COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Alkylation of heme by the antimalarial drug artemisinin

Anne Robert, Yannick Coppel and Bernard Meunier*

Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne 31077 Toulouse cedex 4, France. E-mail: bmeunier@lcc-toulouse.fr

Received (in Cambridge, UK) 26th November 2001, Accepted 4th January 2002
First published as an Advance Article on the web 31st January 2002

The peroxide function of artemisinin has been activated by iron(II)-heme generated *in situ* from iron(III)-protoporphyrin-IX and glutathione, a biologically relevant reductant. In mild conditions, this reaction produced a high yield (85%) of heme derivatives alkylated at α-, β-, and δ-*meso* positions by a C4-centered radical derived from artemisinin.

Artemisinin (**1**, Fig. 1) and its derivatives are highly active against multidrug resistant strains of malaria parasites. Their pharmacological activity is undoubtfully due to the endoperoxide function, since an artemisinin derivative lacking the peroxide function (namely deoxyartemisinin) is devoid of antimalarial activity.[1] Within red blood cells, *Plasmodium falciparum* digests the host hemoglobin to provide itself with aminoacids and to build its own proteins. Then, to avoid the accumulation of the released and potentially toxic iron(II)-heme, *Plasmodium* polymerizes this redox active species as a microcrystalline, non-toxic, iron(III) polymer, called hemozoin or malaria pigment.[2] The possible reactivity of the peroxide function of artemisinin with this unique metabolic pathway has been considered as the key factor of the pharmacological activity.[3,4]

The heme-promoted reductive homolysis of the peroxide function of artemisinin and structurally related trioxanes leading to C4-centered alkylating radicals has been documented in studies on the mechanism of action of these antimalarial drugs.[5,6] The alkylation of proteins,[7] or heme[8] was observed after incubation of pharmacologically relevant concentrations of radiolabelled artemisinin derivatives in cultured parasites, but no structure was proposed for these heme–artemisinin or protein–artemisinin adducts. The *in vitro* alkylation of a synthetic metalloporphyrin used as a heme model by artemisinin and other synthetic pharmacologically active trioxanes has been reported[9,10] and, more recently, alkylation of the dimethylester derivative of heme has also been evidenced.[11] These alkylation reactions using heme models involve a C-centered radical **3** generated by reductive activation of the peroxide bond of artemisinin, followed by C3–C4 β-scission.[12] The alkylation of glutathione by the primary C4-centered radical derived from artemisinin to produce a thioether adduct has been recently reported.[13]

Here, we report the alkylation of heme itself, one of the main targets of artemisinin, in the presence of glutathione, a reducing agent present in high concentration in erythrocytes. Artemisinin was incubated for 1 h at 37 °C with iron(II)-heme **2** generated *in situ* by reduction of hemin with glutathione in dimethyl sulfoxide (final concentrations: [artemisinin] = [hemin] = 30 mM, [glutathione] = 300 mM). Under these mild conditions, quantitative alkylation of three of the *meso* positions of the protoporphyrin-IX ligand was readily achieved. Water was added to the reaction mixture, the precipitate so obtained was filtered, washed with water and acetonitrile. The crude product

