Characterization of the First Covalent Adduct between Artemisinin and a Heme Model

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The incidence of malaria is dramatically increasing since many *Plasmodium falciparum* strains are now resistant to widely-used drugs like chloroquine. The discovery of artemisinin[1,2] (**1**; Figure 1), a potent chinese antimalarial drug extracted from *Artemisia annua*, has drawn the attention to drugs having an endoperoxide function.[3,4] The mechanism of action of **1** probably involves the reductive activation of the endoperoxide function, leading to dioxygen-derived radicals responsible for an oxidative stress[5] within infected erythrocytes or *C*-centered radicals derived from artemisinin and acting as alkylating agents.[3,6] Alkylation of heme[7] and specific parasite proteins[8] by artemisinin is supposed to be more pharmacologically significant than the oxidative stress,[2] but no structure of an artemisinin biologically relevant adduct has been described up to now. Here we report the characterization of a covalent adduct between artemisinin and a heme model, manganese(II) *meso*-tetraphenylporphyrin. This chlorin adduct resulted from a *C*-alkylation of the porphyrin cycle by radical **3** produced by reductive homolytic cleavage of the peroxide bridge of **1**.

The digestion of hemoglobin of infected erythrocytes by the parasite produces free heme. To study a possible adduct formation between a reduced heme (intracellular glutathione concentration is 1−5 mM) and artemisinin, we decided to use a hydrophobic heme model, since artemisinin is insoluble in water, Fe^{II}(TPP), generated in situ by borohydride reduction of Fe^{III}(TPP)Cl (TPP = *meso*-tetraphenylporphyrin dianion). A chlorin-type covalent adduct was obtained between the macrocycle and artemisinin. Unfortunately, the strong paramagnetism of the iron compound did not allow a detailed characterization of this drug−macrocycle adduct (for partial MS data, see below). Removal of iron from porphyrin or chlorin ligands requires drastic conditions which might strongly denature the adduct structure. Consequently we decided to use the manganese analogue Mn^{III}(TPP)OAc, which can be demetalated under milder conditions.[9] Furthermore, the reduction of this manganese complex is easily monitored by visible spectroscopy (Soret bands of Mn^{III}TPP and Mn^{II}TPP being at 472 and 444 nm in dichloromethane, respectively). Artemisinin was incubated for 80 min with Mn^{II}TPP generated *in situ* by reduction with tetra-*n*-butylammonium borohydride under nitrogen at room temperature in dichloromethane (final concentrations: [artemisinin] = 15 mM, [Mn(TPP)OAc] = 4.5 mM, [BH_4^-] = 45 mM). The reaction mixture was then exposed to air, and by addition of diethyl ether a dark-green product containing a manganese(III) compound was precipitated. The UV−vis spectrum of this complex [λ_{max} (nm) (rel intens) 378 (0.95), 479 (1.0), 646 (0.25)] strongly suggested a chlorin-type macrocycle. By mass spectrometry (DCI/NH_3^+), intense peaks were observed at *m/z* = 876 (70), 894 (100), and 936 (20). This product was therefore not the result of a simple addition of the two components (M = 667 and 282 for Mn^{III}TPP and **1**, respectively), but a covalent adduct between a modified artemisinin and a metallochlorin without extensive degradation of both partners. For a complete characterization by NMR spectroscopy the demetalation of the manganese(II) adduct was performed directly after its formation. Therefore, after 80 min of incubation of artemisinin with Mn^{II}-TPP, the reaction mixture was transferred under nitrogen into an acetic acid/12 M hydrochloric acid mixture (95/5, v/v) and stirred for 15 min at 15 °C to ensure a complete demetalation. After neutralization with a 28 wt% aqueous solution of NH_4-OH and extraction with dichloromethane, the covalent adduct artemisinin−macrocycle **4-H_2** (Figure 2) was purified by a chromatography column of neutral alumina with dichloromethane as eluent (yield 25%/metalloporphyrin).

