Reaction of artemisinin with haemoglobin: implications for antimalarial activity

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Elucidation of the principal targets of the action of the antimalarial drug artemisinin is an ongoing pursuit that is important for understanding the action of this drug and for the development of more potent analogues. We have examined the chemical reaction of Hb with artemisinin. The protein-bound haem in Hb has been found to react with artemisinin much faster than is the case with free haem. It appears that the uptake of Hb and the accumulation of artemisinin into the food vacuole, together with the preferred reactivity of artemisinin with haem in Hb, may make Hb the primary target of artemisinin's antimalarial action. Both monoalkylated (HA) and dialkylated (HAA) haem derivatives of artemisinin have been isolated. These 'haemarts' bind to PfHRP II (*Plasmodium falciparum* histidine-rich protein II), inhibiting haemozoin formation, and possess a significantly decreased ability to oxidize ascorbic acid. The accelerated formation of HAA from Hb is expected to decrease the ratio of haem to its alkylated derivatives. The haemarts that are generated from 'haemartoglobins' may bring about the death of malaria parasite by a two-pronged effect of stalling the formation of haemozoin by the competitive inhibition of haem binding to its templates and creating a more reducing environment that is not conducive to the formation of haemozoin.

Key words: artemisinin, haemoglobin, haemozoin, *Plasmodium falciparum* histidine-rich protein II (PfHRP II), redox activity.

INTRODUCTION

Malaria, one of the commonest diseases in tropical countries, is responsible for more than 1 million deaths worldwide each year [1]. Widespread resistance of *Plasmodium falciparum* to quinoline-based drugs has made the disease situation difficult to manage in endemic malaria areas. In spite of serious efforts, a successful malaria vaccine has remained a distant dream, and therefore development of new antimalarials is crucial to the control and management of the disease. For this, novel drug targets and pathways governing the life cycle of the parasite, preferably those that are unique to the parasite, need to be characterized. With the availability of the complete genome sequence of *P. falciparum*, several new drug targets have already been characterized [2]. However, a clear understanding of the mechanism of action of the proven antimalarial drugs can also help in the development of new antimalarials. Since the most commonly used antimalarials target the blood stages of the parasite, a proper understanding of drug action in the infected red blood cell assumes great importance.

During the blood stages, the malaria parasite utilizes host Hb as a major source of amino acids for its own protein synthesis [3,4]. However, the degradation of Hb in the food vacuole is accompanied by the release of free haem, which is extremely toxic to the parasite [5]. There is concomitant production of superoxide anion; this dismutates to H_2O_2 with almost $\sim$15 mmol/litre H_2O_2 being produced, resulting in exposure of the parasites to high fluxes of reactive oxygen species [6]. The malaria parasite, which lacks haem oxygenase enzymes [7], averts the toxicity of the liberated haem mostly by converting it into haemozoin or malarial pigment. Haemozoin, which was previously thought to be a polymer, is now known to be a dimer with hydrogen bonding between the dimer units in the crystal. Several antimalarial drugs, including chloroquine and artemisinin, recognize haem as a target molecule and are known to inhibit the formation of haemozoin [8,9]. The mechanism of biosynthesis of haemozoin, a process unique to the parasite, is not clearly understood, and the search for a haem polymerase has not been fruitful. The HRPs (histidine-rich proteins) of *P. falciparum* (PfHRP II, III, IV and membrane-associated histidine-rich protein-1 etc.), which readily bind multiple haem monomers [10–12], have been suggested to play a crucial role in the haem detoxification process. These HRPs may also be involved in the neutralization of toxic haem in the parasite cytoplasm and infected red blood cells by removing haem that is membrane bound [13]. However, the survival of PfHRP II and III knockout strains of the malaria parasite may suggest that alternative mechanisms of haem detoxification exist.

Artemisinin and its derivatives are effective against multidrug-resistant *P. falciparum* strains, and have been in use for more than two decades, mainly in Southeast Asia and more recently in Africa, without any reported cases of resistance [14]. Artemisinins owe their antimalarial activity to the presence of an endoperoxide bridge, since deoxyartemisinin, which lacks the bridge, is devoid of antimalarial activity [15]. The infected red blood cells, with extensive modifications in the plasma membrane, facilitate the active uptake of artemisinin into the food vacuole, creating a nanmolar to micromolar concentration gradient across the food vacuole membrane [16,17]. Several studies have suggested that the haem-promoted cleavage of the peroxide in artemisinin, leading to the formation of C-radicals which alkylate some proteins of the malaria parasite [18], also contributes to its antimalarial action. Specific reactions of artemisinin with TCTP (translationally controlled tumour protein) [19], inhibition of the SERCA

Abbreviations used: DAB, diaminobenzidine; DMA, dimethyl acetamide; HA, monoalkylated haem–artemisinin adduct; HAA, dialkylated haem–artemisinin adduct; HRP, histidine-rich protein; MALDI, matrix-assisted laser-desorption ionization–time-of-flight; PfHRP II, *Plasmodium falciparum* histidine-rich protein II; Mb, myoglobin; OPD, *o*-phenylene diamine; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; TCTP, translationally controlled tumour protein.

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(sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase) orthologue (PfATP6) of *P. falciparum* [20] and inhibition of *P. falciparum* cysteine proteases have also been proposed to contribute to its drug activity [21]. On the other hand, Hong et al. [22] reported that artemisinin forms covalent adducts with haem in artemisinin-treated parasite cultures.

We have shown previously that the haem–artemisinin adduct, or 'haemart', is a crucial mediator of the ability of artemisinin to inhibit haem polymerization [9]. Here we show that the reaction of artemisinin with haem in Hb is much faster than with free haem. This reaction of Hb with artemisinin has been found to give rise to mono- and di-alkylated derivatives of haem (HA and HAA respectively). These adducts inhibit PfHRP II-mediated haemozoin synthesis. In addition, the redox properties of haemarts are quite distinct from those of haem. The antimalarial action of artemisinin may originate from (a) the ability of these adducts to inhibit haemozoin synthesis and (b) altered redox properties of haemarts.

EXPERIMENTAL

Materials

Artemisinin, L-ascorbic acid, β-mercaptoethanol, DAB (diaminobenzidine), DMA (dimethyl acetamide), DMSO, haemin, Hb, Mb (myoglobin), OPD (*o*-phenylene diamine) and SDS were from Sigma-Aldrich (St. Louis, MO, U.S.A.). Octadecyl silica (C_{18}, 55–105 μm, 125 Å), used for the purification of alkylated products, was from Waters Millipore Corp. (Milford, MA, U.S.A.). Acetonitrile, propan-2-ol, methanol, hydrogen peroxide and Giemsa stain were from Merck. All other reagents were of analytical grade. Protein estimation was done using the bicinchonic acid method (Pierce, Rockford, IL, U.S.A.). Haemin was recrystallized as described previously [9]. Human Hb used in the experiments was purified from blood following the procedure described by Roy and Acharya [23]. Protein concentration was estimated by measuring absorbance at 540 nm (0.8 absorbance unit = 1 mg/ml). Recombinant PfHRP II was expressed and purified as described previously [9].

