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Spectrophotometric and ESI-MS/HPLC Studies Reveal a Common Mechanism for the Reaction of Various Artemisinin Analogues with Hemin

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Abstract—The reaction of hemin with three well known artemisinin analogues, namely dihydroartemisinin, artemether and artesunate, was independently analysed by visible spectrophotometry and by ESI-MS/HPLC. A very similar reaction pathway emerges for all these compounds that matches closely the reaction profile previously described for artemisinin. In the course of the reaction characteristic isomeric 1:1 drug–hemin adducts are formed as in the case of artemisinin; eventual disruption of the porphyrin ring takes place in all cases, most likely through oxidative degradation.

The efficacy of artemisinin and its congeners as antimalarial agents has received considerable attention in recent years, in particular for the treatment of the chloroquine-resistant forms.[1] Artemisinin is a sesquiterpene lactone containing an unique endoperoxide bridge that is primarily responsible for the antimalarial activity.[2] The mechanism of action of artemisinin, still object of much debate,[3] most likely involves the formation of free radical intermediates, originating from the direct interaction of the endoperoxide group with the heme iron.[4–8] It is still controversial whether the plasmocidal effect derives from inhibition of hemin polimerisation into non-toxic hemozoin or from direct damage to *Plasmodium* proteins through alkylation.

Unfortunately, both the low solubility and the poor oral bioavailability of artemisinin severely impair its therapeutic efficacy. Chemical modifications of artemisinin have resulted into new derivatives that combine higher antimalarial activity and higher solubility. For instance, derivatization of artemisinin (Fig. 1a) at C_{10} has yielded compounds such as artemether and sodium artesunate that have already entered the clinics[9,10] (Fig. 1b and c). Another important derivative, in clinical use, is dihydroartemisinin (Fig. 1d).[11] All these drugs are characterised by important plasmocidal activities with IC_{50} values falling in the micromolar range.[12]

We have recently described the direct reaction of artemisinin with hemin, carried out in the absence of reducing agents (thus with the iron center in the oxidation state +3), and gained new insight into the underlying molecular mechanisms.[13] Isomeric artemisinin–hemin adducts were clearly identified; in addition, our studies showed that the reaction eventually leads to hemin degradation, most likely through an oxidative pathway.[13]

To further support the results obtained on the artemisinin/hemin system, we have analysed in detail, by joint use of spectrophotometry and ESI-MS/HPLC, the corresponding reactions of dihydroartemisinin, artemether and artesunate with hemin.

At 37 °C, the reaction is very slow for all derivatives and does not reach completion even within 24 h (data not shown). To reduce the duration of the experiments, the

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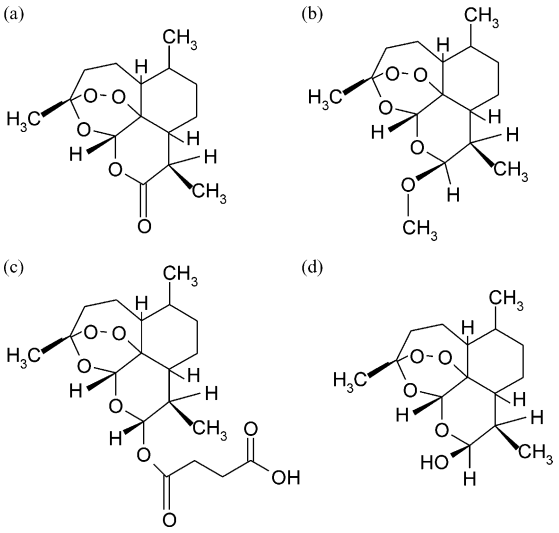

Figure 1. Schematic drawing of artemisinin, artemether, artesunate and dihydroartemisinin.

spectrophotometric determinations were carried out at higher temperature (70 °C). Remarkably, no significant modifications of the spectral shapes were observed compared to the experiments performed at 37 °C.

