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Heme activates artemisinin more efficiently than hemin, inorganic iron, or hemoglobin

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Abstract—Artemisinin derivatives appear to mediate their anti-malarial effects through an initial redox-mediated reaction. Heme, inorganic iron, and hemoglobin have all been implicated as the key molecules that activate artemisinins. The reactions of artemisinin with different redox forms of heme, ferrous iron, and deoxygenated and oxygenated hemoglobin were analyzed under similar in vitro conditions. Heme reacted with artemisinin much more efficiently than the other iron-containing molecules, supporting the role of redox active heme as the primary activator of artemisinin.

1. Introduction

A multi-step mechanism has been proposed for the anti-malarial action of artemisinin and other related endoperoxides.[1] In the first step, reductive cleavage of the endoperoxide bridge of artemisinin occurs,[2] followed by intramolecular electronic rearrangements that produce carbon-centered radicals.[3] Subsequent reactions, including alkylation of proteins, lead to death of the parasite. Because Fe^{2+} is a catalyst that can generate free radicals from peroxides and large amounts of iron accumulate in the food vacuole, hemoglobin, heme, and free iron have been studied as key molecules participating in the initial artemisinin interaction and cleavage event.[4] Artemisinins are effective at reducing parasite levels within a short time after administration, killing all stages of the malaria parasite.[5,6]

Hemin (Fe^{3+} protoporphyrin-IX) has been reported to react directly with artemisinin by spectroscopic analysis,[7] and by analysis using ESI-MS/HPLC.[8] However, the reaction between hemin and artemisinin was very slow at 37 °C (not reaching completion in 24 h) and experiments were performed at a non-physiological temperature of 70 °C casting doubt on the biological significance of the findings.[9] Heme (Fe^{2+} protoporphyrin-IX) has been reported to activate artemisinin through in situ reduction of hemin by glutathione.[10] Within 1 h at 37 °C in these reducing conditions, a high yield of covalent heme–artemisinin adducts formed through a carbon-centered radical generated at the C4 position of artemisinin.

Other studies support a role for non-heme (inorganic) iron in an artemisinin activation process.[1] For example, iron chelators antagonize the anti-malarial effect of artemisinin,[11,12] although such chelators may be effective against both heme and inorganic iron.[13] In addition, carbon-centered radicals generated from artemisinins by Fe^{2+} (or heme) are then oxidized to carbocations by the subsequent Fe^{3+} that is formed by reductive cleavage of the peroxide.[14] This has led to the suggestion that interaction of artemisinin with free iron (II) represents a competitive decomposition pathway.[15] Other forms of iron, such as hemoglobin,[16] in particular ferrous but not ferric hemoglobin, have been reported to react with artemisinin.[17]

Given the disparate results reported for the roles of heme, free iron, and hemoglobin on the initial activation of artemisinin, we examined the interaction of the different redox forms of these molecules with artemisinin under defined conditions in a short time frame. The results support heme as the primary iron molecule that activates artemisinin. In addition to a role in the activation of artemisinin in an anti-malarial mechanism, a central role for heme may have relevance to the anti-cancer effects of artemisinin.[18,19]

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2. Results

2.1. Reaction of artemisinin with hemin and heme

The Soret and Q absorption bands of heme serve as observable markers of the reaction with artemisinin.[17,20] The absorption spectra of ferric heme (hemin) and ferrous heme (heme) were compared at a 20 μM final concentration with or without artemisinin at various molar ratios immediately after mixing in 0.25 M sodium acetate buffer, pH 5.0. DMSO (22% final concentration) was used in the assay buffer to maintain the hydrophobic heme or hemin and artemisinin in solution.[21] A pH of 5.0 was selected to reflect the acidic conditions of the parasite food vacuole[22,23] and the heme concentration of 20 μM was selected because it was much lower than may be expected in the food vacuole,[24] but was sufficient to provide adequate signal detection. As seen in Figure 1a, hemin displayed its characteristic Soret band at 402 nm that did not change with the addition of artemisinin, even in large excess, in agreement with previous observations.[17] We conclude that no redox-mediated interaction between (ferric) hemin and artemisinin occurred using these conditions. Essentially identical results were obtained at pH 7.0 in phosphate buffer (data not shown).

Hemin was then converted into heme by the addition of dithionite[25] with the expected shift in the Soret band to the characteristic 415 nm (Fig. 1a). Dithionite was used as a reducing agent to ensure full reduction of ferric heme and because it does not absorb at 415 nm as does heme or hemin. Under these low pH and reducing conditions, artemisinin caused an instant decrease of the heme A_{415} peak in a concentration-dependent manner, along with the appearance of a new absorption peak at 476 nm. At a (ferrous) heme:artemisinin molar ratio of 4:1, virtually complete disappearance of the A_{415} peak occurred. The new absorption peak observed at 476 nm following the reaction of heme and artemisinin was found to have a half-life of about 25 min (Fig. 1b and c). Essentially identical results were obtained at pH 7.0 in phosphate buffer (data not shown).

At the point of the loss of the heme Soret band peak and the presence of the full peak at 476 nm, the heme–artemisinin mixture was precipitated with KCl. After recovery by centrifugation and washing, the absorbance spectrum was taken and compared with the spectra of heme and hemin. The Soret peak of the complex was zlocated at 411 nm (data not shown), very different from the Soret peak of either ferrous heme or hemin, but similar to the Soret peak of a 'hemart' complex previously reported.[26] Addition of dithionite did not restore the

