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Reaction between Ferriprotoporphyrin IX and the Antimalarial Endoperoxide Artesunate Gives an Intermediate Species with Enhanced Redox Catalytic Activity

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Abstract

The kinetics of the reaction between ferri(Fe(III) protoporphyrin IX (haemin) and the potent sesquiterpene endoperoxide antimalarial artesunate are shown to be consistent with a three-step, two-intermediate mechanism, with the final product possessing a degraded tetrapyrrole ring system. Microscopic rate constants for the mechanism have been evaluated. The redox catalytic capability of the haem artesunate complex is shown to be approximately fourfold that of haemin alone, suggesting a possible mechanism of action of the drug.

Malaria remains a serious world health problem. It affects approximately 200 million people, mostly young children and nonimmune visitors to tropical countries of whom 1–2 million die annually. The problem of treatment has been exacerbated in recent years by the emergence of parasite strains resistant to many conventional forms of therapy, including quinoline antimalarials such as chloroquine. Hence artemisinin (**1**), a novel sesquiterpene endoperoxide isolated from an ancient Chinese herbal remedy, has, along with its derivatives, been hailed as the most important antimalarial compound isolated since quinine (Johnson 1993). The mechanism by which this potent antimalarial acts, is, however, ill-understood. There is considerable evidence (Meshnick et al 1991) that the target of both artemisinin and its derivatives, as well as the quinoline antimalarials, is haemin, formed in the parasite food vacuole by digestion of haemoglobin. Haemin is intrinsically toxic to most biological systems, by virtue of its ability to generate active oxygen species from molecular oxygen (Adams & Berman 1982), and whereas in most organisms, including humans, haemin is efficiently degraded to bile pigments, malaria parasites convert haemin to the insoluble, biologically inactive polymer, haemozoin (malaria pigment), in which an FeOC covalent bond is formed between the iron atom of one haem and the propionyl side chain of its neighbour (Slater et al 1991). Haemin and artemisinin have been shown to form a covalent complex of unknown structure, but with molecular mass close to that expected for a 1 : 1 adduct (Meshnick et al 1991; Hong et al 1994). Reaction between these two species could thus prevent formation of haemozoin, allowing the toxic ferriprotoporphyrin to remain soluble and thus continue to exert its toxic effect. However, no definitive study appears to have been carried out to elucidate the kinetic mechanism of the reaction between haemin and artemisinin and the mode of action of this antimalarial remains a subject of speculation.

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Materials and Methods

Artesunate was obtained from Guilin No 2 Pharmaceutical Factory, Quangxi, China. Chloroquine, quinine and ferriprotoporphyrin IX were obtained from the Sigma Chemical Co (St Louis, MO) the latter being recrystallized (XI) from pPyridine/chloroform. Spectra and absorbance changes with time were obtained using a Hewlett Packard HP4850 diode-array spectrophotometer with a constant-temperature cell holder.

Kinetics of the interaction between haemin and artesunate

Precise UV/visible spectrophotometry—generally the most useful tool for elucidating the kinetics of reaction between haem-containing compounds and peroxo species—is precluded in aqueous solution by the extensive aggregation of haemin. Accordingly, we have investigated the reaction between haemin and artesunate in 0·1 M phosphate buffer, pH 7·4, containing 25% dimethylsulphoxide. In this solvent, haemin exhibits a sharp Soret peak (max 399 nm) with an absorbance coefficient of $\sim 1{\cdot}2 \times 10^5$ M^{-1} cm^{-1} at a haemin concentration of 1×10^{-6} M. Artesunate is an active water-soluble derivative of artemisinin, in which a succinate group is esterified with the dihydro derivative of artemisinin at the position indicated by * in 1. Addition of artesunate in the concentration range $5{\cdot}10^{-6}$–10^{-4} M, causes the Soret region of the haemin spectrum to undergo major changes. Careful examination of the temporal evolution of the difference spectrum (Fig. 1) shows that the reaction is triphasic. An initial small increase in absorbance is observed at 416 nm over a period of $\sim 10^3$ s, followed by partial collapse of the Soret maximum at 399 nm and a synchronous increase at 424 nm. This second phase occurs over a time span of approximately 10^4 s and results in a red-shift of the Soret