Reactions of artemisinin with Hb and haem

Comparison of the reactivity of artemisinin with free haem, Hb and Mb

Hb (0.64 mg; 0.01 μmol = 0.04 μmol of haem), Mb (0.68 mg; 0.04 μmol = 0.04 μmol of haem) and haem (26 μg; 0.04 μmol) were mixed in separate experiments with artemisinin (0.11 mg; 0.4 μmol) in a final volume of 1 ml. The solvent used for the reactions was 1:1 (v/v) acetonitrile/sodium phosphate buffer (100 mM, pH 7). The reaction mixtures were incubated at 37 °C for 1 h under stirring. The measurement of the shift in λ_{max} was by spectral recording (700–300 nm) of 20 μl of the reaction mixture diluted to 1 ml with the same reaction solvent.

Synthesis of HA and HAA obtained from the reaction of Hb with artemisinin

Hb (2.5 mg; 0.038 μmol = 0.152 μmol of haem) was mixed with 1.9 μmol of artemisinin in 1:1 (v/v) acetonitrile/sodium phosphate buffer (100 mM, pH 7). For reverse-phase HPLC analysis, the reaction of Hb was stopped at different time points by adding 1 ml of reaction mixture to 15 ml of ice-cold acid acetone (0.5 %, v/v). The precipitated haem/haemarts were quantified by reverse-phase HPLC [μ-Bondapak C_{18} column (7.8 mm × 300 mm); flow, 2 ml/min; gradient, 30 % acetonitrile/0.1 % trifluoroacetic acid to 100 % acetonitrile/0.1 % trifluoroacetic acid in 40 min; detection at 400 nm].

Reaction between free haem and artemisinin

Haem (130.2 mg, 0.125 mmol) and artemisinin (226.3 mg, 0.5 mmol) were dissolved in 1.6 ml of DMA and incubated with stirring at 37 °C for 24 h. The reaction mixture was then subjected to reverse-phase chromatography on a glass column with 20 g of C_{18} resin pre-equilibrated with 30 % (v/v) acetonitrile/0.1 % trifluoroacetic acid. Chromatographic elution was done by using step gradients in the range 30–100 % (v/v) acetonitrile. The column allowed visible separation of haem (the first to elute) from mono- and di-alkylated products. HAA was subjected to re-chromatography under the same conditions to remove traces of HA. Under these conditions, the yields of homogeneous haemarts were: HAA, 84 mg (79 μmol); HA, 30 mg (36 μmol).

Spectroscopic characterization of HAA

MALDI (matrix-assisted laser-desorption ionization–time-of-flight) MS was carried out at Lab India (New Delhi, India) and at the Indian Institute of Science (Bangalore, India). Visible spectra were recorded on a Hitachi-557 double-beam spectrophotometer. CD spectra were recorded on a Jasco J-720 spectropolarimeter with attached data processor at the Central Instruments Facility [National Institute of Immunology (NII), New Delhi, India]. CD measurements were made after dissolving HAA in DMSO.

Determination of molar absorption coefficients for HA and HAA

Recrystallized haem and purified HA and HAA were dried over KOH in a vacuum until constant weight. Stock solutions of each were prepared in DMSO and methanol. Spectra were recorded on a Hitachi 557 double-beam spectrophotometer using DMSO/methanol as solvents against respective solvent blanks. The molar absorption coefficients reported are means of three independent observations, which differed marginally from each other.

Spectroscopic estimation of binding of HA/HAA to PfHRP II

Stock solutions of HA and HAA were made by dissolving the purified products in methanol. The solutions were then filtered through a 0.45 μm-pore-size syringe filter. Concentrations of these stock solutions were determined spectroscopically by using values of $\varepsilon_{407} = 98\ mM^{-1} \cdot cm^{-1}$ for HA and $\varepsilon_{417} = 48\ mM^{-1} \cdot cm^{-1}$ for HAA. Difference spectroscopic ligand binding titrations were done as described in [24]. Solutions of 1 mM HA/HAA were prepared in methanol from their respective stocks and the two ligands were titrated at room temperature (~26 °C) for binding to PfHRP II in two separate experiments. The ligand solutions were added simultaneously into the sample cuvette containing 20 μg (0.6 μM) of PfHRP II in 2 ml of Tris/HCl (100 mM, pH 7) and the reference cuvette containing the buffer alone. The ligand solutions were added in 3 μM increments, and difference absorption spectra were recorded after each addition, after mixing and allowing a 5 min incubation for complete binding. The spectra had maxima at 426 and 430 nm for HA and HAA respectively and a minimum at 380 nm for both. The binding curves were constructed by plotting A_{426} or A_{430} against the concentration of HA or HAA respectively.

Inhibition of haemozoin formation

Haem (final concentration 380 μM) was aliquotted into Eppendorf tubes, and HA or HAA was added at various concentrations (10–100 μM). Then PfHRP II (3 μg; final concentration 0.09 μM) was added and finally the volume was made up to 1 ml with 200 mM sodium acetate buffer, pH 5.5. Two controls, i.e. haem alone (negative control) and haem + PfHRP II (positive

control), were always run simultaneously. Basal haemozoin synthesis in the 'haem alone' control was typically $< 10\,\%$ of the value in the presence of PfHRP II. This basal value was subtracted from all measurements of assisted haemozoin synthesis. Each assay was set up in triplicate and incubated at 37 °C for 6 h on a rotatory shaker (260 rev./min). The reaction was stopped by centrifugation at 16060 ***g*** for 15 min. Washing and estimation were performed as described previously [9].

Effects of HA and HAA on parasite cultures

Various concentrations of HA, HAA and artemisinin were prepared in DMSO. *In vitro* antimalarial activity against *P. falciparum* (3D7 strain) was assessed by incubating HA/HAA/artemisinin with synchronized parasite cultures at 1 % of initial parasitaemia (ring stage) in 96-well plates for 48 h (until the next ring stage) in an incubator (5 % O_2, 5 % CO_2 and 90 % N_2 atmosphere). After a 48 h incubation, thin blood films were prepared and stained with Giemsa. Percentage parasitaemia was calculated by counting the number of parasitized red blood cells per 5000 red blood cells.

Measurement of the peroxidase activity of haem, HA and HAA

The peroxidase-like activity of haem, HA and HAA was monitored by following the oxidation of OPD. Various concentrations of each were incubated with 2 mM OPD at 37 °C for 15 min in sodium phosphate buffer (100 mM, pH 7). The reaction was initiated by the addition of H_2O_2 (5 mM). Formation of the oxidation product was monitored at 492 nm for OPD after stopping the reaction by the addition of 100 μl of 3 M HCl.