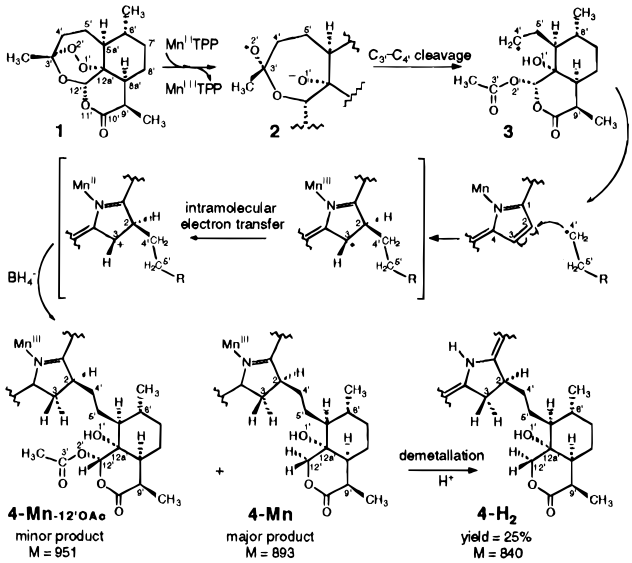

Figure 1. Mechanism of alkylation of the heme model Mn^{II}TPP by artemisinin **1** in the presence of borohydride.

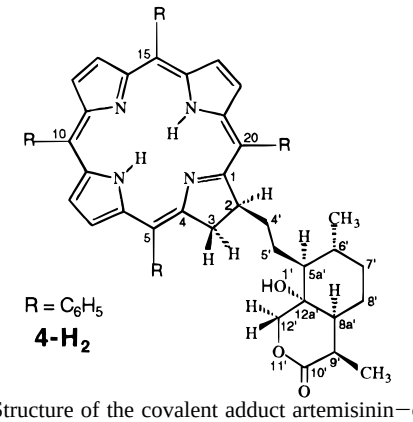

Figure 2. Structure of the covalent adduct artemisinin−chlorin **4-H_2** [the only (2*S*) stereoisomer is depicted]. Significant NMR chemical shifts (CD_2Cl_2, δ in ppm with respect to external TMS). ^{1}H-NMR (400.14 MHz): 4.70 ($H_{2\alpha}$), 4.37 ($H_{3\alpha}$), 3.98 ($H_{3\beta}$), 1.46 and 1.69 ($H_2C_{4'}$), 1.00 and 1.25 ($H_2C_{5'}$), 0.52 ($H_{5a'}$), 1.06 ($H_{6'}$), 0.69 (H_3C-$C_{6'}$), 0.80 and 1.52 ($H_2C_{7'}$), 1.30 and 1.69 ($H_2C_{8'}$), 1.30 ($H_{8a'}$), 3.09 ($H_{9'}$), 1.05 (H_3C-$C_{9'}$), 3.79 and 4.10 ($H_2C_{12'}$), 0.95 (*H*O-$C_{12a'}$), - 1.50 (N*H*). ^{13}C-NMR (100.62 MHz): 44.70 (C_2), 41.65 (C_3), 35.46 ($C_{4'}$), 23.25 ($C_{5'}$), 53.20 ($C_{5a'}$), 34.31 ($C_{6'}$), 19.78 (H_3C-$C_{6'}$), 33.48 ($C_{7'}$), 23.33 ($C_{8'}$), 47.18 ($C_{8a'}$), 33.37 ($C_{9'}$), 12.06 (H_3C-$C_{9'}$), 172.66 ($C_{10'}$), 71.95 ($C_{12'}$), 72.13 ($C_{12a'}$).

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The UV−visible spectrum of the demetalated adduct **4-H$_2$** was characteristic of a chlorin-type macrocycle [λ_{max} (nm) (rel intens) 408$_{sh}$, 418 (1.0, Soret), 520 (0.11), 546 (0.08), 596 (0.07), and 654 (0.15) with a ratio $\epsilon_{654}/\epsilon_{418} = 0.15$]. NMR analyses were performed in CD_2Cl_2 using $\delta^1_H - \delta^{13}_C$ GE-HMQC (^1J) and long-range (^3J) proton−carbon correlations. The multiplet signals corresponding to the aromatic protons (7.70−8.60 ppm) indicated the loss of C_4 symmetry. The two intracyclic N*H* protons were detected at −1.50 ppm (compared to −2.83 ppm in the case of H_2TPP), indicating a diminution of the ring current of the macrocycle. Three dihydropyrrole protons were detected at 4.70 ($H_{2\alpha}$), 4.37 ($H_{3\alpha}$), and 3.98 ($H_{3\beta}$), typical chemical shifts for chlorin derivatives.[10] The proton at 4.70 ppm was coupled with the two protons (1.69 and 1.46 ppm) of the 4′-C*H*$_2$ group of the artemisinin moiety. This provided confirmation of the *C*-alkylation and the reduction of one pyrrole ring of the starting porphyrin ligand.