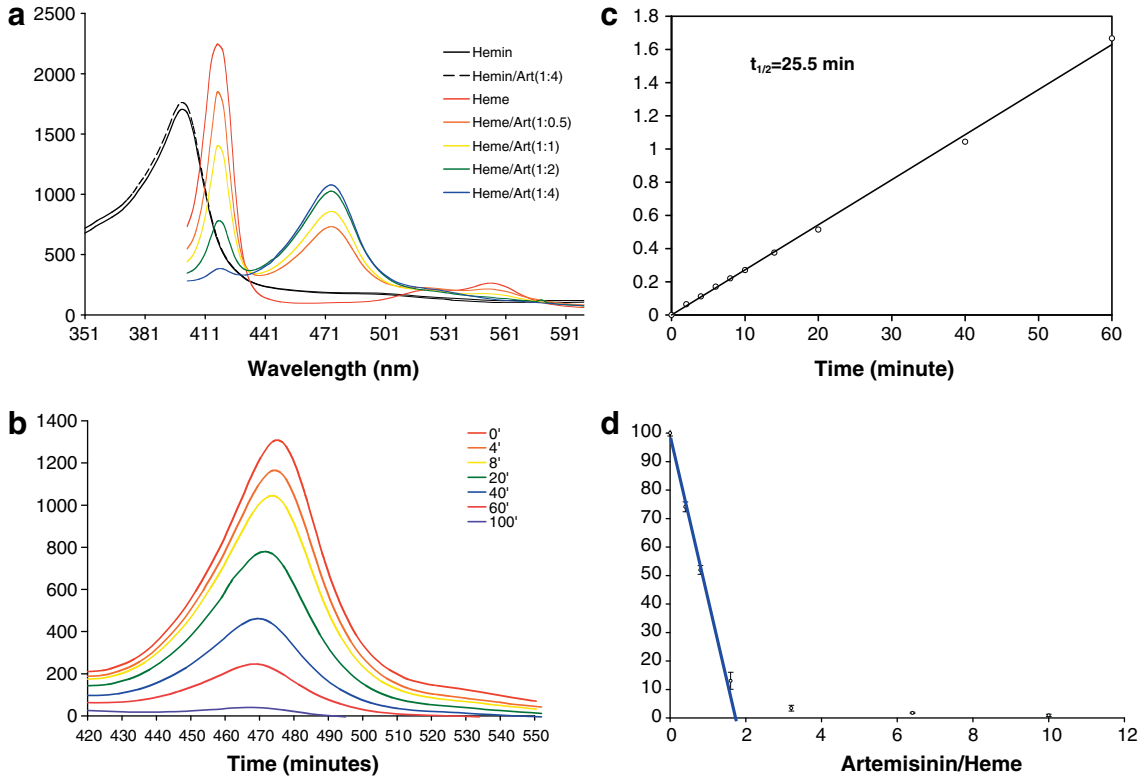

Figure 1. (a) Activation of artemisinin by (ferrous) heme but not (ferric) hemin. Absorption spectra of hemin and heme in the presence of increasing molar ratios of artemisinin at pH 5.0. Hemin (black solid line) was converted to heme (red solid line) by the addition of dithionite. Heme (solid red line) absorbance was dramatically by reaction with artemisinin. (b) The heme–artemisinin complex is unstable. The absorbance of the heme–artemisinin peak observed at A_{476} is almost completely gone by 100 min. (c) Determination of half-life of heme–artemisinin peak observed at A_{476}. The half-life is estimated to be 25.5 min. (d) Plot of peak absorbance at 415 nm at different molar ratios of artemisinin–heme to estimate stoichiometry. Using the A_{415} obtained with 20-μM heme as the 100% value, approximately 2 molecules of artemisinin were estimated to interact with one molecule of heme.

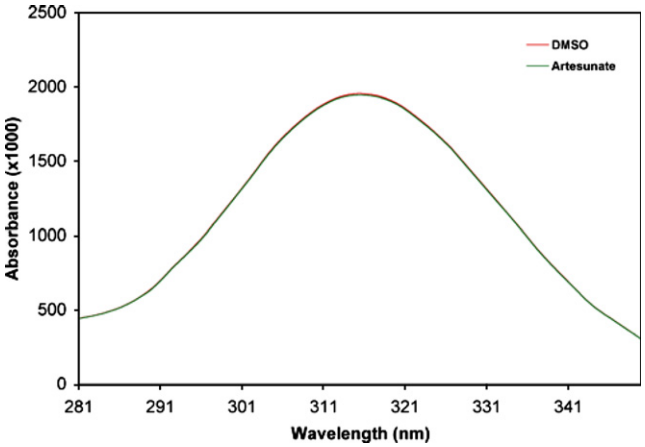

Figure 2. Absorption spectra of dithionite with (black line) or without (red line) artesunate. The molar ratio of artesunate to dithionite was approximately 3:1 in 0.25 M acetate buffer, pH 5.0, with a final DMSO concentration of 20%. The dithionite absorbance spectrum is the same with (artesunate) or without (DMSO) artesunate.

Soret peak to 476 nm, but caused a red shift to 429 nm, as would be observed for a bathochromic shift when ferric hemin is reduced to ferrous heme, also described for 'hemart'.[26] Based upon the extent of reaction at increasing molar ratios, the stoichiometry of the artemisinin–heme reaction was estimated to be two molecules of artemisinin that react with one molecule of heme (Fig. 1d).

To exclude the possibility that artemisinin was first activated by dithionite, which then reacted with heme, the spectra of dithionite with and without artemisinin were compared since oxidation by artemisinin would abrogate the dithionite absorption peak.[27,28] As shown in Figure 2, the presence of artemisinin did not cause a change in the dithionite absorption spectrum, indicating no redox reaction occurred between these two molecules.

2.2. Reaction of artemisinin with inorganic ferrous iron

The interaction of ferrous iron with artemisinin was determined using ferrocyanide as the source of Fe^{2+} because it does not absorb at 419 nm but its oxidized form ferricyanide does, which can be used to follow the oxidation state of the iron during its reaction with artemisinin.[29] Hydrogen peroxide (H_2O_2) was used as a positive control for the generation of ferricyanide. The time course of ferrocyanide oxidation in the presence of artemisinin was compared to the rate of reaction of ferrocyanide with hydrogen peroxide at both pH 5.0 and 7.0 using a 4:1 molar ratio of 8 mM artemisinin or H_2O_2 and 2 mM ferrocyanide (a concentration needed for sufficient signal strength) in a reaction volume that included 30% DMSO (Fig. 3a). No oxidation of the ferrocyanide occurred with either artemisinin or H_2O_2 at pH 7.0. At pH 5.0, however, over 50% of the ferrocyanide was oxidized by H_2O_2 within 12 min, but only about 0.6% with excess artemisinin over the same time frame. These results suggest that the endoperoxide structure of artemisinin is much less effective than H_2O_2 in oxidizing inorganic ferrous iron under these conditions. The relative time course of the reaction of inorganic ferrous iron with artemisinin occurred at a much slower rate than the reaction with (ferrous) heme under similar conditions.