Native PAGE and peroxidase staining

A non-reducing 15 % (w/v) polyacrylamide native gel was used. The stacking gel was at pH 6.8 and the resolving gel was at pH 8.8. Portions of 10 μg of each sample (haem/HA/HAA) were diluted into sample buffer containing 10 % (v/v) glycerol, 0.1 % Bromophenol Blue and 50 mM Tris/HCl, pH 6.8, and were loaded without boiling. Following electrophoresis, all manipulations were done in the dark at 25 °C. The gel was soaked for 60 min in a 3:7 (v/v) mixture of DAB (6.3 mM in methanol) and sodium acetate buffer (0.25 M, pH 5). To this mixture was added H_2O_2 to a final concentration of 30 mM. The gel was incubated for $\sim$30 min. When the brown-coloured bands showed up with good contrast, the gel was washed with propan-2-ol/acetate buffer (3:7, v/v).

Effects of haem, HA and HAA on the auto-oxidation of ascorbate

Different concentrations of haem, HA and HAA (3–12 μM) were incubated with the sodium salt of ascorbate (200 μM) at 37 °C for 5 h in PBS. The decay of ascorbate was followed at 265 nm.

RESULTS

Reactivity of artemisinin with free haem and with protein-bound haem in Hb/Mb

Due to the poor solubility of artemisinin in water, its reaction with Hb was carried out in 1:1 (v/v) acetonitrile/sodium phosphate buffer (100 mM, pH 7). The Hb used was obtained from Sigma, and is likely to be predominantly met-Hb. The reddish brown Hb solution turned to yellowish upon addition of artemisinin. The progress of the reaction was followed by progressive red shifts in the λ_{max} of the Soret band due to the haem moiety in Hb. We observed an initial immediate reaction characterized by a red shift of $\sim$6 nm (401 to 407 nm), followed by another 6 nm shift that was more gradual (411 to 413 nm) (Figure 1). The shift in the Soret λ_{max} suggests that haem in Hb has undergone a chemical reaction with artemisinin. In an analogous reaction, we reacted free haem with artemisinin under otherwise identical conditions. As shown in Figure 1, unlike the great proclivity of haem in Hb to react with the endoperoxide, we found no evidence for reactivity between free haem and artemisinin in a reaction monitored for 1 h with aqueous acetonitrile as the solvent. It may be pointed out that in our previous study [9], where the reaction between haem and artemisinin was studied with DMA as the solvent, HA was indeed obtained in good yield. This apparent contradiction is resolved because the great solvating properties of DMA allowed us to perform the reaction at 200 mM. In contrast, the haem–artemisinin reaction in the present study was performed at a concentration of 40 μM in aqueous acetonitrile. Surprisingly, when we did an analogous reaction with Mb, we observed that the haem of Mb is not reactive with artemisinin.

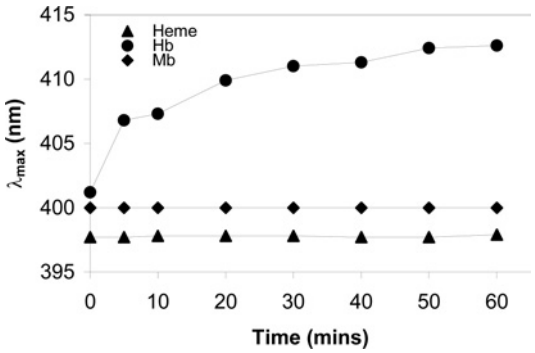

Figure 1 Reactivity of artemisinin with free haem and with protein-bound haem in Hb/Mb

In aqeous acetontrile, artemisinin shows different reactivities with free haem compared with haem bound to proteins. Note that artemisinin-dependent red shifts are exhibited by Hb, but not by free haem or Mb.

Synthesis, purification and characterization of HAA

In order to investigate this reaction further, it was performed between freshly purified Hb and artemisinin on a four times larger scale, and the kinetics of the reaction were monitored for 6 h (Figure 2A). The Hb used here has its haem in the Fe^{2+} state, as shown by its red-shifted Soret band at 406 nm and the presence of pq bands. A rapid reaction associated with a red shift of 5 nm was observed in the first 30 min. Subsequently the red shifts showed a more gradual rise, reaching a plateau at approx. 5 h. In order to be sure that the observed shifts originated from a reaction between protein-bound haem and artemisinin, we isolated the porphyrin reaction products at different time points by acid acetone precipitation. When these were analysed by reverse-phase HPLC, a time-dependent decrease was seen for the haem peak (Figure 2B), along with the emergence of progressively increasing peak intensities for the artemisinin alkylated haem derivatives. The pattern of the decrease in the haem peak (Figure 2B) is nearly a mirror image of the increasing pattern of red shifts seen in Figure 2(A). Figure 2(D) shows the chromatogram after 6 h of the reaction. By this time, when barely 5 % of the haem was left, the reaction between haem and artemisinin appeared to be over. Mass spectral analysis of the reaction mixture (Figure 2E) showed major peaks at 838 and 1060 Da. The difference in mass values between haem (616 Da) and HA (838 Da) is 222 Da. The magnitude of the difference between the mass values of HA (838 Da) and HAA (1060 Da) is also 222 Da. The mass value of