The proton signals of the linked artemisinin, being close to the macrocycle, were significantly shielded compared to the corresponding protons in free artemisinin: $\Delta\delta = -0.89$ ($H_{5a'}$), −0.71 and −0.60 (*H*$_2$C$_{4'}$), −0.35 (*H*-C$_{6'}$), and −0.30 (*H*$_3$C-C$_{6'}$). The signals of protons $H_{12'}$ and *H*$_3$C-C$_{3'}$ (5.85 and 1.42 ppm, respectively, in **1**) disappeared. The presence of two protons at $\delta = 3.79$ and 4.10 ppm ($^2J_{HH} = 13$ Hz) indicated the reduction of carbon $C_{12'}$, the proton corresponding to the signal at 4.10 ppm being coupled with a broad singlet at 0.95 ppm assigned to the hydroxyl function at the 12a′ position. The other signals of the artemisinin moiety were not extensively modified; $H_{9'}$ was present at 3.09 ppm (compared to 3.33 ppm in **1**), attesting the presence of the lactone function confirmed by IR ($\nu_{C=O} = 1734$ cm^{-1}, KBr pellet).

The mass spectrum (DCI/NH_3^+) of **4-H$_2$** exhibited two intense peaks at *m/z* = 841 (MH^+) and 823 [$(M - 18)H^+$, loss of a water molecule]. The molecular mass of this demetalated adduct (M = 840) had 53 mass units less than that of the metalated compound (M = 893), corresponding to the removal of manganese and introduction of two hydrogens. These MS data confirmed that demetalation did not induce other chemical changes. Optical rotation was only measurable at 578 nm where the transmittance was above 70%: $[\alpha]_{598} = -1110$ ($c = 48.5 \times 10^{-6}$, CH_2Cl_2]. It should be noted that both (2*R*) and (2*S*) stereoisomeric configurations were obtained for **4-H$_2$**.

The formation of this alkylation product can be explained by the mechanism proposed in Figure 1. The reductive activation of the peroxidic bond of artemisinin by the manganese(II) porphyrin induced the homolytic cleavage of this O−O bond, the RO$^\bullet$ radical being formed on either the $O_{1'}$ or the $O_{2'}$ atom. The RO$^\bullet$ radical **2** can be quickly isomerized by homolytic cleavage of the $C_{3'}-C_{4'}$ bond to produce the nonsterically hindered *C*-centered radical **3**.[3] The addition of this radical on the β-pyrrolic carbon atom C_2 allowed, after an intramolecular electron transfer from the C_3 position to the manganese(III), the generation of a carbocation at C_3. The attack of borohydride at this position gave the dihydropyrrole ring. Borohydride also mediated the reduction at $C_{12'}$ of the artemisinin fragment and the loss of the acetate side chain to get the metalated adduct **4-Mn** [DCI/NH_3^+-MS: *m/z* = 894 (MH^+, 100), 876 [$(M - 18)H^+$, 70]]. The complex **4-Mn−12′-OAc** was also detected as a minor product in the DCI/NH_3^+ mass spectrum of the crude metalated product [*m/z* = 936 $(M - CH_3)^+$, 20].

