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Interaction of Artemisinin Based Antimalarial Drugs with Hemin in Water-DMSO Mixture

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Abstract: The interaction of hemin with artemisinin, artesunate and dihydroartemisinin was investigated by UV-Visible Spectroscopy at pH 9 and High Performance Liquid Chromatography/Diode Array Detector/Mass Spectrometry (HPLC/DAD/MS) for their reactivity with hemin. It has been showed that artesunate and dihydroartemisinin interacted more strongly with Fe (III) PPIX that artemisinin did. The reported results showed too that hemin and endoperoxide lactone derived antimalarials slowly react to give rise to several stereoisomers supramolecular adducts (three for artesunate, seven for artemisinin and eight isomers for dihydroartemisinin) while in contrast, only heme (Fe^2) was found to react with artemisinin based drugs in previous studies. Based on this result, our work confirmed the mechanism in which the artemisinin derivates approach hemin by pointing O1 at the endoperoxide linkage toward iron center, a mechanism that is controlled by steric hindrance. After that C3-C4 bond is cleaved to give rise carbon radical at C4 as predicted by automated calculation of docking of artemisinin to heme.

Key words: Malaria, artemisinin derivates, hemin, complex, DMSO

INTRODUCTION

Malaria, tuberculosis, AIDS and malnutrition remain the important health problems of the developing countries and contribute significantly to their poverty. According to the estimation of the World Health Organization, each year, more than 500 million people are infected. Actually, all over the world, malaria is killing one people after 30 sec (WHO, 2005). With fast spreading multidrug resistance to commonly used quinoline-based antimalarial drugs, especially chloroquine by *Plasmodium falciparum* (human malaria parasite) (Egan, 2004; Mpiana, 2003), it is necessary to understand their mode of action-and the factors responsible of the resistance in order to improve the antimalarial activity of the existing drugs and to design new drugs (Dechy-Cabaret *et al*., 2003; Gong *et al*., 2001; Tekwan and Walker, 2005).

Historically, since 1940, chloroquine was the most effective drug for treatment and prophylaxis because of its strong therapeutic activity, low toxicity and low cost. A perfect substitute for chloroquine should exhibit the qualities of chloroquine and if possible, be even better. Among the few new drugs against malaria, the promising, from considerations of potency, low toxicity, resistance, cost and rapidity of action, is artemisinin, a non-alkaloidal drug from an ancient Chinese herbal drug *Qinghao* (sweet wormwood) (Klayman, 1985). Artemisinin is a saturated endoperoxide lactone molecule and has been used by the Chinese for 2 milleniums as a folk remedy against fever. Lack of chemical resemblance to the quinoline antimalarial might have been the reason of the success of this drug and its congeners including dihydroartemisinin, artesunate, artemether and arteether in treatment of multidrug resistant malaria (Meshnick, 2002). Despite the proven efficacy of artemisinin against severe malaria, the precise mode of action of it and its derivatives, as well as that of quinoline drugs, remains uncertain. Frequently, controversial discussions in the literature are seen (Chou *et al*., 1980; Yayon *et al*., 1985; Constantinidis and Satterlee, 1988; Egan *et al*., 1996; Cazelles *et al*., 2001).

It has been established that hemin (ferro-or ferriprotoporphyrin IX or intraparasitic heme) is present in high amounts in malaria parasites as a result of hemoglobin digestion (Sherman, 1984). From this, hemin is thought of as having critical role in the mechanism of action of quinolines and artemisinin compounds (Chen *et al*., 2001; Mavakala *et al*., 2003; Meshnick *et al*., 1996; Mpiana, 2003; Robert *et al*., 2002; Yang, 1994).

The interaction between hemin and some antimalarias was investigated in aqueous medium (Chou *et al*., 1980). This medium, however, presents some limitations in regard to the solubility of the reacting partners. Hemin is

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soluble in alkaline aqueous solution because a stable state of hemin in neutral or weakly acid aqueous solution cannot be achieved because of its tendency to dimerize. Whereas as reported (Bilia *et al*., 2002; Kannan *et al*., 2002). Artemisinin compounds are insoluble in aqueous solutions but they are soluble in ethanol, in dimethyl sulfoxide (DMSO), methanol, acetone, influence of hemin dimerization and the prerequisite that reacting partners must be soluble in the medium when studying hemin-drug interactions.

The interactions of hemin with artemisinin compounds were qualitatively investigated in the past using mediums such as water-acetonitrile, dimethyl sulfoxide, -ethanol mixtures and dimethylacetamide medium. It was found that artemisinin reacted with heme (Fe^2) but do not react directly with metal (3) porphyrins like hemin (Fe^3) or (Mn^3) tetraphenylporphyrin (Berman and Adams, 1997; Cazelles *et al*., 2001; Robert *et al*., 2002; Yang *et al*., 1994).