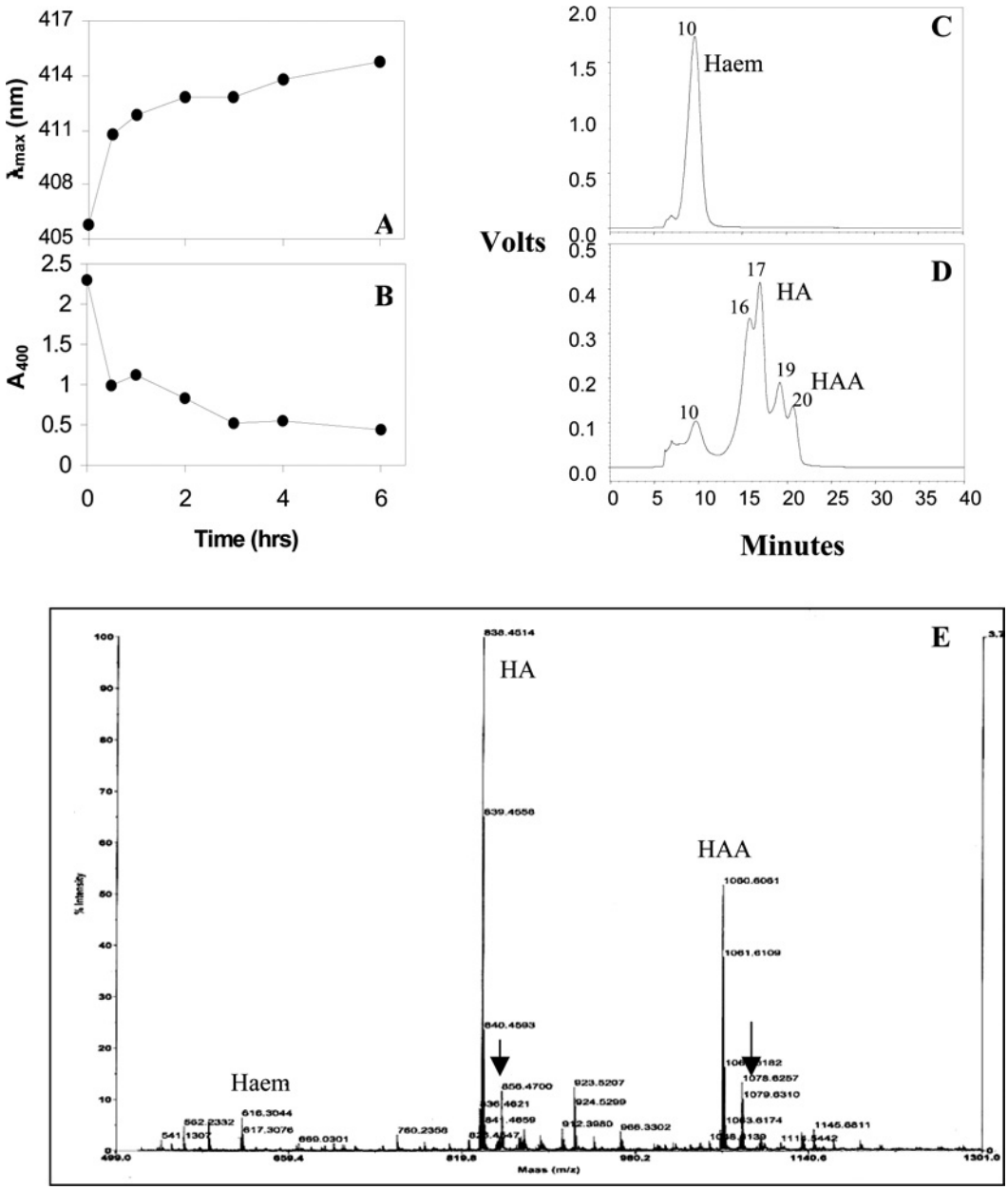

Figure 2 Reaction of artemisinin with Hb

(**A**) The reaction of artemisinin with Hb causes a shift in the λ_{max} of Hb (from 406 to 415 nm). Panel (**B**) shows the decrease in the haem peak as the reaction proceeds. Relative amounts of haem were quantified by reverse-phase HPLC. The ordinate represents the value of the absorbance of the reverse-phase HPLC peak at its maximum. Note the decrease in absorbance due to the haem peak as a function of time. Reverse-phase HPLC profiles of haem extracted from reaction mixtures of Hb alone and Hb incubated with artemisinin for 6 h are shown in (**C**) and (**D**) respectively. Note the decrease in the haem peak (retention time 10 min) and the appearance of haem artemisinin adducts (HA and HAA) at later time points. (**E**) MALDI spectrum of the sample from (**D**). Note the adduct peaks at *m*/*z* 838 (HA) and 1060 (HAA). Arrows represent species corresponding to the addition of a molecule of water to the parent masses.

222 Da corresponds to the artemisinyl group, and the presence of species of 838 and 1060 Da suggests the formation of mono- and di-artemisinyl derivatives of haem.

In order to confirm that the products of the reactions between artemisinin and protein-bound or free haem were identical, and to obtain them in larger amounts, we carried out the reaction of free haem with artemisinin in a molar ratio of 1:4 using DMA as a solvent, in which both the reactants are highly soluble. The reaction mixture was separated by reverse-phase chromatography (Figure 3), which showed a major peak (retention time 17 min) of HA flanked by a small peak of unreacted haem (11 min) and another small peak (21 min) eluting later than HA. Mass spectral analysis of the separated products indicated that the two peaks (retention times 17 and 21 min) corresponded to the monoalkylated (840 Da; HA) and dialkylated (1062 Da; HAA) products of haem. These values are nearly identical to the values obtained for the two adducts upon reaction of Hb with artemisinin (Figure 2E). The relative mass yield of HAA and HA was ~2:1, suggesting that the reaction proceeded smoothly from the mono- to the di-substitution stage. Reaction of purified HA with artemisinin in DMA proceeded to almost complete conversion of HA into HAA (results not shown).

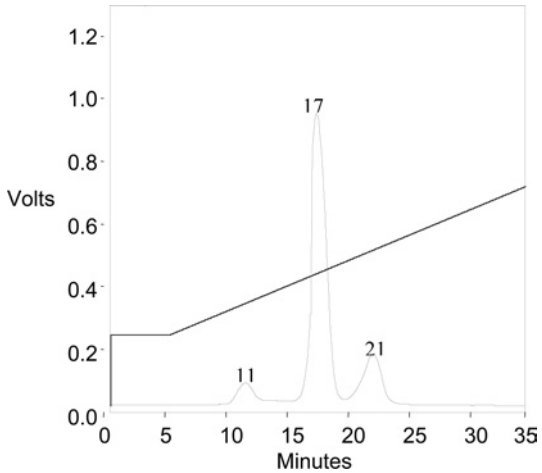

Figure 3 Reverse-phase HPLC profile of the reaction between haem and artemisinin in 1:4 molar ratio in DMA

Reaction mixture equivalent to 50 μg of haem was injected. The numbers 11, 17 and 21 represent the retention times in min for haem, HA and HAA respectively. The gradient was 30 % acetonitrile/0.1 % trifluoroacetic acid to 100 % acetonitrile/0.1 % trifluoroacetic acid in 40 min; detection was at 400 nm.

The estimated molar absorption coefficients ($M^{-1} \cdot cm^{-1}$) for haem, HA and HAA were 137 000, 127 000 and 23 000 respectively in DMSO, and 135 000, 98 000 and 48 000 respectively in methanol. The difference in the molar absorption coefficients of HA and HAA may account for the deceptive appearance of the relative yields of the two when based on peak areas in reverse-phase HPLC (Figure 3) compared with the actual yields. The λ_{max} values for haem, HA and HAA in DMSO were 403, 410 and 417 nm respectively (Figure 4A). The CD spectrum of HAA showed a 1.7-fold higher positive ellipticity at 415 nm compared with HA at 407 nm (Figure 4B, Table 1).

Effects of HA and HAA on *P. falciparum* culture

Metalloporphyrins and protoporphyrins are known to inhibit haemozoin formation and to show antimalarial activity in *in vitro* culture assays [25–27]. Artemisinin is also known to inhibit parasite growth in the nanomolar concentration range. The antimalarial activities of HA and HAA were assessed in *in vitro* *P. falciparum* culture, and we found that both HA and HAA inhibited parasite growth, but at micromolar concentrations, with IC_{50} values ranging from 50 to 100 μM (Table 2).