The introduction into the covalent adduct of two H atoms from borohydride (at C_3 and $C_{12'}$) has been confirmed by using tetra-*n*-butylammonium borodeuteride. In this case, the mass spectrum of the deuterated analogue of **4-H$_2$** exhibited a MH^+ peak at *m/z* = 843 as expected. The positions of these deuterium atoms were confirmed by NMR: proton $H_{3\alpha}$ (4.37 ppm) disappeared from the NMR spectrum, being completely replaced by deuterium. This fact indicated that reduction occurred in the *trans* position with respect to the artemisinin addition. On the contrary, the $H_{12'\alpha}$ and $H_{12'\beta}$ intensities were both 50% of the expected intensity for one proton. The introduction of one deuterium atom at this $C_{12'}$ position with loss of the stereochemistry supported a reduction of the acetoxy group at 12′ involving probably an opening−recyclization process of the lactone ring. The experiment with borodeuteride provided a further piece of evidence for the major loss of the 12′-OAc group before the acidic demetalation treatment. The mass spectrum of the metalated covalent adduct obtained in the presence of BD_4^- was found at *m/z* = 896, instead of 894, when the reaction was performed with BH_4^-. These MS data confirmed that this deuterated analogue of **4-Mn** already contained two deuterium atoms and was therefore reduced at 12′ before demetalation.

This demetalated chlorin-type adduct can be oxidized to the corresponding porphyrin by refluxing it for 30 min in dichloromethane with 3 molar equiv of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The product, recovered after chromatography (SiO_2, CH_2Cl_2/CH_3OH, 99/1, v/v), presented a typical UV−vis spectrum for a porphyrin ($\lambda_{max} = 418$ nm, no absorption at 650 nm). The reaction was monitored by HPLC (analytical Nucleosil 10 μm column; eluent, 15% 1 M CH_3COONH_4, 85% a mixture of CH_3OH/CH_3CN, 90/10; 1 mL/min, detection at 420 nm; the retention time of the porphyrin adduct was identical to that of the chlorin adduct at 26.6 min, but the product was differentiated by its UV−vis spectrum with a diode array detector). The ^{1}H NMR spectrum (250 MHz, CD_2Cl_2) confirmed the structure of the covalent adduct: N*H* resonance was detected at −2.85 ppm, and the region 3.5−5 ppm was cleared from signals except the two protons of the 12′ position of the artemisinin moiety ($\delta = 4.42$ and 4.17 ppm, $^2J_{HH} = 13$ Hz). The 4′−C*H*$_2$ of the artemisinin fragment, being linked to an aromatic structure, was detected as a triplet at 2.95 ppm, strongly deshielded, compared to the two complex signals at 1.46 and 1.69 ppm for this 4′-C*H*$_2$ in the chlorin derivative. Other protons of the porphyrin−artemisinin adduct were present with correct integral values, attesting that no other modifications occurred during the dihydropyrrole oxidation.

In the conditions described above with Mn^{III}(TPP)OAc, a reaction was performed with Fe^{III}(TPP)Cl, artemisinin, and borohydride (1/3/10 molar equiv). After 3 h of reaction, the dichloromethane solution was washed with a 1.5 M HCl solution and water and dried over sodium sulfate (UV−vis: λ_{max} (nm) (rel intens) = 388 (0.93), 414 (1.0), 600 (0.23)). The mass spectrum (DCI/NH_3^+) of this crude product exhibited a peak at *m/z* = 953 (M + 1) as expected for the iron analogue of **4-Mn−12′-OAc** (Figure 1). We are currently trying to find a mild iron removal method to establish the structure of this adduct. This method should also be applicable to the demetalation of heme, one of the biological targets of artemisinin.

The characterization of the first covalent adduct artemisinin−heme model, obtained by alkylation of a porphyrin ring by an artemisinin *C*-radical derivative, is a key step in the understanding of the mode of action of this antimalarial drug. The formation of this chlorin−artemisinin adduct confirmed that the methylene radical **3** is a powerful alkylating agent which can be responsible for the formation of artemisinin−heme[7] and artemisinin−protein[8] radioactive adducts previously evidenced when *P. falciparum* infected erythrocytes have been incubated *in vitro* with radiolabeled **1**. The inhibition of hemozoin formation by **1**[11] might be due to the direct alkylation of the histidine-rich protein "HRP",[8] responsible for the production of this inert crystalline polymeric form of heme,[12] or to an artemisinin−heme adduct acting as inhibitor of this parasite protein.

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