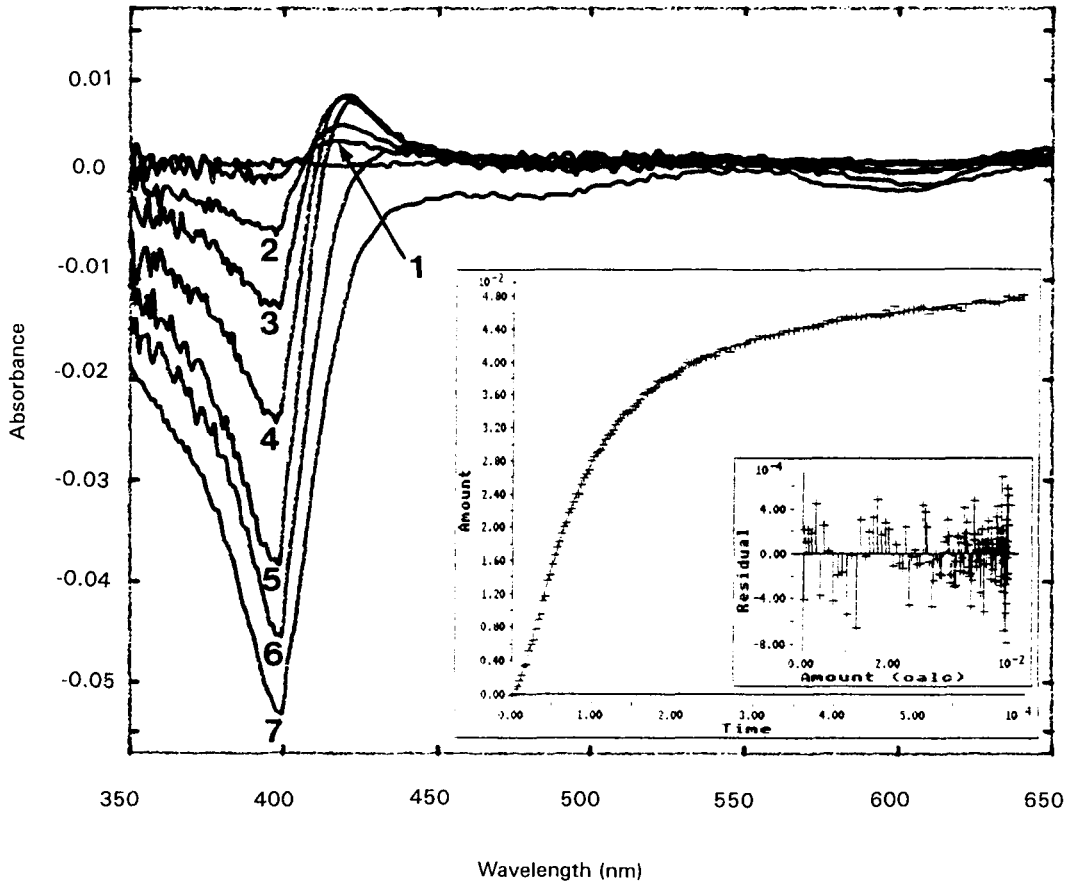


FIG. 1. Difference spectra for reaction between ferriprotoporphyrin IX (1 μM) and artesunate (20 μM) in aqueous 25% dimethylsulphoxide pH_{app} 7·4. T = 30·0°C. 1–7 were obtained at 300, 1800, 4500, 8000, 18 000, 80 000 and 150 000 s, respectively. The 416-nm peak is indicated by an arrow. The inset to the Fig shows the variation of (A399nm–A650nm) with time, over a period of 65 000 s on reaction between haemin (1 mM) and artesunate (20 mM) at 30·0°C. The data clearly shows the triphasic nature of the reaction. The least squares fit value for the macroscopic pseudo first-order rate constants are, for the data shown: $k_1^* = 5{\cdot}27\ (\cdot 73) \times 10^{-4}\ s^{-1}$; $k_2^* = 1{\cdot}70\ (\cdot 17) \times 10^{-4}\ s^{-1}$; $k_3^* = 2{\cdot}9\ (\cdot 6) \times 10^{-5}\ s^{-1}$.

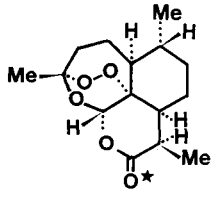
maximum to ~402 nm, coupled with a reduction in absorbance coefficient to approximately 60% of the original value. The final stage of the reaction occurs over a period of approximately 24–36 h and involves complete disappearance of the Soret maximum indicating disruption of the delocalized π-electron system of the porphyrin tetrapyrrole ring.

