Alkylation of human hemoglobin A_0 by the antimalarial drug artemisinin

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Abstract In vitro, the heme cofactor of human iron(II) hemoglobin was efficiently and quickly alkylated at *meso* positions by the peroxide-based antimalarial drug artemisinin, leading to heme–artemisinin-derived covalent adducts. This reaction occurred in the absence of any added protease or in the presence of an excess of an extra non-heme protein, or even when artemisinin was added to hemolysed human blood. This activation of artemisinin by the heme moiety of non-digested hemoglobin clearly indicates the high affinity of this drug for heme, and its efficient alkylating ability under very mild conditions.

Key words: Artemisinin; Heme alkylation; Hemoglobin; Malaria

1. Introduction

Plasmodium falciparum malaria is a major health scourge in more than 90 countries and 40% of the global population is vulnerable to this potentially deadly disease, due to global resistance to the two most widely used antimalarial drugs, chloroquine and the antifolate sulfadoxine/pyrimethamine. Artemisinin and its hemisynthetic derivatives (artemether, arteether and artesunate, which are all metabolized into dihydroartemisinin) have been increasingly used over the two past decades in the treatment of multidrug-resistant *P. falciparum* malaria. They are active against all malarial blood stages [1] and induce a rapid clearance of the blood parasitemia. They are also well tolerated and, to date, no significant resistance has been reported either in clinical isolates or in laboratory experiments. The combination of artemisinin derivatives with conventional antimalarials with a longer half-life is increasingly seen as a preferred way to efficiently treat malaria while, at the same time, minimizing the risk of developing drug resistance [2,3].

Within red blood cells, *Plasmodium* parasites digest the host hemoglobin to obtain amino acids to build their own proteins. Then, to avoid the accumulation of the released and potentially toxic iron(II)-heme, *Plasmodia* polymerize this redox-active species as a microcrystalline, non-toxic, iron(III) polymer, called hemozoin or malaria pigment. The reactivity of the peroxide function of artemisinin with heme derivatives has been considered a key factor to explain the pharmacological activity of these endoperoxide drugs [4–7].

The mechanism of action of artemisinin derivatives involving the iron(II)-heme promoted reductive homolysis of the peroxide function, and subsequent formation of drug-derived C4-centered alkylating radicals has been documented [8]. The alkylation of heme [9] was observed after incubation of pharmacologically relevant concentrations of radiolabelled artemisinin derivatives in cultured parasites, and we have recently reported the complete characterization of the heme–artemisinin adducts [10–12]. Under these conditions, heme is both the trigger and the target of artemisinin. But maybe this mechanism is not the only possible one [13]. Proteins are other possible targets of the artemisinin-derived alkylating species and the alkylation of specific parasite proteins has also been proposed [14,15], but no structure has been proposed up to now for these protein–artemisinin adducts. It is noticeable that this latter enzyme inhibition seems also to be dependent on an iron(II)-mediated activation of artemisinin [15]. In this alkylation of proteins by artemisinin, the trigger may be either heme [14] or an unidentified reducing iron species [15], the target being the close protein environment of these iron activators.

Beside the activation of artemisinin by heme itself, the alkylation of the heme residue of microperoxidase-11 (MP11, a heme undecapeptide obtained by proteolytic digestion of cytochrome *c*) has also been reported [16]. The heme moiety of MP11 was found to be dialkylated by artemisinin, thus confirming the ability of artemisinin peroxide to be activated by heme, even when linked to a small protein fragment. However, in MP11, heme is covalently bound to the peptide through two thioether bonds, unlike hemoglobin. Since artemisinin is pharmacologically active on young stages of *P. falciparum*, when hemoglobin is not yet extensively degraded, it was necessary to investigate its reactivity with intact hemoglobin or partly digested hemoglobin, provided that the heme residues are still coordinated to a protein fragment. We report here the efficient alkylation at *meso* positions of the heme residues of human ferrous hemoglobin A_0 (OxyHb) in the presence of artemisinin (only 1 mol equiv with respect to heme) at 37°C. This reaction occurs in the absence of any added protease, or in the presence of an excess of an extra non-heme protein, or even when artemisinin was added to hemolysed whole human blood.