The ability of inorganic ferrous iron to compete with (ferrous) heme in the activation of artemisinin was also determined. Reactions of artemisinin and heme with or without ferrocyanide (or ferrous chloride) were run with the same final concentration of DMSO under reducing (2 mM dithionite) and acidic (acetate buffer pH 5.0) conditions. Absorbance readings at 415 nm (to monitor heme) were taken within one minute after the addition of excess dithionite to reduce heme. Even at 80-fold excess, ferrocyanide was not able to interfere with the reaction between heme and artemisinin (Fig. 3b). Similar

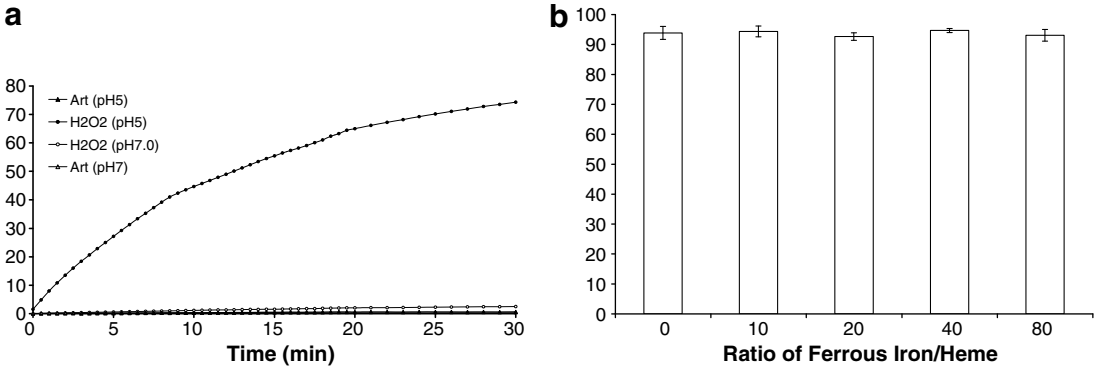

Figure 3. (a) Activation of artemisinin by inorganic ferrous iron. The percent conversion to ferricyanide (measured at 419 nm) was used to determine the time course of oxidation of ferrocyanide by artemisinin and H_2O_2. Only H_2O_2 at pH 5.0 exhibited significant oxidation of ferrocyanide. Artemisinin had little effect over the 30-min incubation period. (b) Competition of ferrous iron with (ferrous) heme in the activation of artemisinin. The molar ratio of artemisinin to heme was 2:1 with molar ratios of inorganic ferrous iron to heme that ranged from 0 to 80. Absorption measurements were taken at 415 nm within one minute after the addition of dithionite to reduce hemin to heme. The A_{415} of the reaction mix without heme was used as complete inhibition (100% reduction) of artemisinin activation, and the absorbance of the reaction mix without artemisinin (but with heme and ferrocyanide) was taken as no (0%) reduction in activation. Even at 80-fold excess, ferrous irons were not able to interfere with the reaction between heme and artemisinin. Results are expressed as means of triplicates with SD.

results were obtained using ferrous chloride as the source of inorganic iron (data not shown).

2.3. Reaction of artesunate with hemoglobin

Conflicting data have been reported on the activation of artemisinins by hemoglobin in neutral or weakly basic (pH 7.0–8.0) buffer conditions.[30,31] The interaction of artesunate with either metHb, deoxyHb, or oxyHb was therefore determined. Because hemoglobin is sensitive to changes in protein conformation in non-aqueous environments, an aqueous buffer system was used. Sodium artesunate, a derivative of artemisinin, was used because it is more soluble in aqueous solutions[17] but has similar reactivity as artemisinin toward (ferrous) heme (data not shown). All assays involving hemoglobin therefore had only ~1% DMSO unless otherwise specified.

The reaction of sodium artesunate and metHb was determined spectrophotometrically at neutral pH in phosphate buffer. No reaction was observed with or without artesunate over 30 min (Fig. 4a). The reaction of artemisinin with deoxyHb was then determined. deoxyHb was produced by reducing metHb with dithionite as described in Materials and Methods. Its presence was confirmed by the Soret band peak at 429 nm and a broad single Q band at 555 nm (Fig. 4a). A decrease in Soret band absorbance was used as the indicator of the reaction between deoxyHb and artesunate. At pH 7.0, deoxyHb exhibited only a very slight decrease in the Soret band absorption (Fig. 4a). The same experiments were then performed at pH 5.0 (Fig. 4b). At this pH, similar results were obtained for metHb, consistent with little or no reaction after 30 min of incubation with artesunate. The Soret band absorption of deoxyHb, however, decreased more than when measured at pH 7.0. The kinetics of reaction between deoxyHb and artesunate at the two pH levels was then determined (Fig. 4c). Most of the reaction occurs within the first minute of incubation. To exclude the possibility that the presence of dithionite may have altered the structure or conformation of the Hb exposed the heme moiety, deoxyHb was preincubated with dithionite for 30 min prior to the addition of artesunate, which had no effect on the rate of reaction (data not shown).

With little reaction occurring with metHb or deoxyHb, oxyHb was next studied. A significant decrease in the Soret peak absorbance of oxyHb after a 24-h incubation with artesunate at 37 °C was previously reported and the results were interpreted as an artesunate–oxyHb interaction.[17] This result may be somewhat unexpected because the heme iron in oxyHb is tightly bound with oxygen and therefore is unlikely to be accessible for activation of artemisinins. The previous report also did not include an oxyHb only control. We replicated the experiments using the same conditions, but included an oxyHb only control. The presence of oxyHb was confirmed by the absorption spectrum pattern (Fig. 5a) of the Soret band at 414 nm and two Q bands at 541 nm and 576 nm, respectively.[32] The spectra of oxyHb were recorded over 24 h at 37 °C using conditions similar to those that were