In this study, the interaction of endo per oxide lactone based antimalarias, i.e., artemininin (ART), dihydroartemininin (DHA) and artesunate (AST), with hemin were then investigated using UV-Vis Spectroscopic and HPLC-MS methods in water-DMSO mixture where the dimerization process of hemin could be well controlled. The relatively nontoxicity property and miscibility with water of DMSO allow it to have wide applications in cell biology as solvent for drugs (Yu and Quinn, 1994), then is well suitable studying of the systems containing hemin and artemisinin derivates. For this exploratory study, only the interaction of hemin with artemisinin, artesunate and dihydroartemisinin in 40% aqueous DMSO at pH 9 and at 37°C were reported.

MATERIALS AND METHODS

Hemin and artemisinin based drug solutions: Forty percent aqueous DMSO stock solution of Hemin (Sigma), 306 μmol L^{-1} in concentration was prepared by first dissolving 10 mg of it in 20 mL of DMSO (Beijing Xing Jin Chemical Factory), followed by addition of 30 mL of Tris buffer. Tris buffer stock solutions were prepared by mixing different amounts of 0.2 mol L^{-1} tris (hydroxymethyl)-methylamine (Beijing Yili Fine Chemical Reagents Company) and 0.2 mol L^{-1} HCl (Beijing Chemical Factory) to give required pH. Then, 0.1 mol L^{-1} of Tris-HCl buffer was used as buffer for all 40% aqueous DMSO solutions. For HPLC-MS analysis, DMSO stock solutions of hemin (1.0 mmol L^{-1}) were prepared by dissolving 6.52 mg of it in 10 mL of DMSO. All stock solutions of hemin were refrigerated under 4°C and stored in the dark and were stable for at least two weeks.

In 40% DMSO aqueous solutions, artesunate, dihydroartemisinin and artemisinin stock solutions (0.002 mol L^{-1}) were prepared by dissolving 19.2, 14.2 and 14.1 mg of them, respectively, in 10 mL of DMSO, completed with addition of 15 mL of buffer. For HPLC-MS measurements, in DMSO solutions, artesunate, dihydroartemisinin and artemisinin stock solutions (2 mmol L^{-1}) were prepared by dissolving 7.69, 5.69 and 5.65 mg of them, respectively, in 10 mL of DMSO.

pH and optical absorption measurements: The pH values were determined with pH meter PHS-2C (Shanghai Dapu Instruments Limited company) equipped with glass electrode which was kept soakep in 4 mol L^{-1} KCl solution and calibrated with aqueous standard buffers. A Shimadzu UV-Visible 21OOS Spectrophotometer Coupled to CPS-260 thermostat was used for titration. Temperature was controlled at (37±0.1)°C. Due to its lack of chromophores group, artemisinin and its derivates absorb weakly in the low wavelength region and made his quantification more difficult. Then, the titration was carried out at the hemin characteristic Soret band at 402 nm by mixing a constant volume (0.1 mL) of hemin solution with various volumes of drug solutions and then diluted to 10 mL by addition of Tris-DMSO mixtures. Thereby, hemin remains at constant concentration (3 μmol L^{-1}) while drugs concentrations are changed in the range of 0-200 μmol L^{-1}. Before each measurements of absorbance, the work solutions were incubated at 37°C. UV-Visible spectra were carried out after 10 or 24 h of incubation and were recorded in the range 200-450 nm. Products were detected at 412 nm in order to follow the modification of the porphyrin chromophore.

HPLC-MS measurements: HPLC/DAD/MS analysis was performed on Agilent 1100 series LC/MSD Trap. The Column was a 4.6×250 mm (5 μm), SB-C18 Zorbax (Hewlett and Packard, USA); the open temperature was 30°C. The eluents were A: H_2O adjusted to pH 3.2 by CH_3COOH and B: methanol. The following gradient was applied: from 50% A and 50% B to 25% A and 75% B within 70 min. Flow elution was 1 mL min^{-1}, 20 μL of samples were injected. MS spectra were registered in positive ion mode. The positive MS spectra were performed on an LCQ electrospray directly coupled to the HPLC. For all HPLC-MS analysis, 1 mL of 2 mmol L^{-1} DMSO solution of artemisinin compounds was mixed with 1 mL of 1 mmol L^{-1} DMSO hemin solution and incubated at (37±1)°C over 10 h. All work solutions were mixed under magnetic stirring for 5 min and prepared, daily before each experiment or analysis and protected from light.

Samples were prepared in DMSO solutions and incubated at 37°C over 12 h. For the HPLC/DAD/MS analysis, 0.002 mol L^{-1} DMSO drug solutions and 0.001 mol L^{-1} DMSO hemin solutions were used.

This study was conducted in the Laboratory of bioorganic phosphorous chemistry biology, Tsinghua University, Beijing, China.

RESULTS

Binding reaction of hemin with artemisinin, dihydroartemisinin and artesunate in water-DMSO mixture: Typical spectral changes observed upon hemin-sesquiterne endoperoxide lactone-based drugs complexation are shown in Fig. 1.