Interaction of HAA with PfHRP II, and its effect on haemozoin formation mediated by PfHRP II

We have shown previously that both haem and HA bind PfHRP II with high affinity, and that the binding is characterized by a shift of λ_{max} from 382 to 415 nm for haem and from 407 to 426 nm for HA [9]. Difference spectroscopy revealed a shift of λ_{max} of the Soret band due to HAA from 415 to 430 nm in the presence of PfHRP II. The molar absorption coefficients of HA–PfHRP II and HAA–PfHRP II were found to be 29 820 $M^{-1} \cdot cm^{-1}$ and 3580 $M^{-1} \cdot cm^{-1}$ respectively. Figure 5 shows the binding of HA and HAA to PfHRP II. The end point of saturation was attained at $\sim$28 molecules of HA or $\sim$23 molecules of HAA per molecule of PfHRP II.

PfHRP II binds and plays a catalytic role in the conversion of haem into haemozoin, and HA has been shown to inhibit PfHRP II-mediated haemozoin formation [9]. We found that the haem bound to PfHRP II was displaced by the addition of HAA in a dose-dependent manner (results not shown). We then studied the effects of different concentrations of HAA on PfHRP II-mediated haemozoin formation, and found that both HA and HAA inhibited haemozoin formation, with IC_{50} values of $\sim$30 μM and $\sim$55 μM respectively (Figure 5C). Artemisinin, a known inhibitor of haemozoin formation, had an IC_{50} value of 66 μM in this assay [9].

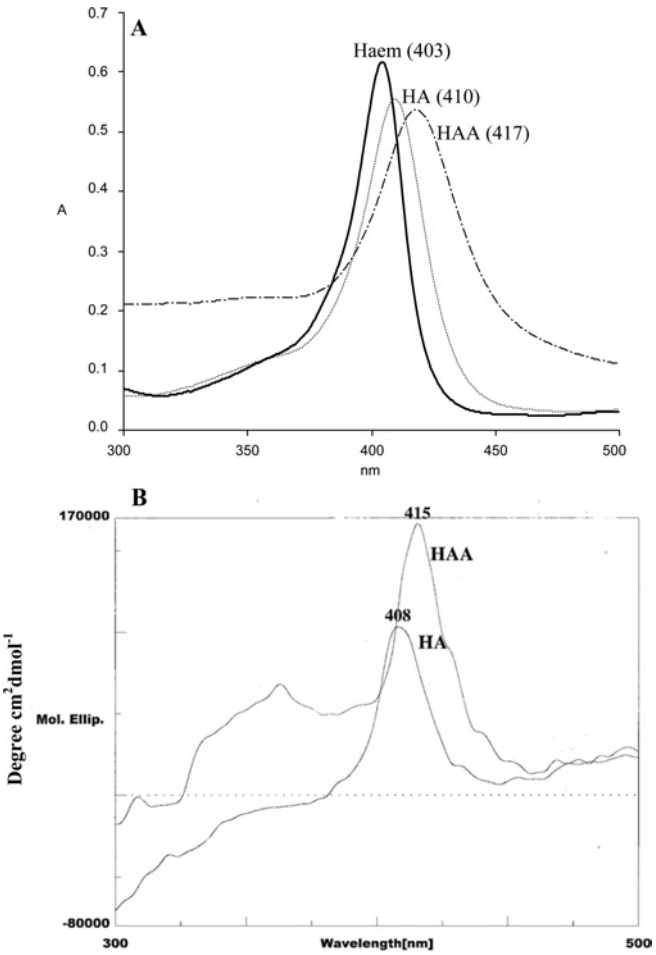

Figure 4 Absorbance and CD spectra of haem and its artemisinin alkylated derivatives

(**A**) Visible spectra of haem (5 μM), HA (5 μM) and HAA (20 μM) in DMSO; numbers in parentheses indicate λ_{max} in nm. (**B**) CD spectra of HA (81 μM) and HAA (336 μM) in DMSO. Note the 1.7-fold increment in the molar ellipticity of HAA compared with HA.

Table 1 Spectral characterization of haem, HA and HAA

	Haem	HA	HAA
Visible spectrum			
λ_{max} in DMSO (nm)	403	410	417
λ_{max} in methanol (nm)	398	407	417
Mass (Da)	652	838	1060
Retention time on reverse-phase HPLC (min)	11	17	21
CD spectrum	Nil	+ ve ellipticity (408 nm)	+ ve ellipticity (415 nm)
θ (degree $\cdot$ cm^2 $\cdot$ $dmol^{-1}$)	–	100 000	170 000
Molar absorption coefficient ($mM^{-1} \cdot cm^{-1}$)			
DMSO	137	127	23
Methanol	135	98	48

Table 2 Antiparasitic effects of HA and HAA in *in vitro P. falciparum* culture

Values represent the number of non-parasitized red blood cells as a percentage of total cells. IC_{50} values determined were ~50 μM (HA and HAA) and <7.5 nM (artemisinin).

	Concn	Non-parasitized red blood cells (%)
Artemisinin	30 nM	88
	15 nM	77
	7.5 nM	58
HA	200 μM	77
	100 μM	60
	50 μM	34
HAA	200 μM	79
	100 μM	77
	50 μM	35

Redox properties of haem, HA and HAA

Haem is known to react with H_2O_2, and possesses both catalase-like and peroxidase-like activities [28,29]. In order to determine whether alkylation of haem by artemisinin affects the peroxidase activity of haem, we used OPD and DAB, commonly used substrates for measuring peroxidase activity. As shown in the Figure 6(A), HAA showed considerably less peroxidase activity than haem and HA. Likewise, while both haem and HA produced a strong brownish-coloured stain on a 15% native gel with DAB, there was only a faint stain with HAA (Figure 6B). These results suggest that there is a significant loss in the peroxidative activity of haem after its dialkylation with artemisinin. Haem has been shown to catalyse the auto-oxidation of ascorbate [30]. We tested this property for both HA and HAA by incubating them with ascorbate and measuring the absorbance at 265 nm. As shown in Figure 6(C), haem exhibited greater oxidizing activity than HA or HAA; the ED_{50} values for haem, HA and HAA were 1.9, 7.4 and 12.3 μM respectively.