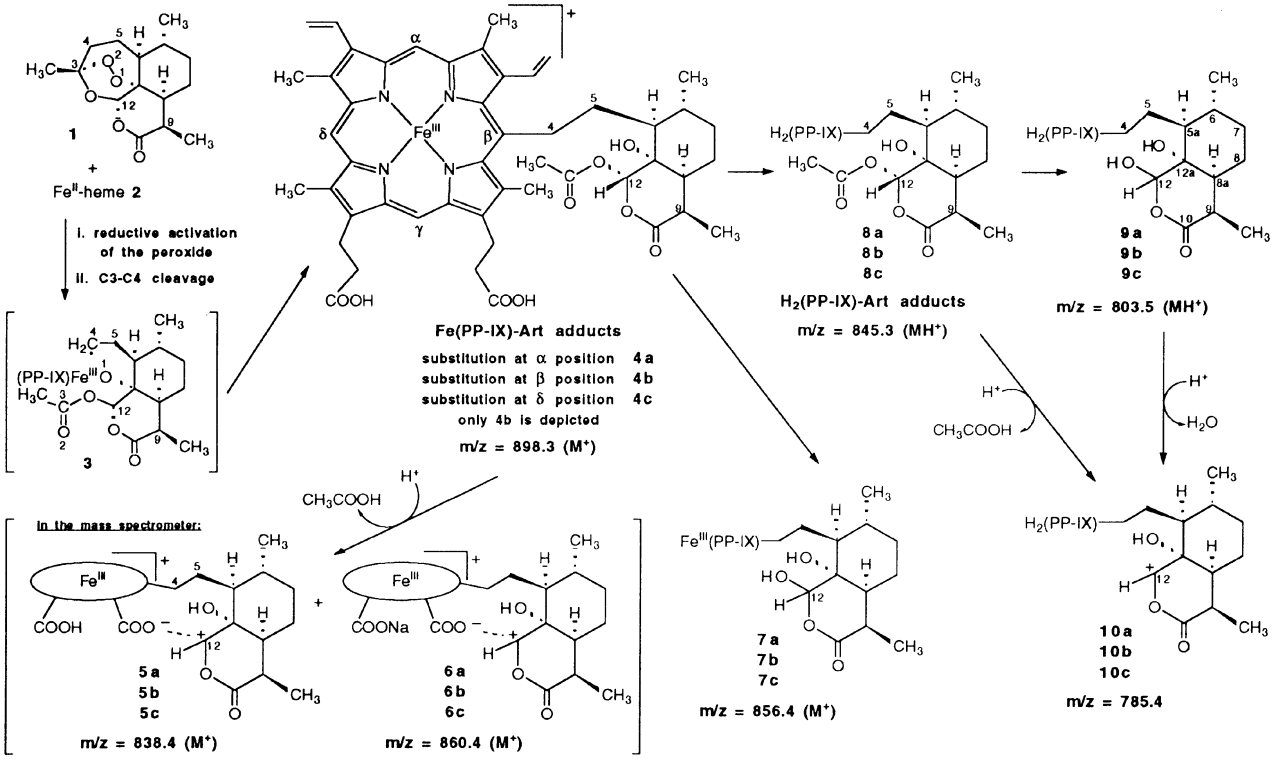

Fig. 1 Alkylation of iron(II)-heme by artemisinin in the presence of glutathione. In mass spectrometry, M$^+$ is detected for iron(III) complexes without axial ligand. The oval stands for the protoporphyrin-IX macrocycle.

DOI: 10.1039/b110817b

was analyzed by ES$^+$-MS. The molecular peak was detected at m/z = 898.3 (M$^+$, compound **4**), corresponding to the expected mass for a 1 : 1 iron(III)-heme–artemisinin adduct. The identification of the alkylation positions was carried out after demetallation reaction (see below). The parent peak (m/z = 838.4, M$^+$ for compound **5**) was due to the loss of an acetic acid molecule from **4** within the mass spectrometer, and the sodium carboxylate derivative **6** (m/z = 860.4, M$^+$) was also detected. Only a small amount of non-alkylated hemin (11% of the parent peak, m/z = 616.2) was detected. As minor compounds ($\leqslant$5%), the C12-methoxylated derivative (m/z = 870.3, M$^+$ for the iron(III) complex, not shown in the Figure) and its monosodium carboxylate analogue (m/z = 882.4) were also detected. These two latter compounds were generated within the spectrometer by nucleophilic attack at C12 of the methanol used as solvent for the MS analyses.

For further characterization, the crude mixture of heme–artemisinin regioisomer adducts was submitted to demetallation with iron(II) sulfate (20 mol. equiv.) and HCl 12 M in glacial acetic acid at room temperature.[14] Demetallation was immediate as attested by UV-visible spectroscopy. The reaction mixture was then diluted with water and the alkylated protoporphyrin-IX ligand **8** was extracted with ethyl acetate, and precipitated by addition of hexane. The covalent adducts **8** were characterized by UV-visible spectroscopy [λ_{max} (rel. intensity) in acetone: 410 (100), 510 (8), 542 (4), 584 (3)] and ES$^+$-MS spectroscopy [m/z (rel. intensity) = 845.3 (6, MH$^+$), 785.4 (100, MH$^+$–CH_3COOH). As in the case of the iron adducts, the nucleophilic attack of methanol in MS conditions gave rise to the C-12 methoxylated adduct with m/z = 817.4 (5, MH$^+$)]. The structure of **8** was confirmed by ^{1}H NMR (250 MHz, acetone-d_6) with intracyclic NH detected at $-$3.03 ppm and vinyl protons at 5.86–6.34 (H_a + H_b) and 7.96–8.24 (H_c). The covalent coupling between artemisinin and heme moieties was attested by the resonance of H_2C4 at 5.05 and 5.37. The resonance of H12 as a sharp singlet at 5.62 ppm comfirmed the retention of the ortho ester structure of the artemisinin derived fragment. The molar ratio between covalent adducts and non-alkylated protoporphyrin-IX was 85 : 15.