2. Materials and methods

2.1. Materials

OxyHb, pronase E (protease type XIV from *Streptomyces griseus*, EC 3.4.24.31), trypsin (TPCK-treated from bovine pancreas, EC

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3.4.21.4), α-chymotrypsin (type I-S from bovine pancreas, EC 3.4.21.1), and all chemicals were purchased from Sigma-Aldrich.

High performance liquid chromatography (HPLC) analyses were performed using a 5 μm C4-QS-uptisphere 300 column, 250×4.6 mm (Interchim, France). Eluents were (A) 0.05 vol% trifluoroacetic acid in water, (B) 0.05 vol% trifluoroacetic acid in acetonitrile. The gradient was the following: from A/B = 63/37 to A/B = 52/48 in 30 min, then A/B = 52/48 for 15 min. Flow rate was 1 ml/min, and UV detection at 220 and 400 nm. Under these conditions, hemoglobin is denatured and heme released from globins. Retention time (min): 12.0 (heme, λ_{max} = 398 nm), 15.4 (β-globin), 21.5 (α-globin), 22.7, 25.0, 26.9, 27.5 (four heme–drug adducts, relative ratio 1/3/3/3, λ_{max} = 406–408 nm, Fig. 1).

2.2. Standard conditions for the reaction of hemoglobin with artemisinin

OxyHb (2.5 mg, 0.039 μmol) was dissolved in CH_3COONH_4 10 mM, pH 8. Artemisinin (0.188 μmol in 5 μl dimethyl sulfoxide (DMSO)) was then added. Final hemoglobin concentration was 300 μM, heme/artemisinin molar ratio = 1/1.2, and H_2O/DMSO ratio = 96/4 v/v. The solution was stirred and allowed to stand at 37°C. After 1 h, an aliquot (20 μl) was injected for HPLC analysis. When specified, the following changes were made: (i) CH_3COONH_4 10 mM, pH 8 was replaced by CH_3COONH_4 10 mM, pH 6, or by (ii) H_2O/NaOH, pH 8; (iii) the H_2O/DMSO ratio varied from 99/1 to 1/1 v/v; (iv) glutathione (GSH) was added: heme/GSH molar ratio = 1/10; (v) bovine methemoglobin (MetHb) was used instead of OxyHb; (vi) bovine serum albumin (BSA, 12.5 mg, OxyHb/BSA weight ratio = 1/5) was added.

2.3. Reaction of OxyHb with artemisinin in the presence of a protease

OxyHb (2.5 mg, 0.039 μmol) and pronase E, trypsin or α-chymotrypsin (0.125 mg) were dissolved in CH_3COONH_4 10 mM, pH 8 (125 μl). After 1 h, artemisinin (0.188 μmol in 5 μl DMSO) and glutathione (1.56 μmol in 5 μl water) were added and the reaction mixture was kept at 37°C for 1 h before HPLC analysis. Final hemoglobin concentration was 300 μM, heme/artemisinin/GSH molar ratio = 1/1.2/10).

2.4. Reaction of artemisinin with human blood

Human blood (O+) was hemolysed just after withdrawal by addition of an equal amount of distilled water and centrifuged. An aliquot of the supernatant (100 μl) was further diluted with water (300 μl), artemisinin was added (0.625 μmol in 18 μl DMSO), and the mixture was kept at 37°C for 1 h before HPLC analysis. Final hemoglobin concentration was 390 μM, heme/artemisinin molar ratio = 1/1.

3. Results and discussion

3.1. Hemoglobin and reaction medium

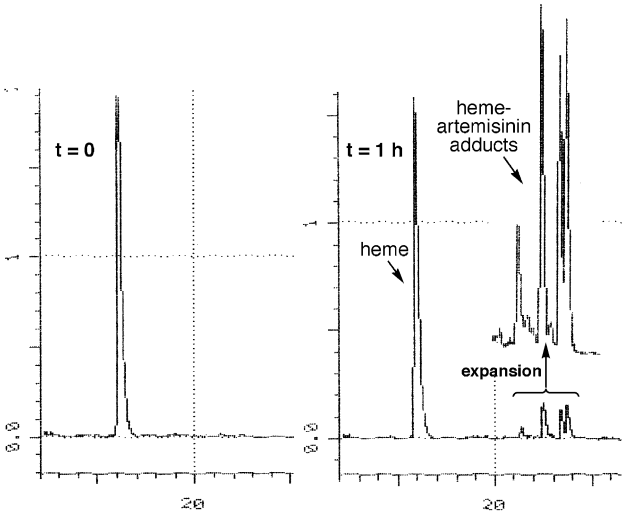
Normal OxyHb was used in ferrous state as confirmed by its UV-visible spectrum, with characteristic absorbances at 542 and 576 nm (Soret at 412 nm, ammonium acetate 10 mM, pH 8) [17,18]. It was checked that OxyHb was stable for several hours at room temperature, even in solution as diluted as 5 μM. This is consistent with the reported slow rate of deoxygenation (2–4 days) and the limited autoxidation of oxyhemoglobin or oxymyoglobin solutions at pH 6–8 [17,19,20].