DISCUSSION

While the mechanisms of action of artemisinin have remained uncertain, it is generally believed that cleavage of the peroxide bond, giving rise to transient radical intermediates, is a prerequisite for its action [31]. This cleavage of the peroxide is mediated by iron, which makes iron a necessity in the early onset of artemisinin action [32,33]. In fact, the oxidizing effects of artemisinin on parasitic membranes are greatly enhanced in the presence of haem [34], and haem itself is known to react with artemisinin [9,22,35]. Although a general notion about artemisinin action is that haem iron is central to its action, the relative effects of free haem and protein-bound haems with regard to reactivity with artemisinin have remained unclear. Here we have investigated the reaction of artemisinin with Hb, and observed a markedly higher rate of reaction with haem in Hb than was the case with free haem. The reactivity of haem in Hb with artemisinin is significant in the context of malaria, since the parasite in its blood stage feeds on Hb.

The Soret band shifts observed upon reaction of Hb with artemisinin are typical of the reaction of haem with alkylating agents such as endoperoxides or alkyl and aryl hydrazines [9,36]. Separation of the reaction products followed by mass spectral analysis indicated the formation of mono- and di-alkylated derivatives of haem. Mass spectral analysis showed that these derivatives were the same as are obtained upon reaction of free haem with artemisinin. The 2-fold higher yield of HAA in comparison with HA appeared to be in contradiction with the analytical reverse-phase HPLC profile of the reaction mixture, where the peak for

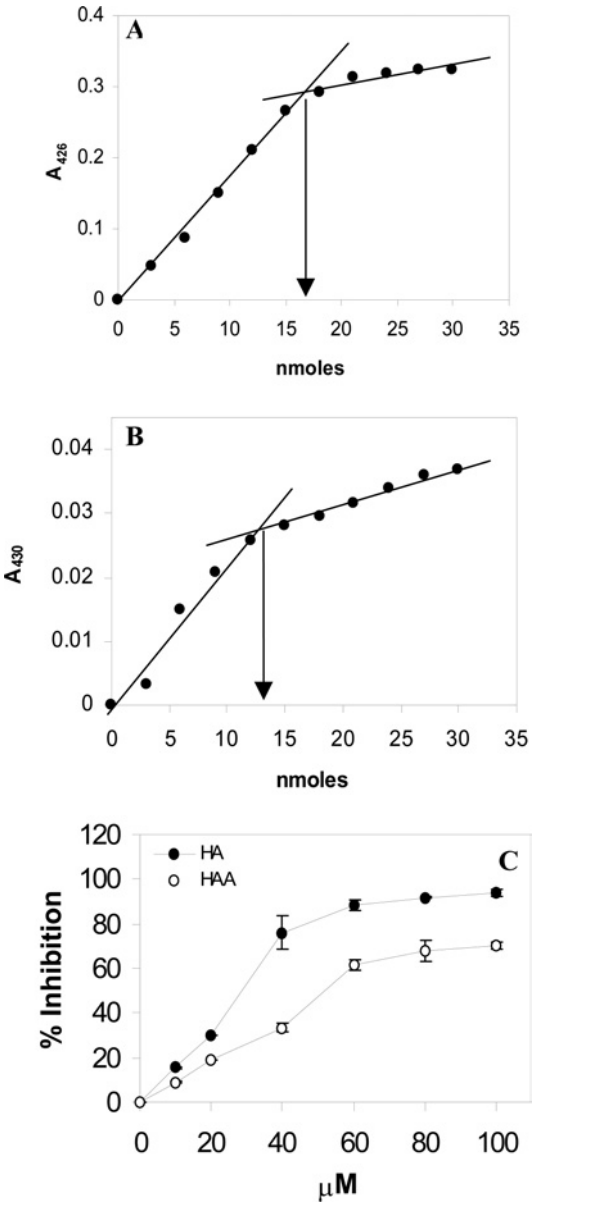

Figure 5 Binding of HA and HAA to PfHRP II and corresponding inhibitory effects on haemozoin formation

Increasing concentrations of (**A**) HA and (**B**) HAA were titrated with a constant amount of PfHRP II (0.6 μM) in the difference spectroscopy mode in Tris/HCl buffer (100 mM, pH 7). Arrows indicate the saturating concentrations of HA/HAA. The estimated number of ligand binding sites per molecule of PfHRP II was 28 and 23 for HA and HAA respectively. Volume increases at the end of the titrations were <5%. The increases in absorbance at 426 nm (A_{426}) for HA and at 430 nm (A_{430}) for HAA were recorded at room temperature. (**C**) Haemozoin formation was measured as described in the Experimental section. The IC_{50} values for HA and HAA are 30 and 55 μM respectively.

HA was significantly more intense than that for HAA (Figure 3). Determination of the molar absorption coefficients of the alkylated haem products accounted for the discrepancy, since that of HAA was about five times lower than that of HA (Table 1). The visible spectrum of HAA was further red-shifted to 417 nm compared with HA at 410 nm. The red shift may arise due to the effect of in-plane nuclear reorganization, defined as changes in porphyrin bond lengths and bond angles induced by interactions between the substituents and the porphyrin macrocycle [37]. Alkylation of the optically inert porphyrin ring of haem by the large,

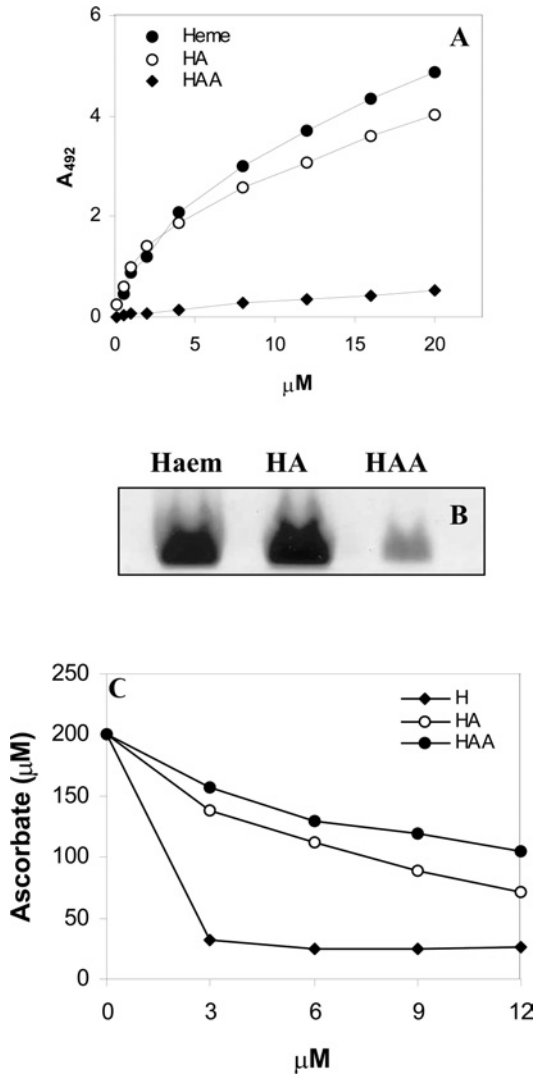

Figure 6 Decrease in the redox activities of haem upon alkylation by artemisinin

(**A**) Different concentrations of haem, HA and HAA were incubated at 37 °C with the OPD reaction mixture. Peroxidase activity was measured at 492 nm. Experiments were done in triplicate, and S.D. values are within the points. (**B**) Portions of 10 μg of haem, HA and HAA were loaded on 15 % native PAGE and stained for peroxidase activity using DAB. (**C**) Ascorbic acid (200 μM) was incubated (37 °C, 5 h) with 3–12 μM haem, HA or HAA in PBS (pH 7). Oxidation was monitored by measuring the decrease in absorbance at 265 nm. The ordinate shows the conversion of A_{265} values to ascorbate concentration.

