Alkylation of Microperoxidase-11 by the Antimalarial Drug Artemisinin

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alkylation · artemisinin · heme proteins · malaria · mass spectrometry

Artemisinin (**1**, Scheme 1) is an antimalarial drug extracted from the Chinese wormwood *Artemisia annua*. Artemisinin and its hemisynthetic derivatives artemether and artesunate are effective against multi-drug-resistant *Plasmodium falciparum* strains. They have been used for twenty years to cure more than two million people, mainly in southeast Asia, without any serious side-effects or reported cases of resistance.[1] These peroxide-based drugs kill all stages of the malaria parasite erythrocyte life cycle, including "ring" forms, by interaction with heme to produce carbon-centered radicals[2] that are able to alkylate both the heme moiety itself and parasite proteins.[3, 4] The reductive activation of the peroxide function of artemisinin by iron(II) heme produces a high yield of heme derivatives alkylated at the *meso* positions by a C-centered radical derived from artemisinin. The resulting heme – artemisinin covalent adducts have recently been characterized.[5] The alkylating ability of artemisinin toward heme groups or parasite proteins has been considered responsible for the parasiticidal activity of the molecule.[6] In addition, it was recently reported that these heme – artemisinin adducts are able to inhibit heme polymerization but cannot self-polymerize.[7]

The ring and trophozoite stages of the *Plasmodium* life cycle are actively involved in the digestion of hemoglobin, which leads to the release of free heme into the food vacuole of the parasite. The heme group is then polymerized to form an iron(III) complex called hemozoin. We investigated the reactivity of artemisinin with a heme group bound to a peptide fragment in order to mimic the possible interaction of artemisinin with a partially degraded hemoglobin molecule. Microperoxidase-11 (MP11) is a heme undecapeptide prepared by proteolytic digestion of horse heart cytochrome *c* that retains the sequence Val11 – Glu21 of the starting protein.[8] The heme group is covalently bound to Cys14 and Cys17 through thioether linkages. The coordination of His18 as an axial ligand of the iron atom occupies one face of the porphyrin macrocycle and allows the peroxide function of artemisinin to interact with the iron atom on the other face.

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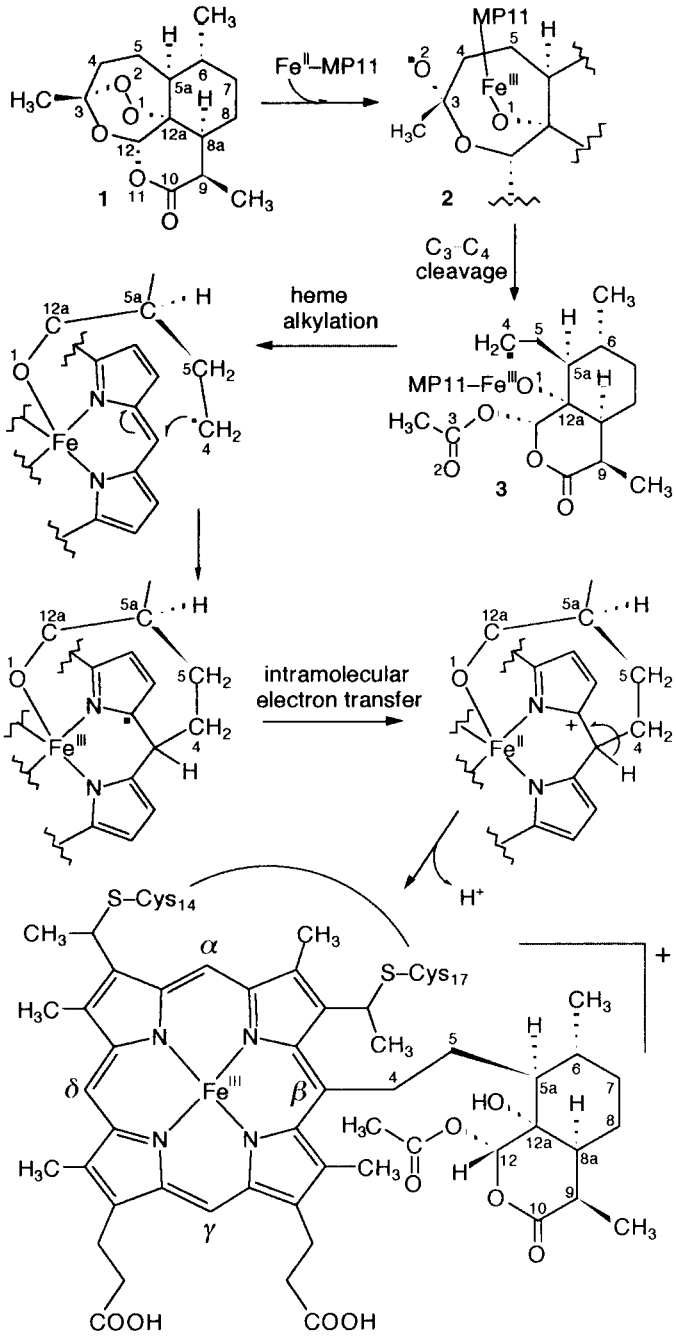