The time dependent spectrophotometric profiles are shown in Figure 2. Notably, the spectrophotometric patterns obtained for the three derivatives are very similar to one another implying a virtually identical reaction mechanism. In all cases, addition of the trioxane drugs produces a progressive decrease in intensity of the Soret band until disappearance. At 70 °C the reaction is complete within ca. 12 h for all compounds, being sligthly faster for dihydroartemisinin. In all cases, a clear isosbestic point is detected at 340 nm; a modest red shift of the Soret band is observed as well. On turn, the present spectral profiles match very closely those of the direct reaction of artemisinin with hemin, previously reported.[10,13]

The reactions of the three artemisinin derivatives with hemin were independently assayed by the ESI-MS/HPLC technique, an approach particularly suitable to analyse complex reaction mixtures.[14] Samples were analysed at various time intervals over 24 h working again at 70 °C. In all cases the reaction results into slow formation of a series of characteristic drug-hemin adducts. Representative profiles of the different drug/hemin samples, analysed 3 h after mixing, are shown in Figure 3. Remarkably, the peak of hemin, the first component to elute ($R_t = 12.7$ min), progressively decreases in intensity with time whereas new peaks appear with longer retention times, characterised by molar masses that roughly correspond to the 1:1 adducts of the individual drugs with hemin. Details of the HPLC–MS results of the three investigated systems are provided in ref 15.

The visible spectra of these adducts exhibit modest but significant red shifts of the Soret band in line with the above spectrophotometric results. On turn, after a few hours, the peaks of the 1:1 adducts decrease in intensity,

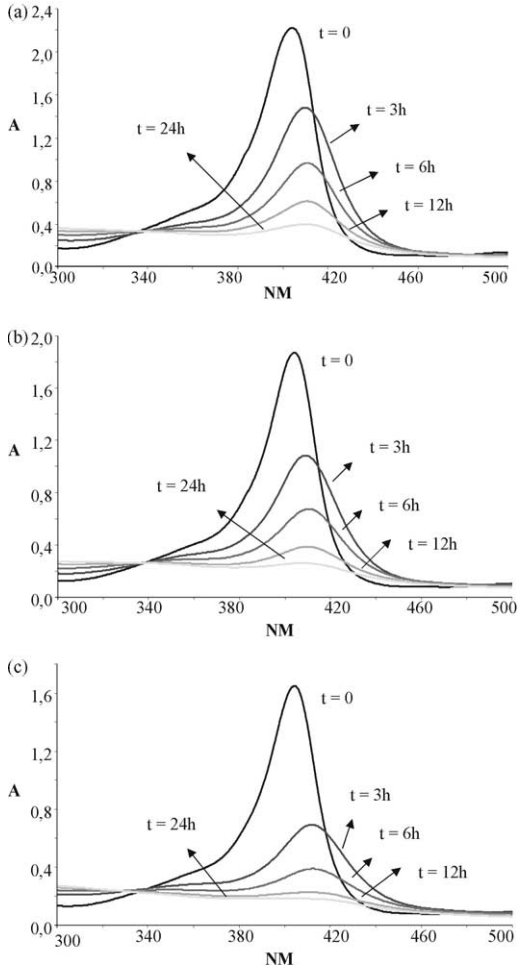

Figure 2. Spectrophotometric profiles of the reaction between hemin (1×10^{-5} M) and the three artemisinin analogues (2×10^{-5} M) in DMSO at 70 °C followed during 24 h: (a) artemether; (b) artesunate and (c) dihydroartemisinin.

in agreement with the progressive decrease in intensity of the Soret band. We interpret this peculiar behavior in terms of progressive disruption of the porphyrin ring, most likely through oxidative degradation.[16,17] Again, strict similarities in the behavior of these three compounds are evident as well with the behavior of artemisinin.[13]

Thus, we can state that all the artemisinin analogues considered in this work manifest very similar spectral features in their reaction with hemin. The observed reaction profiles match quite closely that of artemisinin, implying a virtually identical interaction mechanism. The reaction essentially consists of two steps: first 1:1 drug–hemin adducts are formed that conserve the Soret band; then these adducts further evolve with concomitant loss of the Soret band. In our opinion the modest differences observed in the respective reactivities with hemin do not account for the important differences observed in their biological activity. It is likely that the improved pharmacological properties of these artemisinin analogues must be primarily referred to differences in bioavailability and pharmacokinetics.