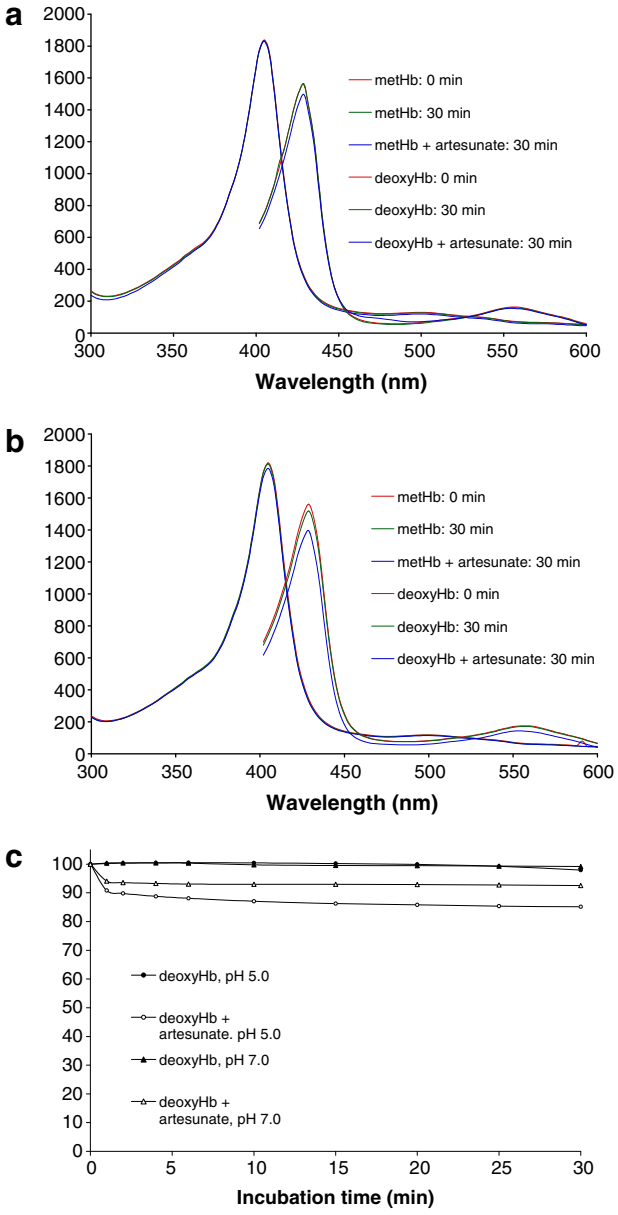

Figure 4. (a) Little interaction occurs between artesunate and deoxyhemoglobin. Absorption spectra of metHb and deoxyHb with artesunate at pH 7.0 in phosphate buffer at a 1:2 molar ratio. MetHb has no effect on artesunate, while deoxyHb has only a slight interaction based upon Soret band absorbance after 30 min. (b) Absorption spectra of metHb and deoxyHb with artesunate at pH 5.0. The results obtained for metHb were similar to those at neutral pH, while deoxyHb had an increased, but still small, degree of interaction with artesunate. (c) Quantitative comparison of interaction of deoxyHb and artesunate at two different pH levels based upon absorbance at 429 nm. The interaction between deoxyHb and artesunate is greater at pH 5.0, but still very small as compared to the interaction of artesunate with heme.

previously found to show an interaction between oxyHb and artesunate.[17,30] A 10 mM ammonium acetate buffer was used at pH 8.0 with 4% DMSO. At 37 °C, the Soret band absorbance of oxyHb did indeed decrease, but this occurred whether artesunate was present or not (Fig. 5a vs Fig. 5b).

Because specific reaction conditions could affect the interaction of artesunate and oxyHb, in particular,

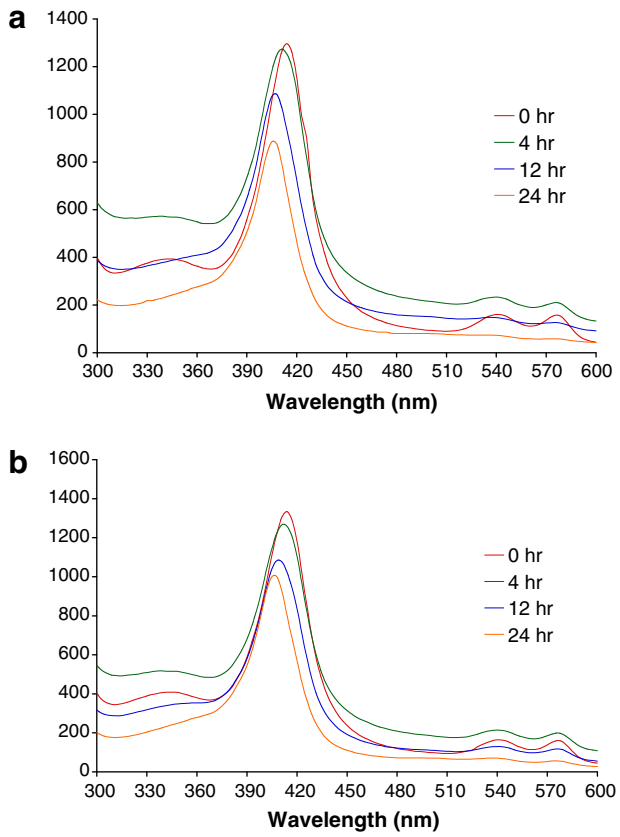

Figure 5. (a) No interaction occurs between artesunate and oxyHb at pH 8.0. Absorption spectra of oxyHb at pH 8.0 (ammonium acetate buffer with 4% DMSO) over 24 h at 37 °C. OxyHb undergoes a spontaneous decay over 24 h. (b) Absorption spectra of oxyHb and artesunate at pH 8.0 (acetate buffer with 4% DMSO) over 24 h at 37 °C. The absorption spectra with artesunate are essentially unchanged over an 8:1 molar ratio of artesunate to heme (results for 2:1 shown), indicating little or no interaction between oxyHb and artesunate.

DMSO and pH levels could alter the protein microenvironment/structure to render the heme group more accessible or reactive to artesunate,[33] experiments were performed using phosphate buffer with minimal DMSO at neutral pH, also similar to conditions previously used.[17] In phosphate buffer at pH 7.0, some spontaneous decay in the oxyHb absorbance was observed during the period of incubation, with a much slower rate at room temperature (Fig. 6a) than at 37 °C (Fig. 6c). Under both temperature conditions, similar decreases in the oxyHb absorption spectra were observed in the samples including artesunate (Fig. 6a vs Fig. 6b and Fig. 6c vs Fig. 6d) up to an 8:1 molar ratio (data not shown). Similar results were obtained with a 2:1 molar ratio of ascorbic acid (data not shown), as used in previous studies.[17] These results indicate that artesunate does not react with oxyHb to cause the observed spectral changes, which are likely due to spontaneous changes in oxyHb.

3. Discussion

Artemisinin drugs harbor a unique endoperoxide bridge that undergoes a redox-mediated activation[34,35] that is essential for their anti-malarial activity. Derivatives without the endoperoxide structure (e.g., deoxyartemisinin) lack anti-malarial activity.[36] Activation of artemisinin involves reductive homolysis of the endoperoxide bridge by a reducing equivalent obtained from the parasite's microenvironment, followed by formation of C4-centered carbon radicals that can alkylate other molecules.