On monitoring the time course of the reaction in dual wavelength mode, using 650 nm as the invariant reference wavelength and 399, 416 and 424 nm as the test wavelengths, the triphasic nature of the reaction was confirmed. A typical kinetic trace is shown as the inset to Fig. 1. A statistically acceptable fit to the data,in terms of random distribution of residuals, was only attainable for these traces using a triexponential function. Pseudo first order rate constants k_1^*, k_2^* and k_3^* for each phase can be evaluated by non-linear least squares regression (Leatherbarrow 1987). These observations suggest a two intermediate three-step kinetic mechanism for the interaction as shown in Scheme 1:

$$\text{Artesunate} + \text{Haemin} \quad \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I_1 \quad \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I_2 \xrightarrow{k_3} P$$

Scheme 1

where I_1 and I_2 are intermediate species and P is the final product. This kinetic mechanism (scheme 1) leads to a triexponential model (eqn 1) relating absorbance change to time (Bernasconi 1976):



$$\text{Abs} = a_0 + a_1 e^{-k_1^* t} + a_2 e^{-k_2^* t} + a_3 e^{-k_3^* t} \qquad (1)$$

where the values of k_1^*, k_2^* and k_3^* are as given by Bernasconi (1976). The values of k_1^*, k_2^* and k_3^* for this triexponential model were evaluated by non-linear regression (Leatherbarrow 1987) at a number of artesunate concentrations in the range 1×10^{-5} M – 8×10^{-5} M with a haemin concentration of 1×10^{-6} M, between 100 and 200 Abs/time data points were used in each evaluation. k_3^* was found to be independent of the concentration of artesunate and had a value

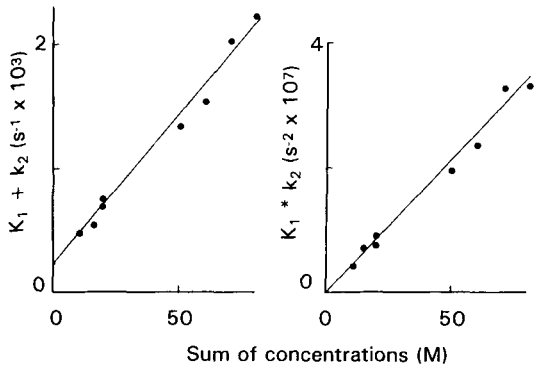


FIG 2. The variation of $k_1^* + k_2^*$ and $k_1^* . k_2^*$ with Σ. Microscopic rate constants evaluated from the figures are: $k_1 = 25(1)\,M^{-1}s^{-1}$; k_{-1} (max) $= 4{\cdot}2 \times 10^{-5}\,s^{-1}$; $k_2 = 1{\cdot}7(\cdot2) \times 10^{-4}\,s^{-1}$; $k_{-2} = 0$; and $k_3 = 2(\cdot3) \times 10^{-5}\,s^{-1}$.

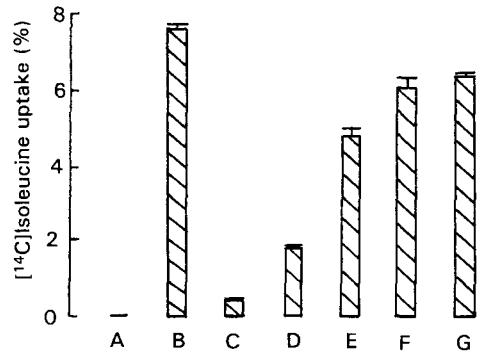


FIG. 4. The effect of preincubation of artesunate with haemin upon the plasmodiotoxic activity of artesunate, parasite viability was estimated by the ability to uptake ^{14}C-labelled isoleucine. A. Non-infected red blood cells (RBC), B. parasite-infected RBX (PIRBC), C. PIRBC + 10^{-7} M artesunate; D. PIRBC + 10^{-7} M artesunate precincubated with haemin (2 mM) for 30 min; E. as for D, with t = 5 h; F. as for D with t = 20 h; G. PIRBC + haemin alone.

approximately 10% that of k_2^* (min). Thus the third step of the reaction is effectively uncoupled from the first two and the dependence of k_1^* and k_2^* on concentration can be used to evaluate the microscopic rate constants for Scheme 1 (Bernasconi 1976). Fig. 2 shows plots of $k_1^* + k_2^*$ and $k_1^* . k_2^*$ {[Art] + [Haemin]} $- \Sigma$. For these plots k_1 is the slope of the straight line in Fig. 2a; furthermore the intercept in Fig. 2a is equal to $k_{-1} + k_2 + k_{-2}$; the slope in Fig. 2b is $k_1(k_2 + k_{-2})$ and the intercept $k_{-1} . k_{-2}$ (Bernasconi 1976). Thus all microscopic rate constants may in principle be evaluated, however since the intercept of Fig. 2b is not significantly different from zero, only an upper limit can be obtained for $k_{-1} . k_{-2}$. Since there are good chemical reasons for believing $k_{-2} = 0$ (see discussion) we can thus obtain an upper bound for k_{-1}, and these values are given in the legend to Fig. 2.