optically active artemisinyl groups could induce non-planarity in the porphyrin. Indeed, while haem is planar and lacks chiroptical properties, the molar ellipticity values of HA and HAA were $\sim$100 000 and 170 000 respectively. Assuming that non-planarity is introduced via alkylation with the chiral artemisinin, the increment in ellipticity in going from HA to HAA suggests further enhancement in non-planarity upon dialkylation.

In order to obtain a better understanding of the reaction between Hb (tetramer) and artemisinin, we also studied the reaction of artemisinin with Mb (monomer). Contrary to our expectation, haem in Hb turned out to be far more reactive than haem in Mb. The greater reactivity of haem in Hb compared with free haem seemed to suggest that docking of haem on proteins makes it more reactive with artemisinin. However, the poor reactivity of haem in Mb suggests that docking *per se* may not be sufficient. It is worth remembering that the two reactants, i.e. haem and artemisinin, are fairly hydrophobic. While haem in haemoproteins, by virtue of binding to hydrophobic pockets, manages to go into water, artemisinin is incapable of docking to protein-bound haem unless the milieu flanking the haem excludes water. We believe that the haems in Hb are anchored in such hydrophobic pockets that exclude water, facilitating the partitioning of artemisinin into the haem pockets. Such partitioning may trigger an already strong penchant for reaction between artemisinin and haem. Unlike in Hb, the haem binding pocket of Mb is unlikely to be efficiently insulated from water. Some indication of the more polar mileu of haem in Mb than in Hb comes from the more red-shifted Soret seen in Hb. Further, the presence of four subunits in the tetrameric organization of Hb may provide a cage enclosing the four haems. Increased local concentrations of haem and artemisinin in the apolar mileu of the caged haem moieties in Hb could accelerate the reaction of haem with artemisinin. Being monomeric, this cannot be the case with Mb.

It may be noted that while the antimalarial IC_{50} for artemisinin is in nanomolar range, the IC_{50} values for HA and HAA are in the micromolar range. This discrepancy may be due to a far more efficient uptake of artemisinin (neutral) and a poor uptake of haemarts (negatively charged) by the infected red blood cells. Conversion of toxic haem into non-toxic haemozoin is crucial for parasite survival and is mediated by highly unusual HRPs of *P. falciparum* [10]. Inhibition of haemozoin formation is a major mechanism of the antimalarial action of both chloroquine and artemisinin [8,9,38]. We have shown previously that HA binds to PfHRP II and inhibits the formation of haemozoin [9]. In the present study, we found that, like HA, HAA also binds to PfHRP II. For the series haem, HA and HAA, the number of binding sites on PfHRP II is highest ($\sim$50) for haem, intermediate ($\sim$28) for HA and lowest ($\sim$23) for HAA. This phenomenon of a decreasing number of ligand binding sites on PfHRP II with increasing number of alkyl substituents may be related to the masking of haem binding sites by the large artemisinyl groups. HA and HAA showed characteristic red shifts of their λ_{max} upon binding to PfHRP II; HA showed a shift from 407 to 426 nm (19 nm), and HAA showed a shift from 415 to 430 nm (15 nm). The corresponding shift observed when haem binds to PfHRP II is 30 nm. The unequal shifts observed upon binding to PfHRP II may suggest either that PfHRP II interacts with the altered ligands differently or that the response of the altered ligands for binding with the PfHRP II is different.

It is believed that artemisinin interferes with the process of haem detoxification, and this may constitute at least one of the many mechanisms of the action of this drug [9,22]. Alkylation of haem to HA introduces a haem mimic that interferes with the formation of haemozoin [9]. Since HAA binds to PfHRP II in the same manner as HA, we wondered if it would also inhibit PfHRP II-mediated haemozoin formation. Our results showed that, like HA, HAA also inhibited the conversion of haem into haemozoin. The IC_{50} values for HA and HAA in the PfHRP II-mediated haemozoin synthesis assay were 30 and 55 μM respectively. Since dialkylation did not result in a lower IC_{50} than that for HA, this suggests that more potent inhibitors of haemozoin synthesis may not result from higher degree of alkylation. Rather, they are more likely to come from better analogues of artemisinin.

The formation of haemozoin is a highly ordered process in which the shape and planarity of the haem monomers appears to be crucial in the initial stacking of the haem monomers to form dimers, which are then held together by a network of hydrogen bonds to form haemozoin [39]. Diverse metalloporphyrins are known to be efficient inhibitors of haemozoin formation, and it is believed that they do so by forming π–π complexes with haem