Actually, these alkylation adducts were found to be unstable because of their ortho ester structure, and hydrolyzed to the mixture of adducts **9**, even in mild conditions, *e.g.* a mixture acetone–water. Adducts **9** were characterized by ES$^+$-MS [m/z (rel. intensity) = 803.5 (15, MH$^+$), 785.4 (100, MH$^+-$ H_2O)]. It should be mentioned that the only molecular peak allows the spectra of **8** and **9** to be distinguished. In fact, the fragment cation at m/z = 785.4 (compound **10**, Fig. 1) present in both spectra can be produced by protonation and loss of acetic acid or water from **8** and **9**, respectively. However, the three major adducts **9** were characterized by ^{1}H NMR at 500 MHz (acetone d_6, 318 K). They correspond to the alkylation of heme at the α-, β- and δ-*meso* positions without regioselectivity (25%, 28%, 33%). The amount of adduct at γ positions was low (13%). The NH resonances were detected at $-$2.99 ppm, strongly deshielded with respect to the protoporphyrin-IX ($-$4.12 ppm). The *meso* protons were detected in the region 10.1–10.3 ppm, with a low resolution. However, the methylene group H_2C4 was detected at 5.24 and 5.50 (broad signals) as expected for binding to *meso* positions of the porphyrin ring, and these signals exhibited NOE with the β-pyrrolic methyls (3.58–3.81 ppm) indicating the alkylation at α-, β-, and δ- *meso* positions. Vinyl H_c resonances resulted in a complex pattern in the range 8.16–8.40 ppm, and H_a and H_b appeared as six different multiplets, confirming the presence of three different major adducts (5.98, 6.18, 6.23, 6.27, 6.35, and 6.44 ppm). On the artemisinin moiety, H_3C–C9 resonance was detected as three doublets at 1.03, 1.05, and 1.06 ppm (^3J = 7.8 Hz) coupled with H9 (2.80 ppm). For the minor adduct resulting from alkylation at γ, the resonances of H_3C–C9 and H9 were detected at 1.02 and 2.56 ppm, respectively, the other signals being overlapped with those of the three major adducts.

Some of the products characterised in the present work have been obtained but not identified by Meshnick *et al*. when artemisinin was incubated with hemin, isolated hemozoin, or cultured *P. falciparum*.[8] These authors isolated adducts with m/z = 838, 856, 857, and 871, corresponding to adducts **5**, **7**, the iron(II) analogue of **7** (MH$^+$), and the iron(II) complex bearing a methoxy substituent at C12 (MH$^+$), respectively, all these compounds resulting from alkylation of heme by the drug [reduction of iron(III) porphyrin complexes to iron(II) analogues is frequently observed under mass spectrometry conditions].

Both structures **7a–c** and **9a–c** have been depicted with a lactone cycle, but an open form with an aldehyde function at C12 and a free acid at C10 is also possible. Further NMR investigation will answer this question.

It should be noted that the alkylation of heme by artemisinin is observed in high yield in the presence of an excess of glutathione, a compound which has been proposed as a key target of artemisinin.[13] This result indicates that, in a mixture where heme and glutathione are possible competitive targets, 85% of artemisinin preferentially alkylates heme instead of glutathione.

In infected erythrocytes, free heme is generated by hemoglobin digestion in the presence of glutathione, which is present at millimolar concentration. Under these conditions, the reductive activation of artemisinin may produce covalent heme–artemisinin adducts in high yield in a very short time (compatible with the short half-life of artemisinin in plasma) *via* a carbon-centered radical generated at C4 position of artemisinin. This alkylation process should be toxic for *Plasmodium* by accumulation in the parasite of non-polymerizable redox-active heme–drug adducts. Such alkylation process can also probably be extented to essential proteins of the parasite.

Notes and references

1 D. L. Klayman, *Science*, 1985, **228**, 1049–1055.
2 S. Pagola, P. W. Stephens, D. S. Bohle, A. D. Kosar and S. K. Madsen, *Nature*, 2000, **404**, 307–310.
3 S. R. Meshnick, T. E. Taylor and S. Kamchonwongpaisan, *Microbiol. Rev.*, 1996, **60**, 301–315.
4 A. V. Pandey, B. L. Tekwani, R. L. Singh and V. S. Chauhan, *J. Biol. Chem.*, 1999, **274**, 19383–19388.
5 G. H. Posner, C. H. Oh, D. Wang, L. Gerena, W. K. Milhous, S. R. Meshnick and W. Asawamahasakda, *J. Med. Chem.*, 1994, **37**, 1256–1258.
6 C. W. Jefford, F. Favarger, M. G. H. Vicente and Y. Jacquier, *Helv. Chim. Acta*, 1995, **78**, 452–458.
7 J. Bhisutthibhan, X.-Q. Pan, P. A. Hossler, D. J. Walker, C. A. Yowell, J. Carlton, J. B. Dame and S. R. Meshnick, *J. Biol. Chem.*, 1998, **273**, 16192–16198.
8 Y.-L. Hong, Y.-Z. Yang and S. R. Meshnick, *Mol. Biochem. Parasitol.*, 1994, **63**, 121–128.
9 A. Robert and B. Meunier, *J. Am. Chem. Soc.*, 1997, **119**, 5968–5969.
10 J. Cazelles, A. Robert and B. Meunier, *J. Org. Chem.*, published on the web 21st January 2002.
11 A. Robert, J. Cazelles and B. Meunier, *Angew. Chem., Int. Ed.*, 2001, **40**, 1954–1957.
12 A. Robert, O. Dechy-Cabaret, J. Cazelles and B. Meunier, *Acc. Chem. Res.*, manuscript accepted for publication.
13 D.-Y. Wang and Y.-L. Wu, *Chem. Commun.*, 2000, 2193–2194.
14 J.-H. Fuhrhop and K. M. Smith, in *Porphyrins and Metalloporphyrins*, ed. K. M. Smith, Elsevier, Amsterdam, 1975, pp. 757–869.