Scheme 1. *Alkylation of the heme cofactor of MP11 by reductive activation of artemisinin, which leads to an MP11 – artemisinin adduct (only monoalkylation at the β position is depicted).*

Alkylation of MP11

MP11 (disodium salt, 1 mg; 0.53 μmol) and artemisinin (10 mol equiv) were dissolved in a mixture of dimethyl sulfoxide (DMSO), acetic acid, and water (25/0.2/1.8 v/v/v, 70 μL) and the solution was degassed. A reducing agent (hydroquinone, 10 mol equiv) dissolved in the same solvent mixture (5 μL) was added in order to reduce the iron(III) heme moiety to the ferrous state and transfer one electron to the drug (final concentration of MP11 = 7.1 mM).[4] HPLC monitoring indicated more than 65 % conversion

of MP11 in 30 min at 30 °C and five peaks that correspond to less polar products were detected.[9] The reaction was stopped by addition of dichloromethane and subsequent precipitation of MP11 derivatives. The mixture was centrifuged, washed with dichloromethane, and dried. The λ_{max} value of all products was at 410 nm which, compared to that of MP11 (396 nm), suggested modification(s) of the heme moiety of MP11 had occured.

The positive ESI mass spectrometry analysis of this crude reaction mixture was consistent with the presence of MP11 derivatives that have been mono-, di-, or trialkylated by an alkyl radical produced by reductive activation of artemisinin by the iron(II) heme cofactor. The coordination of the iron(II) heme to the peroxide group followed by homolytic cleavage of the O–O bond gives rise to alkoxy radicals either on O1 or on O2 that are quickly isomerized into secondary or a primary alkyl radicals centered at C4. This C4-centered radical (**3**, Scheme 1) has previously been evidenced by trapping and alkylation of heme groups or by heme models.[3, 4] In the present work, alkylation of the heme residue of MP11 by **3** with conservation of the complete structures of both MP11 and artemisinin entities led to the mono-, di-, or trialkylated adducts termed "full-size" **MP11-(art)$_1$**, **MP11-(art)$_2$**, and **MP11-(art)$_3$**, respectively in Table 1. The mechanism of heme alkylation and the structure of the adduct

Table 1. *Positive ESI MS of the mixture of covalent MP11 – artemisinin adducts:* m/z *values detected.*

		MP11-(art)$_1$ $M = 2144$	**MP11-(art)$_2$** M = 2426	**MP11-(art)$_3$** M = 2708
$z = 2$	"full size"	1072	1213	1354
	$-CH_3COOH$	1042	1183	1324
	$-(CH_3COOH)_2$	-	1153	1295
	$-(CH_3COOH)_3$	-	-	1265
$z = 3$	"full size"	715	809	903
	$-CH_3COOH$	695	789	883
	$-(CH_3COOH)_2$	-	769	863