Artemisinin drugs are toxic to the malaria parasites in their vegetation stage in host red blood cells, where they engulf and digest large amounts of hemoglobin in the food vacuole, releasing very high concentrations of heme that can be further degraded to release iron. The exact distribution of different types of iron-containing molecules in the parasitic food vacuole is not yet known. The parent molecule from which all other iron-containing species are derived is hemoglobin, which harbors (ferrous) heme when it is engulfed into the food vacuole. The known acidic pH and relatively high level of reducing equivalents (i.e., glutathione[37]) would slow the oxidative rates of ferrous iron and ferrous heme. A variety of in vitro studies have implicated hemoglobin, heme, or iron in the activation of artemisinins,[1,17] however their relative reactivities, and therefore their potential pharmacological relevance in mediating artemisinin drugs' anti-malarial function, are still not known. Since artemisinin drugs are effective within a short time after administration the activation of the drug is relatively rapid,[6] thus short-term studies may be most relevant. The results reported here demonstrate that under the in vitro conditions tested, (ferrous) heme reacts with artemisinin much more efficiently than the other iron-containing molecules analyzed.

Previous in vitro studies indicate that heme promotes radical formation in, and forms adducts with, artemisinins.[10,26,38,39] Previous studies using glutathione to maintain the reducing environment with similar conditions as used here, found that the formation of the artemisinin–heme adduct reached 85% in 1 h at 37 °C, with heme derivatives alkylated by artemisinin at different meso-positions.[40] These results suggest that heme may be both the trigger and the target of artemisinins. In vivo, artemisinin alkylated heme was also detected in a mouse model of malaria using pharmacologically relevant doses of the drug.[41]

The potential mechanisms by which heme is involved in the malarial toxicity of artemisinin have also been studied. Heme dramatically increased the inhibitory effect of artemisinin on the parasite's cysteine protease digestion of hemoglobin.[31] Heme–artemisinin adducts have been reported to be incapable of self-polymerization, competitively inhibiting the binding of heme to parasite histine-rich protein II, thereby blocking heme polymerization to form beta-hematin,[26] although other data have been reported that do not support this mechanism.[15]

In addition to (ferrous) heme, activation of artemisinin by hemin (ferric heme) has also been reported, as evidenced by a significant decrease in hemin Soret band absorbance over a 24 h period.[7] These results were not

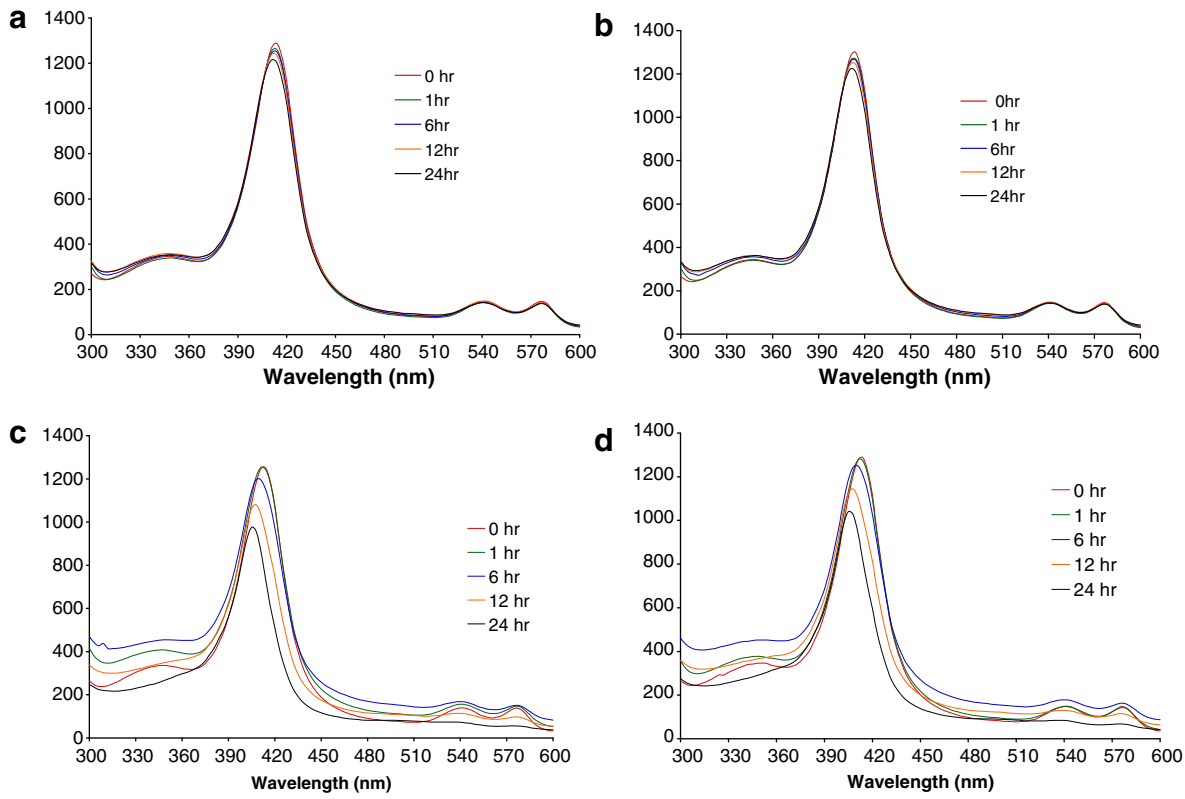

Figure 6. (a) No interaction occurs between artesunate and oxyHb at pH 7.0 in phosphate buffer. Absorption spectra of oxyHb at pH 7.0 over 24 h at room temperature. OxyHb undergoes a slight spontaneous decay over 24 h. (b) Absorption spectra of oxyHb and artesunate at pH 7.0 over 24 h at room temperature. The absorption spectra with artesunate are essentially unchanged from oxyHb by itself (A above) over an 8:1 molar ratio of artesunate to heme (results for 2:1 shown), indicating little or no interaction between oxyHb and artesunate. (c) Absorption spectra of oxyHb at pH 7.0 over 24 h at 37 °C. OxyHb undergoes a greater spontaneous decay over 24 h than at room temperature (A above). (d) Absorption spectra of oxyHb and artesunate at pH 7.0 over 24 h at 37 °C. The absorption spectra with artesunate are essentially unchanged from oxyHb by itself (C above) over an 8:1 molar ratio of artesunate to heme (results for 2:1 shown), indicating little or no interaction between oxyHb and artesunate.