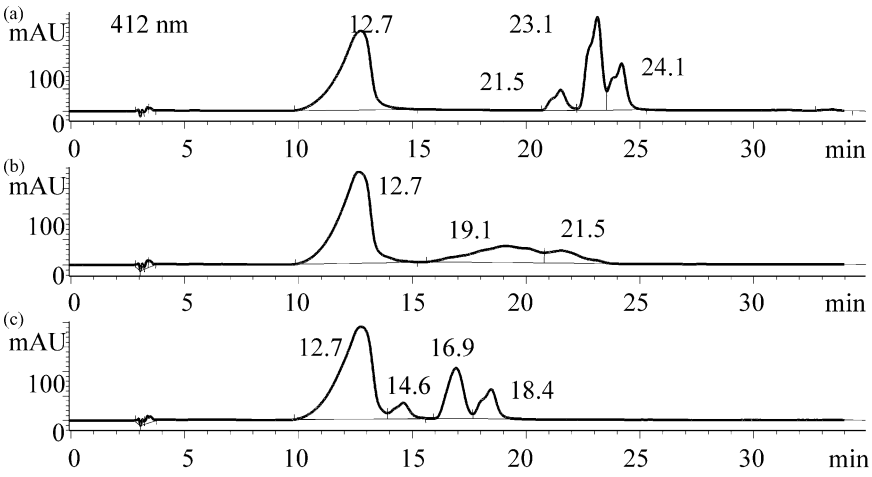

Figure 3. HPLC profiles of the adduct between hemin and artemether (a), artesunate (b) and dihydroartemisinin (c) after 3 h at 70 °C.

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14. **Analytical HPLC/DAD/MS**: HPLC/DAD analysis was performed on a HP 1100L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Hewlett & Packard, Palo Alto, CA, USA). The reverse-phase column was a RP-18 201TP54 (5 μm, 250 mm, 0.5 mm i.d., 300 Å, Vydac Separation Group Hesperia, CA, USA) maintained 30 °C. The eluents were A: H_2O adjusted to pH 3.2 by HCOOH and B: MeOH. The following solvent gradient was applied: from 50% A and 50% B to 25% A and 75% B within 35 min. Flow elution was 1 mL min^{-1}, 20 μL of samples were injected. UV–Vis spectra were recorded in the range 200–450 nm. The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA, USA). MS spectra were registered in positive ion mode. Capillary temperature was 220 °C, capillary voltage 3.0 V, source voltage 4.2 kV, tube lens voltage 30 V and collision energy 35%.
15. **Arthemeter:** three products appear at 21.5, 23.1 and 24.1 min characterised by a similar visible and mass spectrum. One main fragment is observed at 854 *m*/*z*, which corresponds to artemether-hemin adduct lacking a CH_3COOH moiety. **Dihydroartemisinin:** Only two adducts are observed ($t = 19.1$ and 21.5 min).The main fragment at 840 *m*/*z* corresponds to the sum of dihydroartemisinin and hemin lacking a CH_3COOH moiety, the second fragment at 872 *m*/*z* corresponds to the sum of dihydroartemisinin and hemin lacking a H_2O moiety. **Artesunate:** the chromatograms show the formation of three main products ($t = 14.6$, 16.9, 18.4 min). They are characterised by a similar visible spectrum and by the same mass spectrum, which shows three fragments. The one at 940 *m*/*z* appears with little intensity and represents the complex lacking an CH_3COOH moiety. The main fragments appear at 822 and 804 *m*/*z*. The first one corresponds to the adduct lacking, in addition to the CH_3COOH moiety a fragment of 115 *m*/*z*, most likely due to the loss of 4-oxo-pentanoic-acid; and the latter further lacks a fragment of 18 *m*/*z*, thus a molecule of water.
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