MP11-(art)$_1$, are depicted in Scheme 1. The mass spectrum presented peaks at $m/z = 1072$, 1213, and 1354 that were assigned to dicationic species. The molecular masses of these compounds are $(1862 + 283 - 1)$, $[1862 + (2 \times 283) - 2]$, and $[1862 + (3 \times 283) - 3]$, respectively, and correspond to the sum of the masses of MP11 ($M = 1862$), and one, two, or three C-centered radical(s) (**3** after hydrolysis of the Fe–O bond) after removal of one, two, or three hydrogen atoms from MP11. The tricationic peaks of these covalent adducts of MP11 and artemisinin were detected at $m/z = 715$, 809, and 903 for **MP11-(art)$_1$**, **MP11-(art)$_2$**, and **MP11-(art)$_3$**, respectively. Peaks corresponding to the loss of acetic acid from the full-size adducts were also present as expected (loss of 60, 120, and 180 amu from the full-size adducts described in Table 1). The acetate function at C12 of the artemisinin-derived fragment is labile because of its acetal structure, and intramolecular protonation of this fragment by a propionic acid function of the heme moiety leads to the loss of an acetic acid molecule. These results clearly show the polyalkylation of MP11 by an artemisinin-derived alkyl radical.

Alkylation Sites

The samples that gave the peaks corresponding to tricationic adducts were fragmented in order to define the alkylated positions of MP11. The fragmentation of the $m/z = 695$ sample [**MP11-(art)$_1$**, $z = 3$] produced a peak at $m/z = 838$, which arises from the monocationic monoalkylated heme derivative after cleavage of the peptide chain by reduction of the thioether functions. Such dissociation of the heme cofactor and peptide chain has been observed by MS analysis of MP11 itself. A peak at $m/z = 420$ could be assigned to the corresponding dicationic monoalkylated heme adduct.

The fragmentation of the $m/z = 769$ species, the trication of **MP11-(art)$_2$**, produced fragments of $m/z = 1062$ and 531, assignable to a dialkylated heme derivative after loss of the peptide chain and two acetic acid molecules (the 1062 and 531 peaks correspond to the mono- and dicationic species, respectively). Fragmentation of the $m/z = 769$ species also showed the above-mentioned $m/z = 695$ peak from **MP11-(art)$_1$** as the result of the loss of an artemisinin moiety.

Fragmentation of the $m/z = 903$ species, the tricationic trialkylated MP11 derivative [**MP11-(art)$_3$**, $z = 3$], produced $m/z = 769$ and 695 peaks as a result of the presence of **MP11-(art)$_2$** after loss of two acetic acid molecules and **MP11-(art)$_1$** after loss of one acetic acid, respectively. No peak assignable to a trialkylated heme derivative could be detected.

These results indicate that dialkylation of the heme group is possible in the presence of a tenfold excess of artemisinin with respect to MP11 (in the previously reported heme alkylation with a molar ratio heme:artemisinin = 1:1, dialkylated heme derivatives were not obtained and the less reactive *meso* position was the γ position[4b]). In light of this result and the steric constraint of the heme α *meso* position in MP11 (located between the two thioether functions), the most reasonable proposal is that dialkylation of the heme group occurred at the β and δ positions. It should be noted that when heme itself (instead of MP11) was reacted with artemisinin and hydroquinone under the same conditions as above, mono- and dialkylated heme derivatives were identified by positive ESI MS, but no trialkylated adduct could be detected. The site of the third alkylation of MP11 may therefore be on the peptide chain.

