
82. Mirković, B., Sosić, I., Gobec, S. & Kos, J. Redox-based inactivation of cysteine cathepsins by compounds containing the 4-aminophenol moiety. *Plos ONE* **6**, e27197, <https://doi.org/10.1371/journal.pone.0027197> (2011).
83. Ferreira, C. M., Oliveira, M. P., Paes, M. C. & Oliveira, M. F. Modulation of mitochondrial metabolism as a biochemical trait in blood feeding organisms: the redox vampire hypothesis redux. *Cell Biol. Int.* **42**, 683–700 (2018).
84. Xiao, S. H. & Sun, J. *Schistosoma* hemozoin and its possible roles. *Int. J. Parasit.* **47**, 171–183 (2017).
85. Oliveira, M. F. *et al.* Haemozoin formation in the midgut of the blood-sucking insect *Rhodnius prolixus*. *FEBS Lett.* **477**, 95–98 (2000).
86. Kitagishi, H. *et al.* Oxoferryl porphyrin/hydrogen peroxide system whose behavior is equivalent to hydroperoxoferric porphyrin. *J. Am. Chem. Soc.* **132**, 16730–16732 (2010).
87. Herraiz, T. & Galisteo, J. Naturally-occurring tetrahydro-beta-carboline alkaloids derived from tryptophan are oxidized to bioactive beta-carboline alkaloids by heme peroxidases. *Biochem. Biophys. Res. Co.* **451**, 42–47 (2014).
88. Herraiz, T., Guillen, H., & Galisteo, J. Metabolite profile resulting from the activation/inactivation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 2-methyltetrahydro-beta-carboline by oxidative enzymes. *Biomed. Res. Int.*, 248608; <https://doi.org/10.1155/2013/248608> (2013).
89. Tsemakhovich, V. A., Bamm, V. V., Shaklai, M. & Shaklai, N. Vascular damage by unstable hemoglobins: The role of heme-depleted globin. *Arch. Biochem. Biophys.* **436**, 307–315 (2005).
90. Ascoli, F., Fanelli, M. R. & Antonini, E. Preparation and properties of apohemoglobin and reconstituted hemoglobins. *Methods Enzymol.* **76**, 72–87 (1981).
91. Huy, N. T. *et al.* A simple and rapid colorimetric method to measure hemozoin crystal growth *in vitro*. *Anal. Biochem.* **354**, 305–307 (2006).
92. Men, T. T. *et al.* A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity. *Malaria J.* **11**, 272, <https://doi.org/10.1186/1475-2875-11-272> (2012).
93. Josephy, P. D., Eling, T. & Mason, R. P. The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine-free radical and charge transfer complex intermediates. *J. Biol. Chem.* **257**, 3669–3675 (1982).
94. Herraiz, T., Flores, A. & Fernandez, L. Analysis of monoamine oxidase (MAO) enzymatic activity by high-performance liquid chromatography-diode array detection combined with an assay of oxidation with a peroxidase and its application to MAO inhibitors from foods and plants. *J. Chromatogr. B* **1073**, 136–144 (2018).
95. Herraiz, T. & Galisteo, J. Endogenous and dietary indoles: A class of antioxidants and radical scavengers in the ABTS assay. *Free Radical Res.* **38**, 323–331 (2004).

Acknowledgements

The authors thank Spanish Government-FEDER through projects RTI2018-093940-B-I00, RTI2018-095544-B-I00 and SAF-2015-66690-R. We thank Elena Yuste and Fernando Vero for technical assistance. EY and FV are recipients of “Garantía Juvenil” fellowships from Comunidad de Madrid (Fondo Social Europeo-Iniciativa de Empleo Juvenil).

Author contributions

T.H. and V.A. obtained funding, conceived the study, and discussed results. H.G. and D.G. performed experimental work and contributed equally to study. T.H. designed, supervised, and contributed to experimental work. T.H. wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-019-51604-z>.

Correspondence and requests for materials should be addressed to T.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019