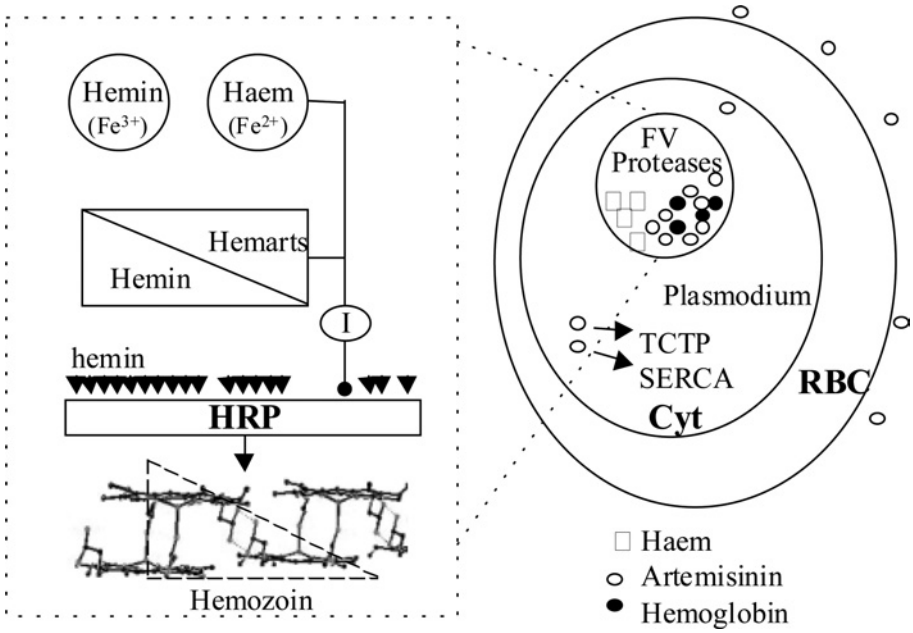

Figure 7 Antimalarial action of artemisinin

The available data suggest that artemisinin may have multiple molecular targets. Our data indicate that haem in Hb may be a prime target of artemisinin action. Note the greater reactivity of artemisinin with haem in Hb compared with free haem. Note also the ability of artemisinin to accumulate in the food vacuole (FV) against a concentration gradient. The accumulation of Hb and artemisinin in the food vacuole coupled with favourable reactivity between the two seems to make the vacuole a major site for artemisinin action. Upon activation, artemisinin alkylates haem in Hb. In addition, the list of proteinaceous targets of artemisinin includes proteases of the food vacuole, as well as TCTP and PfATP6 in the cytoplasm (Cyt). Mono- and di-alkylated haemarts released from haemartoglobin proteolysis are haem mimics, which prevent haemozoin biosynthesis by competitive displacement of haem from templates such as PfHRP II. In addition, HAA has a markedly lowered oxidative potential, which can tilt the haemin (Fe^{3+}) to haem (Fe^{2+}) ratio in favour of the latter. Since haem is a potent inhibitor of haemozoin biosynthesis [45], HAA-mediated inhibition of haemozoin synthesis (indicated by 'I') is likely to occur also by creation of a more reducing environment in the food vacuole. The broken triangle at the bottom suggests the inverse relationship between the concentration of haemarts and the yield of haemozoin biosynthesis. Under such conditions, free haem and haemarts can exert their toxicity, killing the parasite.

[25]. The presence of a bulky artemisinyl group in HA and HAA at *meso* positions of the porphyrin ring of haem is likely to inhibit the stacking of haem molecules. In addition, inhibition of haemozoin formation by HA and HAA may also arise from the fact that they not only bind PfHRP II strongly but can also displace haem bound to the protein. Molecules that can displace haem bound to PfHRP II show antimalarial activity and high-throughput screens based on this principle have been described [40,41].

Deformations of porphyrin are known to result in significant changes in the chemical and spectroscopic properties of the porphyrin macrocycle [37]. Changes in the spectral properties of haem upon alkylation have been described previously [9] and in the present paper. The chemical properties of porphyrin that are modified by non-planar distortion include oxidation potentials, basicity of the inner nitrogen atoms, and axial ligand binding affinity [37]. All of these can influence the biological functions of porphyrin cofactors in proteins. Of the various reactions that may reflect changes in the chemical properties of haem, the simplest and perhaps the most relevant in the present context are the ones that deal with the redox properties of haem [42]. We found that while HA retains a significant amount of the peroxidase activity of haem, HAA is nearly deficient in this regard. Ator et al. [36,43] found that alkylation of the prosthetic haem group at the δ-*meso* position by alkyl and aryl hydrazines leads to complete inactivation of horseradish peroxidase activity. This inactivation was due to steric interference with electron delivery to the haem edge rather than to the intrinsic electronic consequences of *meso* alkylation. Alkylation of haem with two bulky artemisinyl groups at the *meso* positions could interfere with the electron delivery process, leading to a decline in peroxidase activity. Haem is also known to catalyse the oxidation of ascorbate [30], a potent reducing agent found in biological tissues, including malaria parasite-infected red cells, where the concentrations of ascorbic acid are known to increase upon malaria infection [44]. The lowered ability of alkylated haem adducts to oxidize ascorbate (Figure 6C) may increase the ascorbate/dehydroascorbate ratio. A reducing environment created by HA/HAA formed because of the alkylation of haem by artemisinin could shift the haem–haemin equilibrium towards the former. Such a mileu, which causes enrichment of haem, would antagonize haemozoin formation, since haem is known to be a very potent inhibitor of haemozoin synthesis [45].

The alkylation of haem by artemisinin has been well characterized, and it is clear that haem is both the initiator as well as the target of artemisinin action [9,22,35]. Other possible targets of artemisinin are specific parasite proteins, although no details have been reported for such artemisinin–protein adducts. Inhibition of the SERCA orthologue PfATP6 and of the cysteine proteases of *P. falciparum* by artemisinin and its specific reaction with TCTP have been reported, but it is noteworthy that, in all of these cases, the action of the drug was found to be dependent on the presence of haem and/or some unidentified reducing iron species [19,20]. However, a literature review using the ISI Web of Knowledge revealed that carbon-centred radicals are poor protein alkylators [46]. Thus it seems more likely that artemisinin-alkylated haem derivatives obtained upon reaction of protein-bound haem with artemisinin may have a major role in the antimalarial effects of artemisinin. More recently, Meunier and co-workers [47] described the reaction of artemisinin with Hb under reducing conditions in aqueous DMSO and identified monoalkylated haem as the major reaction product. We have shown here that alkylation of haem in aqueous acetonitrile proceeds smoothly to give the

dialkylated product in high yield. It may also be noted that molar absorption coefficient of HAA is much lower than those of haem and HA, a feature that may require special attention with regard to detection by reverse-phase HPLC. Compared with the high reactivity of haem in Hb, the inability of free haem on the one hand and of protein-bound haem in Mb on the other to react with artemisinin holds important clues to our understanding of the mechanism and the specificity of action of this vital antimalarial drug. We have shown that the redox properties of haem are substantially modulated upon its alkylation, and that both HA and HAA possess antiparasitic activity *in vitro*. The enhanced reactivity of artemisinin with Hb in comparison with free haem suggests that Hb itself acts as a nodal target protein for the primary attack of artemisinin. The haemarts that are generated from 'haemartoglobins' may bring about the death of the malaria parasite by a two-pronged effect of stalling the formation of haemozoin by competitive inhibition of haem binding to its templates, and of creating a more reducing environment that is not conducive for formation of haemozoin.

A comprehensive model of the antimalarial action of artemisinin indicating its pleiotropic actions at multiple sites is presented in Figure 7. A highlight of this model is the enhanced reactivity of artemisinin with the largest iron pool (i.e. Hb) in the infected red blood cell. This makes biological sense, since the food vacuole of the malaria parasite becomes the meeting ground for Hb (that is food for the parasite) and artemisinin, which is known to accumulate in the food vacuole. It is likely that artemisinin may have other targets of action, such as TCTP and PfATP6. The exact contributions of the various targets to the antimalarial efficacy of artemisinin remain to be determined. However, any claims about artemisinin's antimalarial effect not requiring haem [20] are premature for two reasons: (a) iron chelators used in such studies are known to chelate haem iron as well, and (b) inhibition of Hb degradation by the inhibition of proteases does not inhibit artemisinin action, since, as shown in the present study, artemisinin reacts preferentially with haem in Hb rather than with free haem.

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