Yield of the Alkylation Reaction

In order to evaluate the yield of the reaction, HPLC spectra were recorded at 396 and 410 nm, the Soret band wavelengths of MP11 and the adducts, respectively. Our hypothesis was that the ε values at λ_{max} would be identical for MP11 and its alkylated derivatives. The alkylation was run with MP11/artemisinin/hydroquinone in molar ratio 1/10/10 and the selectivity of the reaction was in the range 45 – 50 % (35 % yield of alkylated MP11 for 70 % MP11 conversion after 30 min). However, the MP11/artemisinin/hydroquinone molar ratio 1/4/4 gave a selectivity of 90 % after 15 min (40 % yield of alkylated derivatives for 44 % MP11 conversion). The same conversion rates and product distribution were obtained by using the biologically relevant

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reductant glutathione instead of hydroquinone (MP11/artemisinin/glutathione molar ratio = 1/10/10, solvent: DMSO/acetic acid/water, 25/0.2/1.8 v/v/v).

Conclusion

The reductive activation of artemisinin by the heme cofactor of MP11 is able to produce drug–MP11 adducts that result from mono-, di-, or trialkylation of MP11 by a carbon-centered radical derived from the antimalarial drug. The heme moiety itself was dialkylated, likely at the β and δ positions, and a third alkylation position is now under investigation. Alkylation of heme by artemisinin is therefore possible even when the heme group is still partially protected by a protein fragment since one face of the heme moiety is accessible for the reductive activation of the peroxide group of artemisinin. These data indicate that complete digestion of globin in infected erythrocytes may not be required for heme-induced activation of artemisinin. Consequently, artemisinin is probably able to act as a lethal alkylating agent in the early stages of hemoglobin degradation mediated by *Plasmodium*.

[1] P. Newton, M. White, *Annu. Rev. Med.* **1999**, *50*, 179–192.
[2] G. H. Posner, C. H. Oh, D. Wang, L. Gerena, W. K. Milhous, S. R. Meshnick, W. Asawamahasakda, *J. Med. Chem.* **1994**, *37*, 1256–1258.
[3] S. R. Meshnick, T. E. Taylor, S. Kamchonwongpaisan, *Microbiol. Rev.* **1996**, *60*, 301–315.
[4] A. Robert, O. Dechy-Cabaret, J. Cazelles, B. Meunier, *Acc. Chem.* Res. **2002**, *35*, 167–174.
[5] a) A. Robert, J. Cazelles, B. Meunier, *Angew. Chem.* **2001**, *113*, 2008–2011; *Angew. Chem. Int. Ed.* **2001**, *40*, 1954–1957; b) A. Robert, B. Meunier, *Chem. Commun.* **2002**, 414–415; c) A. Robert, Y. Coppel, B. Meunier, *Inorg. Chim. Acta* **2002**, in press.
[6] a) Y.-L. Hong, Y.-Z. Yang, S. R. Meshnick, *Mol. Biochem. Parasitol.* **1994**, *63*, 121–128; b) J. Bhisutthibhan, X.-Q. Pan, P. A. Hossler, D. J. Walker, C. A. Yowell, J. Carlton, J. B. Dame, S. R. Meshnick, *J. Biol. Chem.* **1998**, *273*, 16192–16198.
[7] R. Kannan, D. Sahal, V. S. Chauhan, *Chem. Biol.* **2002**, *9*, 321–332.
[8] H. M. Marques, C. B. Perry, *J. Inorg. Biochem.* **1999**, *75*, 281–291. The sequence of MP11 is: Val11-Gln12-Lys13-Cys14-Ala15-Gln16-Cys17-His18-Thr19-Val20-Glu21.
[9] A 10 mm C18 Nucleosil column was used for the HPLC analysis. The eluent solutions were: A) 20 mM ammonium acetate (pH 4.4); B) methanol. The gradient used was: A/B 50/50 to A/B 42/58 in 55 min, to A/B 75/25 in 10 min, to A/B 50/50 in 10 min. Elution rate: 0.7 mL min^{-1}. Detection at 396 nm (λ_{max} for MP11) and 410 nm (λ_{max} for alkylated MP11). Retention time: 16.4 min (MP11), 18.0, 20.8, 24.5, 29.5, 37.0 min (alkylated MP11).

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