replicated here using an air-tight cuvette with hemin and artemisinin, or with a hemin only control (data not shown). In an another report, hemin–artemisinin adducts were detected in dimethyl acetamide solvent containing 200 mM hemin and artemisinin at 37 °C for 24 h.[26] However, an artemisinin only control was not included in those experiments, and in subsequent studies, adducts were not detected in aqueous acetonitrile after 1 h incubation.[16]

Artemisinin has been reported to interact with deoxyhemoglobin,[42,43] although our results indicate that the extent of reaction is relatively low. Previous studies showed that artemisinin binds only weakly to normal hemoglobin but strongly to abnormal hemoglobin H (HbH), which cannot effectively transport oxygen.[44] The strong binding may be due to better access of the drug to the heme molecule in HbH relative to normal hemoglobin A. In patients with alpha-thalassemia, this creates a sink that partitions artemisinin away from the parasite-infected erythrocytes, which may account for why the drug is less effective in patients with alpha-thalassemia.[44]

An interaction between oxyHb with artesunate has been reported at alkaline pH (8.0) with a 1.2:1 artesunate:oxyHb ratio at 37 °C within 1 h using HPLC.[30] However, the conditions used for HPLC likely denatured the Hb and exposed free heme that could then interact with artesunate. Similar results were reported for metHb and artesunate using HPLC and MS, which would also likely be complicated by denaturation of the Hb with 50% acetonitrile.[16,17] Another question not addressed was the degree of oxygenation of the Hb. In follow-up studies, the spectral absorption of the oxyHb–artesunate reaction was reported.[8,17] However, an oxyHb only control was not included, which, as shown here, would manifest decay of the Soret band absorption even without artesunate.

Our results showed that, while deoxyHb interacts with artemisinin at a slow rate, oxyHb is inert to artesunate. These data may be of physiological relevance, since oxyHb in circulation does not appear to be able to activate artemisinin. Following engulfment into the parasite's food vacuole, oxyHb would be exposed to an acidic pH which, according to the Bohr Effect, would drive the dissociation of oxygen from oxyHb to become deoxyHb. Such spatial restriction of artemisinin activation might be one of the reasons that the drugs are relatively nontoxic to the human host but very potent to the parasite.

Inorganic iron has also been implicated in artemisinin activation.[12] Artemisinin has been reported to oxidize

ferrous ions and reduce ferric ions to produce dihydroartemisinin,[45] although this appears to involve the lactone side groups and not the endoperoxide bridge, thus the relevance to drug activation being not clear. The reaction of artemisinin with ferrous sulfate in aqueous acetonitrile produced a number of stable isomeric rearrangement products.[46] We did not see a significant reaction with ferrous iron, which also could not compete with the interaction of heme and artemisinin.

Alternative redox mechanisms for the activation of artemisinins independent of heme may also exist. For example, the sub-cellular distribution of artemisinins in infected RBCs indicates that artemisinins can be found in multiple organellar compartments in addition to the food vacuole.[47] Redox systems are likely present in other parasite sub-cellular compartments that could serve as an activation mechanism for artemisinin, although the concentration of redox active heme in the food vacuole may be quite high. In addition, artemisinin derivatives are hydrophobic, as is heme, and may also partition into biological membranes such as in the food vacuole.

Malaria infection is associated with severe hemolysis that releases ferrous hemoglobin, which can be readily oxidized. For example, in the presence of reactive oxygen species (ROS) generated by white blood cells, free ferrous hemoglobin can be readily oxidized to ferric methemoglobin (MetHb), the major heme species in chronic hemolysis,[48] from which free hemin is released. Carbon monoxide (CO) has been shown in a mouse model to arrest this process through binding of CO to Fe^{2+} in the heme groups of ferrous hemoglobin, which then prevented hemoglobin oxidation and subsequent release of free hemin from oxidized hemoglobin.[49] The existence of significant circulating levels of free ferrous heme could potentially activate artemisinin during the hemolytic stages of malaria. Pharmacological manipulation of hemoglobin oxidation to increase the amount of free ferrous heme, for example, through scavenging ROS, could thus potentially increase the efficacy of artemisinin. Several previous studies have examined the role of antioxidants as a potential primary and/or adjuvant treatment of malaria.[50] For example, certain compounds with potential antioxidant activity may increase the effectiveness of artemisinin.[51] Our results may point to a focus on heme as the key molecule to modulate, although the pro- and antioxidant defense systems in both the human host and malarial parasite are complex.[52]

The activation of artemisinin by heme may also have relevance in the induction of apoptosis by artemisinin and related compounds.[53] Cytotoxicity of these endoperoxides toward rapidly dividing human cancer cells has been reported,[18,19] and has been hypothesized to be dependent upon activation of the endoperoxide bridge by an iron(II) species to form C-centered radicals that trigger caspase-dependent apoptosis.[18] Exploiting artemisinin activation by heme for apoptosis may be a novel mechanism for developing anti-cancer therapeutics.

4. Experimental

4.1. Chemical reagents

Hemin (ferric heme chloride, >98% HPLC, Fluka 51280), potassium ferrocyanide, ferrous chloride, dimethyl sulfoxide (DMSO), sodium dithionite (>82%), artemisinin, methemoglobin (metHb, from horse hemoglobin), ascorbic acid, and buffers and salts were purchased from Sigma–Aldrich (St. Louis, MO). Sodium artesunate was a gift from Dafra Pharma N.V. (Turnhout, Belgium). Reverse osmosis-purified water was used for all experiments. A hemin stock solution at 2 mM was prepared in 0.05 N NaOH (used immediately) or in DMSO (used immediately or stored at −20 °C for later use). Artemisinin and sodium artesunate stock solutions were prepared in DMSO at 10 mM. A hemoglobin stock solution (0.5 mM) was freshly prepared in phosphate buffered saline (PBS) at pH 7.2 before use. Sodium acetate buffer at pH 5.0 was made at 0.25 M in water with or without DMSO as specified in the assays. Saturated sodium dithionite stock solutions were freshly prepared in 50 mM phosphate buffer, pH 7.0, or in sodium acetate buffer before use. A 10 mM ascorbic acid stock solution was freshly prepared in water and used immediately. Solutions of ferrous chloride and potassium ferrocyanide at 20 mM were freshly prepared in water prior to use.