The interaction of artemisinin with hemoglobin was investigated in conditions that preserve the structure of hemoglobin, at least that maintain the link of heme residues to the globin chains. Ammonium acetate (10 mM) was reported to be a non-denaturing medium at neutral pH, as it allowed mass spectrometry analyses of hemoglobin with a low level of dissociation of the Hb A_0 tetramer, and a small content of apo-α- and apo-β-globins [21,22], even with up to 20% of an added organic cosolvent (methanol) [23]. However, artemisinin is hydrophobic, nearly insoluble in water, but very soluble in polar organic solvents such as methanol or DMSO. The reaction of OxyHb with artemisinin was therefore performed in a mixture of ammonium acetate (10 mM, pH 8) and DMSO (or methanol), 96/4, v/v.

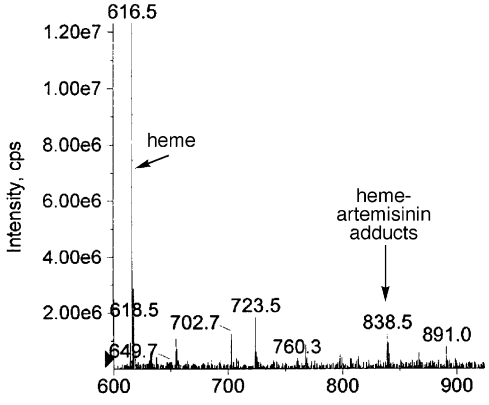
3.2. Reaction of OxyHb with artemisinin

In the presence of 1.2 mol equiv of artemisinin with respect to heme residues of OxyHb, alkylation at α-, β-, γ- and δ-positions of heme occurred, giving rise to four isomeric covalent adducts with overall yield of 31–33% (calculated per heme residue). These heme–artemisinin adducts were identified by comparison (HPLC and LC-MS, see Fig. 1) with reference adducts previously characterized [12]. This alkylation reaction resulted first from the hydrophobic interaction of artemisinin with heme within the non-polar environment of the heme-binding site, followed by the reductive homolysis of the peroxide function of artemisinin **1** by Fe(II)-heme **2**, leading to the addition of the artemisinin-derived C4 radical **3** onto the porphyrin macrocycle (Scheme 1). The same products were obtained with the same yield when using methanol (4 vol%) instead of DMSO.

When OxyHb was replaced by MetHb, no modification of the heme residues occurred, whereas alkylation of heme was

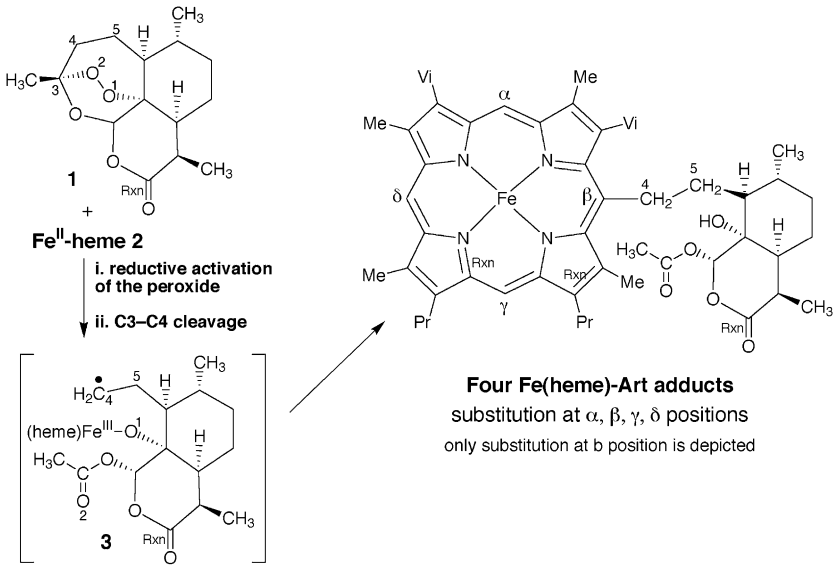


Alkylation of heme of OxyHb by artemisinin, pH 8.
HPLC monitoring at 400 nm (see Experimental Section).