Redox catalytic activity of the haem-artesunate complex (Fig. 3) shows the effect of incubation of haemin (1 μM) with artesunate (1 mM) in 0·1 M phosphate buffer pH 7·02 on the redox catalytic capability of the metalloporphyrin. Redox activity was assessed by the peroxidasic reduction of H_2O_2 (1×10^{-4} M) by the reaction system, using 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate, ABTS) as chromogenic reducing substrate. Clearly, reaction of haemin with artesunate gives rise to an intermediate product with enhanced redox catalytic capability which persists over a period of several hours. The observation that maximum redox activity is reached within minutes for the data shown in Fig. 3 is a consequence of the high artesunate concentration (1 mM) compared with that used in the data shown in Fig. 1 (·02 mM). The initial obligatory second-order interaction between the endoperoxide and the haemin will thus be some fifty times faster (i.e. pseudo first-order rate constant $k_1^* \sim 0{\cdot}026\,s^{-1}$) giving an approximate t ½ for initial complex formation of the order of 30 s. Extended incubation results in a slow decline of this catalytic ability toward zero. Of particular interest is the observation that interaction of the widely used quinoline antimalarial, chloroquine, effectively abolishes redox catalytic activity of ferriprotoporphyrin IX within seconds of mixing. A similar result (not shown) is obtained with quinine.

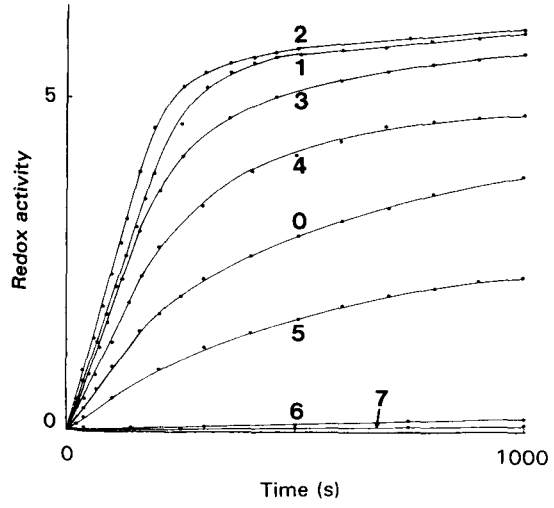


FIG. 3. The effect of incubation of haemin (1 μM) with artesunate (1 mM) on the redox catalytic efficiency of the metalloporphyrin. Curves 0–5 refer to haemin/artesunate reaction times of 0, 60 s, 1 h, 6 h, 22 h and 48 h, respectively. Curve 7 was obtained in the absence of haemin, while curve 6 was obtained when the peroxidasic reaction is initiated 10 s after mixing haemin (1 mM) with the quinoline antimalarial chloroquine.

Effect of preincubation of artesunate with haemin on plasmodiotoxicity of the antimalarial

In Fig. 4 we show that preincubation of haem with artesunate results in a drop in plasmodiotoxicity of the antimalarial on a timescale compatible with formation of the final product (P) in scheme 1 i.e. ~24 h.

Discussion

The endoperoxide bridge is necessary for the antimalarial activity of artesunate (Meshnick et al 1991). Thus on the basis of the known chemistry of interaction of haemin with H_2O_2 and organic hydroperoxides (Adams 1990), and changes in the Soret peak which are diagnostic for the type of intermediates formed on haem-peroxo interaction (Baek & Van Wart 1989; Erman et al 1993; Vitello et al

1993), we propose the following reaction sequence between artesunate and haemin:

1. Coordination of an oxygen atom of the endoperoxide bridge to the axial fifth position of the Fe^{3+} by a donor-acceptor bond utilizing one of the spare sp3 electron pairs of the oxygen atom. This is responsible for the transient absorbance increase at 416 nm in the difference spectrum. A similar spectral change is noted on reaction of H_2O_2 with the haemprotein, methaemalbumin, where a transient increase in A404 occurs prior to formation of a compound I type intermediate and subsequent haem degradation (unpublished results).
2. Dioxygen bond cleavage to yield an intermediate species containing an iron-oxo-carbon covalent bond, and possessing enhanced redox catalytic capability. The close resemblance between the Soret spectrum of this intermediate and that of horseradish peroxidase compound I species, suggest that it forms by heterolytic cleavage of the dioxygen bond (Baek & Van Wart 1989). However it is noted that addition of ABTS without H_2O_2 to this intermediate does not result in ABTS+. cation radical formation, indicating that the intermediate is relatively stable. Furthermore, since the reverse of this step, i.e. reformation of the O–O bond is highly improbable we conclude that k_{-2} in scheme 1 is equal to zero.
3. A very slow oxidative degradation of the porphyrin ring, leading to the redox inactive covalent adduct.