4.2. Artemisinin interaction assays

All assays were done at room temperature or 37 °C. All assays were cuvette-based, with a 1-ml total volume, except for the determination of heme–artemisinin stoichiometry and the inorganic iron and heme-competition experiments, in which microplates with a total volume of 260 μl were used.

4.2.1. Interaction of artemisinin with hemin and heme. Absorption spectra were performed in a 1-ml cuvette using either ammonium acetate or phosphate buffer. For hemin, 10 μl of a 2 mM hemin solution in DMSO (20 μM in final), 10 μl of artemisinin solution at various concentrations in DMSO, and 980 μl of 0.2 M acetate (20% DMSO) pH 5.0 buffer solution or 0.1 M phosphate (20% DMSO) pH 7.0 buffer solution were combined and the spectrum measured. In order to generate heme through reduction of hemin, 2 mM dithionite was included in the buffer.

To investigate the absorbance peak observed at 476 nm after the reaction of heme and artemisinin, a large volume of 1 M KCl solution was added within seconds of the complete disappearance of the Soret band characteristic of heme and the appearance of the full peak at 476 nm, to consume dithionite and precipitate the heme-containing complex. The precipitate was recovered by centrifuging at 13,000*g* followed by three washes with 1 M KCl and then one wash with water. The precipitate was re-dissolved in DMSO for determination of the absorption spectrum in 0.2 M acetate, pH 5 with or without 50 mM dithionite.

4.3. Interaction of artemisinin and inorganic iron

The time course of the reaction (consisting of 2 mM ferrocyanide and 8 mM artemisinin or hydrogen peroxide in ammonium acetate (pH 5.0) or phosphate (pH 7.0) buffer, with 30% DMSO) was recorded at 419 nm in 1-ml containing cuvette. The heme-competition experiments were conducted in ammonium acetate buffer pH 5.0 (22% DMSO in final) in microplates with a total volume of 260 μl containing 20 μM heme, 40 μM artemisinin, and various concentrations of ferrocyanide or ferrous chloride at a range of molar ratios of inorganic iron to heme from 0 to 80.

4.4. Interaction of artesunate with different forms of hemoglobin

MetHb stock solution (0.5 mM in PBS) was used as the initial source of hemoglobin for all experiments. The final concentrations of various forms of hemoglobin in all assays was 5 μM (equivalent to 20 μM hemin or heme iron). The molar ratio of artesunate to hemoglobin was 2:1, similar to that used in previous studies.[17,30] Both deoxygenated hemoglobin (deoxyHb) and oxygenated hemoglobin (oxyHb) were made from metHb using dithionite. For deoxyHb, 10 μl of metHb solution was first added to 980-μl reaction buffer solution, then saturated dithionite stock solution was added. By doing so, deoxyHb was formed because the oxygen in the reaction solution was rapidly consumed by the dithionite, which was confirmed by its characteristic absorption spectrum. In order to make oxyHb, 150-μl concentrated metHb solution (0.5 mM) was added to 2-μl saturated stock dithionite solution to make a concentrated deoxyHb solution. When 10 μl of this solution was added to 980-μl phosphate buffer solution, the deoxyHb was oxygenated to form oxyHb, confirmed by its characteristic absorption spectral pattern. During 24-h incubations, all cuvettes were sealed to prevent exposure to air.

4.5. Spectrophotometry

Assays with cuvettes were recorded on a Spectronic® Genesys™ 5 instrument (Spectronic Instruments, Inc. Rochester, NY) at room temperature. Readings on microplates were done using a Spectra MAX250 microplate reader (Sunnyvale, CA, USA).