Alkylation of heme of OxyHb by artemisinin, pH 8.
ES^+-MS of the reaction mixture (1 h), after dilution with aqueous formic acid, pH 4.

Fig. 1. Alkylation of OxyHb by artemisinin. HPLC and ES^+-MS monitoring of the reaction.



Scheme 1. Mechanism of the reductive activation of artemisinin by iron(II)-heme, leading to covalent heme–artemisinin adducts.

partly restored when MetHb was reduced by 10 molar equivalents of glutathione or sodium ascorbate with respect to heme, thus confirming the role of Fe(II) for the activation of artemisinin. On the other hand, addition of glutathione to OxyHb did not significantly change the alkylation reaction (adduct yield: 29%).

3.3. Reaction of artemisinin with partly digested OxyHb

OxyHb was first exposed to pronase E, trypsin [24], or α-chymotrypsin (5 wt%) at 37°C and the decay of the globin chains was monitored by HPLC. When the globin degradation was in the range of 10–40% (1–2 h), artemisinin and glutathione were added (1.2 equiv and 10 equiv, respectively, with respect to heme), and the mixture was allowed to stand for 1 h at 37°C. With α-chymotrypsin, the digestion of β- and α-globins was 10% and 30%, respectively, and the yield of heme alkylation was 24% when the reaction was performed in CH_3COONH_4 (pH 8) with 4 vol% of DMSO. A similar yield was obtained at pH 6 in the absence of glutathione, or when CH_3COONH_4 was replaced by aqueous sodium hydroxide pH 8 in the presence of 1 vol% or 20 vol% of DMSO (yield 23%). No higher yields were obtained for heme alkylation when partial proteolysis was achieved using either trypsin or pronase E.

3.4. Reaction of artemisinin with OxyHb in the presence of serum albumin

In order to check the specificity of the artemisinin reaction with hemoglobin, a large excess of BSA (OxyHb/BSA weight ratio = 1/5) was added to the reaction medium (CH_3COONH_4 pH 6, and 4 vol% DMSO). This extra non-heme protein did not induce any change in the heme alkylation yield.

3.5. Reaction of artemisinin with human whole blood

Artemisinin was added to an aliquot of hemolysed human whole blood. After 1 h, HPLC analysis indicated that 22% of the total heme content was alkylated as heme–artemisinin covalent adducts (25% when 10 equiv of glutathione were added with respect to artemisinin).

As previously reported for 'free' heme itself, the heme residues of hemoglobin are able to activate the peroxide function of artemisinin, leading to the same characterized heme–artemisinin covalent adducts in yields as high as 22–33%, providing that the iron is in its reduced state. The mild reaction conditions suggested that hemoglobin was not extensively modified. Heme residues are not deeply buried and their hydrophobic sites, able to accommodate artemisinin, may account for this reactivity.

In the reported conditions, the heme alkylation yield did not increase above 22–33%, actually, close to one alkylated heme per hemoglobin tetramer. That may be due to the limited solubility of artemisinin in the highly aqueous reaction mixture. The alkylation of one heme may also induce modifications of the conformation of the hemoglobin tetramer that preclude further alkylation reaction.

However, these results clearly indicated that artemisinin has a high affinity for hemoglobin resulting in efficient heme alkylation by the drug, even in whole blood where a lot of components might disturb the interaction of artemisinin with hemoglobin, and subsequently the activation of the drug. But they do not allow us to draw definitive conclusions about the in vivo mode of action of artemisinin. Within the digestive vacuole of *P. falciparum*, the sequential process of hemoglobin degradation is initiated by aspartic proteases called plasmepsins, one of which, plasmepsin I, starts to cleave the hemoglobin α-chain between Phe-33 and Leu-34 [25]. Interestingly, the use of several usual non-parasitic proteases did not make it possible to increase the yield of heme alkylation by artemisinin. This feature may account for the specificity of artemisinin reactivity toward *Plasmodium* compared to healthy erythrocytes.

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