Our studies reported here provide a rational explanation for a number of published observations relating to the action of artemisinin. Firstly, reaction of artesunate with haemin results in an intermediate species in which the iron possesses enhanced redox catalytic activity. Thus, complex formation could potentiate the ability of haem, formed in the parasite food vacuole, to generate activated oxygen species, explaining both the selective plasmodiotoxicity of artemisinin and the reported oxygen dependence of such toxicity (Krungkrai & Yuthavong 1987). Secondly, quinoline antimalarials, such as chloroquine and quinine—which are known to interact with haemin via π–π association with the delocalized π-electron system of the metalloporphyrin (Constantinidis & Satterlee 1988 a,b)—give adducts which are peroxidasically inactive by comparison with artesunate. The only positions of potential reactivity between peroxo species and the metalloporphyrin are the central iron atom and the porphyrin ligand (Bonnett 1981), the latter via the delocalized π electron systems. The observation therefore that known π–π interactions cause redox inactivation of the haemin, while reaction with artesunate causes redox activation, supports our contention that interaction of artesunate occurs at the iron centre, and that, while both artemisinin and the quinoline antimalarials have food vacuolar haemin as their therapeutic target, their mode of toxic action could thus be fundamentally different.

The final stage of the reaction involving the loss of the Soret peak correlates with the loss of redox activity of the haem-artesunate complex (Figs 3, 4), and explains the observations of ourselves (Fig. 4) and Meshnick et al (1991) that reacting artemisinin with haem for 24 h results in a loss of much (80%) of its plasmodiotoxic activity. Since this final stage corresponds with the total loss of the Soret absorbance of the haemin, we further conclude that an intact π-electron system for the tetrapyrrole ring is essential to antimalarial activity, and thus that ferriprotoporphyrin IX is indeed the primary antimalarial agent.

In what way, then, could the complex be toxic to the parasite? One possibility is that the oxidised intermediate species formed on -O–O- bond cleavage (the compound-I-like species) is able to directly initiate peroxidation of parasite lipid membranes. The observation that this species does not oxidise ABTS renders, in our opinion, this mechanism unlikely. From our data it appears that reaction of artesunate with haemin potentiates the redox activity at the sixth co-ordination position of the iron. Whether this occurs simply through monomerization and solubilization of the haem, or by a specific effect increasing the intrinsic redox activity of the iron, is unimportant. Whatever the mechanism, the complexed haem is more redox active and thus would be more cytotoxic. Furthermore, since the fifth co-ordination position of the iron is occupied by artesunate via the Fe–O–C bond, the haem cannot be polymerized to haemozoin. This prolongs its biological activity by many hours, possibly aiding diffusional transport of the protected haem to the cell membrane where, after solubilization within the membrane, lipid peroxidation could be initiated.

Recent work by Slater & Cerami (Reference needed) indicate the presence of a "haem polymerase" activity in food vacuoles, able to catalyse formation of haemazoin via a C–O–Fe bond, between the iron atom of one haem and the propionyl side chain of the next. They propose that the quinoline antimalarials act by inhibiting this enzyme.

We propose that a similar C–O–Fe bond forms between the iron atom of the haemin and the disrupted endoperoxide group of the artesunate. In what way is the complex toxic to the parasite?

One possibility is that, like chloroquine, it interferes with haem polymerization. Another is that the oxygen-centred free radical, formed by cleavage of the endoperoxide bond, is able to directly initiate peroxidation of parasite lipid membranes and important sulphydryl groups. We believe that this is unlikely as the radical so formed is relatively occluded between the haemin and the artesunate. From our data, what appears most likely is that binding of artesunate potentiates the redox activity of haemin, presumably by the sixth free ligand of the iron atom, so that not only is the complexed haem more redox active, but can also not be polymerized to haemazoin, thereby prolonging its biological activity by many hours.

In this context, it is worth noting that the quinoline antimalarials seem to inhibit the redox activity of haemin. This observation is difficult to reconcile with the proposed mode of action (Slater et al 1991), whereby inhibition of haem polymerization allows redox active haemin to remain in solution and effect oxidative damage to the parasite.

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