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References and notes

1. Meshnick, S. R.; Taylor, T. E.; Kamchonwongpaisan, S. *Microbiol. Rev.* **1996**, *60*, 301–315.
2. Kapetanaki, S.; Varotsis, C. *FEBS Lett.* **2000**, *474*, 238–241.
3. Posner, G. H.; Oh, C. H.; Wang, D.; Gerena, L.; Milhous, W. K.; Meshnick, S. R.; Asawamahasadka, W. *J. Med. Chem.* **1994**, *37*, 1256–1258.
4. Woodrow, C. J.; Haynes, R. K.; Krishna, S. *Postgrad. Med. J.* **2005**, *81*, 71–78.
5. Afonso, A.; Hunt, P.; Cheesman, S.; Alves, A. C.; Cunha, C. V.; do Rosario, V.; Cravo, P. *Antimicrob. Agents Chemother.* **2006**, *50*, 480–489.
6. Alin, M. H.; Bjorkman, A. *Am. J. Trop. Med. Hyg.* **1994**, *50*, 771–776.
7. Bilia, A. R.; Lazari, D.; Messori, L.; Taglioli, V.; Temperini, C.; Vincieri, F. F. *Life Sci.* **2002**, *70*, 769–778.
8. Messori, L.; Piccioli, F.; Eitler, B.; Bergonzi, M. C.; Bilia, A. R.; Vincieri, F. F. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4055–4057.
9. Meshnick, S. R. *Antimicrob. Agents Chemother.* **2003**, *47*, 2712, author reply 2712-3.
10. Robert, A.; Coppel, Y.; Meunier, B. *Alkylation of Heme by the Antimalarial Drug Artemisinin*. In *Chemical Communications*; Cambridge: England, 2002; Vol. 5.
11. Eckstein-Ludwig, U.; Webb, R. J.; Van Goethem, I. D.; East, J. M.; Lee, A. G.; Kimura, M.; O'Neill, P. M.; Bray, P. G.; Ward, S. A.; Krishna, S. *Nature* **2003**, *424*, 957–961.
12. Meshnick, S. R.; Yang, Y. Z.; Lima, V.; Kuypers, F.; Kamchonwongpaisan, S.; Yuthavong, Y. *Antimicrob. Agents Chemother.* **1993**, *37*, 1108–1114.
13. Reeder, B. J.; Wilson, M. T. *Chem. Research Toxicol.* **2005**, *18*, 1004–1011.
14. Szpilman, A. M.; Korshin, E. E.; Hoos, R.; Posner, G. H.; Bachi, M. D. *J. Org. Chem.* **2001**, *66*, 6531–6540.
15. Haynes, R. K.; Monti, D.; Taramelli, D.; Basilico, N.; Parapini, S.; Olliaro, P. *Antimicrob. Agents Chemother.* **2003**, *47*, 1175.
16. Kannan, R.; Kumar, K.; Sahal, D.; Kukreti, S.; Chauhan, V. S. *Biochem. J.* **2005**, *385*, 409–418.
17. Messori, L.; Gabbiani, C.; Casini, A.; Siragusa, M.; Vincieri, F. F.; Bilia, A. R. *Bioorg. Med. Chem.* **2006**, *14*, 2972–2977.
18. Mercer, A. E.; Maggs, J. L.; Sun, X. M.; Cohen, G. M.; Chadwick, J.; O'Neill, P. M.; Park, B. K. *J. Biol. Chem.* **2007**, *282*, 9372–9382.
19. Nam, W.; Tak, J.; Ryu, J. K.; Jung, M.; Yook, J. I.; Kim, H. J.; Cha, I. H. *Head Neck* **2007**, *29*, 335–340.
20. Meshnick, S. R.; Thomas, A.; Ranz, A.; Xu, C. M.; Pan, H. Z. *Mol. Biochem. Parasitol.* **1991**, *49*, 181–189.
21. Egan, T. J.; Ncokazi, K. K. *J. Inorg. Biochem.* **2004**, *98*, 144–152.
22. Hayward, R.; Saliba, K. J.; Kirk, K. *J. Cell Sci.* **2006**, *119*, 1016–1025.
23. Yayon, A.; Cabantchik, Z. I.; Ginsburg, H. *EMBO J.* **1984**, *3*, 2695–2700.
24. Sullivan, D. J., Jr.; Gluzman, I. Y.; Russell, D. G.; Goldberg, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11865–11870.
25. Di Iorio, E. E. Preparation of Derivatives of Ferrous and Ferric Hemoglobin. In *Methods in Enzymology*; Antonini, E., Rossi-Bernadi, L., Chiancone, E., Eds.; Academic Press, Inc.: New York, 1981; pp 57–72.
26. Kannan, R.; Sahal, D.; Chauhan, V. S. *Chem. Biol.* **2002**, *9*, 321–332.
27. Dixon, M. *Biochim. Biophys. Acta* **1971**, *226*, 241–258.
28. Ljones, T.; Burris, R. H. *Anal. Biochem.* **1972**, *45*, 448–452.
29. Konstantinov, A. A.; Vygodina, T.; Capitanio, N.; Papa, S. *Biochim. Biophys. Acta* **1998**, *1363*, 11–23.
30. Selmeczi, K.; Robert, A.; Claparols, C.; Meunier, B. *FEBS Lett.* **2004**, *556*, 245–248.
31. Pandey, A. V.; Tekwani, B. L.; Singh, R. L.; Chauhan, V. S. *J. Biol. Chem.* **1999**, *274*, 19383–19388.
32. Antonini, E.; Brunori, M. Hemoglobin and Myoglobin in their Reactions with Ligands. In *Frontiers of Biology Specific Aspects of the Reactions of Hemoglobin with Ligands*; Neuberger, A., Tatum, E. L., Eds.; North-Holland Publishing Co.: Amsterdam, 1971; pp 235–285.

33. Liu, C.; Bo, A.; Cheng, G.; Lin, X.; Dong, S. *Biochim. Biophys. Acta* **1998**, *1385*, 53–60.
34. Meshnick, S. R. *Int. J. Parasitol.* **2002**, *32*, 1655–1660.
35. Kamchonwongpaisan, S.; Meshnick, S. R. *Gen. Pharmacol.* **1996**, *27*, 587–592.
36. Meshnick, S. R. *Med. Trop. (Mars)* **1998**, *58*, 13–17.
37. Zhang, J.; Krugliak, M.; Ginsburg, H. *Mol. Biochem. Parasitol.* **1999**, *99*, 129–141.
38. Cazelles, J.; Robert, A.; Meunier, B. *J. Org. Chem.* **2002**, *67*, 609–619.
39. Benoit-Vical, F.; Robert, A.; Meunier, B. *Antimicrob. Agents Chemother.* **2000**, *44*, 2836–2841.
40. Robert, A.; Coppel, Y.; Meunier, B. *Chem. Commun. (Camb.)* **2002**, 414–415.
41. Robert, A.; Benoit-Vical, F.; Claparols, C.; Meunier, B. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13676–13680.
42. Bakhshi, H. B.; Gordi, T.; Ashton, M. *J. Pharm. Pharmacol.* **1997**, *49*, 223–226.
43. Yang, Y. Z.; Little, B.; Meshnick, S. R. *Biochem. Pharmacol.* **1994**, *48*, 569–573.
44. Vattanaviboon, P.; Wilairat, P.; Yuthavong, Y. *Mol. Pharmacol.* **1998**, *53*, 492–496.
45. Sibmooh, N.; Udomsangpetch, R.; Kujoa, A.; Chantharaksri, U.; Mankhetkorn, S. *Chem. Pharm. Bull. (Tokyo)* **2001**, *49*, 1541–1546.
46. Creek, D. J.; Chiu, F. C.; Prankerd, R. J.; Charman, S. A.; Charman, W. N. *J. Pharm. Sci.* **2005**, *94*, 1820–1829.
47. Maeno, Y.; Toyoshima, T.; Fujioka, H.; Ito, Y.; Meshnick, S. R.; Benakis, A.; Milhous, W. K.; Aikawa, M. *Am. J. Trop. Med. Hyg.* **1993**, *49*, 485–491.
48. Jeney, V.; Balla, J.; Yachie, A.; Varga, Z.; Vercellotti, G. M.; Eaton, J. W.; Balla, G. *Blood* **2002**, *100*, 879–887.
49. Pamplona, A.; Ferreira, A.; Balla, J.; Jeney, V.; Balla, G.; Epiphanio, S.; Chora, A.; Rodrigues, C. D.; Gregoire, I. P.; Cunha-Rodrigues, M.; Portugal, S.; Soares, M. P.; Mota, M. M. *Nat. Med.* **2007**, *13*, 703–710.
50. Turrens, J. F. *Mol. Aspects Med.* **2004**, *25*, 211–220.
51. Sannella, A. R.; Messori, L.; Casini, A.; Francesco Vincieri, F.; Bilia, A. R.; Majori, G.; Severini, C. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 177–181.
52. Becker, K.; Tilley, L.; Vennerstrom, J. L.; Roberts, D.; Rogerson, S.; Ginsburg, H. *Int. J. Parasitol.* **2004**, *34*, 163–189.
53. Singh, N. P.; Lai, H. C. *Anticancer Res.* **2004**, *24*, 2277–2280.