These spectra are similar to those observed on hemin-artesunate and hemin-artemisinin interactions in other mediums (Berman and Adams, 1997; Bilia *et al*., 2002). The wavelength of 402 nm was selected because of the greatest variation of the optical density observed in the presence of the antimalarial drugs. Complex formation was monitored by the decline in Absorbance (402 nm) whereas absorbance of hemin, incubated with drug, was essentially unchanged after 24 h.

Typical kinetic analysis of the mixture of hemin plus artesiminin and derivatives are shown in Fig. 2. This

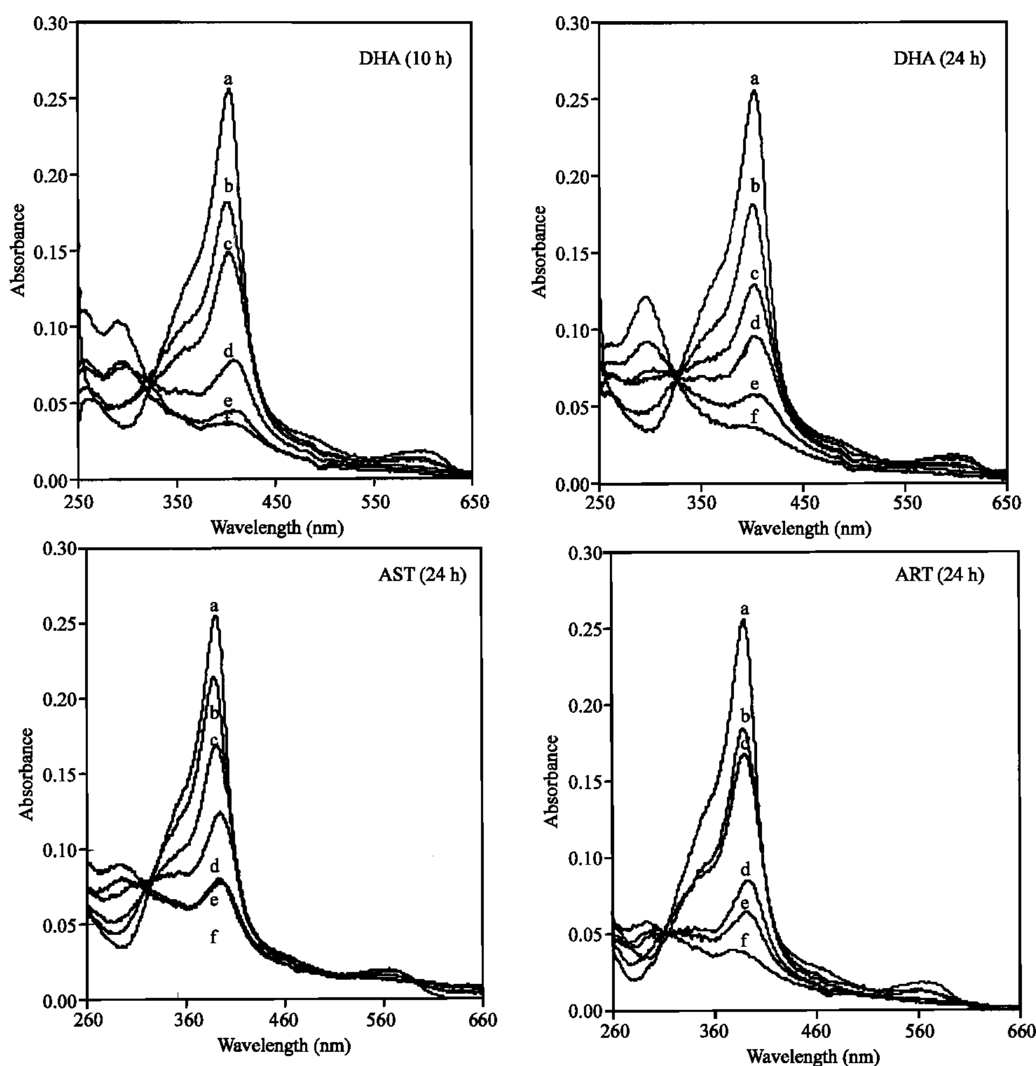

Fig. 1: Spectroscopic changes of hemin as function of increasing concentrations of three drugs.
0 (a): 1.4 (b), 2.4 (c), 70 (d), 100 (e), 160 μmol L^{-1} of DHA (f) after 10 h,
0 (a), 6 (b), 14 (c), 24 (d), 50 (e), 120 μmol L^{-1} of DHA (f) after 24 h,
0 (a), 3 (b), 10 (c), 50 (d), 120 (e), 160 μmol L^{-1} of AST (f) after 24 h,
0 (a), 14 (b), 18 (c), 70 (d), 100 (e), 160 μmol L^{-1} of ART (f) after 24 h with 40% DMSO aqueous as solvent and 0.1 mol L^{-1} Tris-HCl buffer (pH 9)

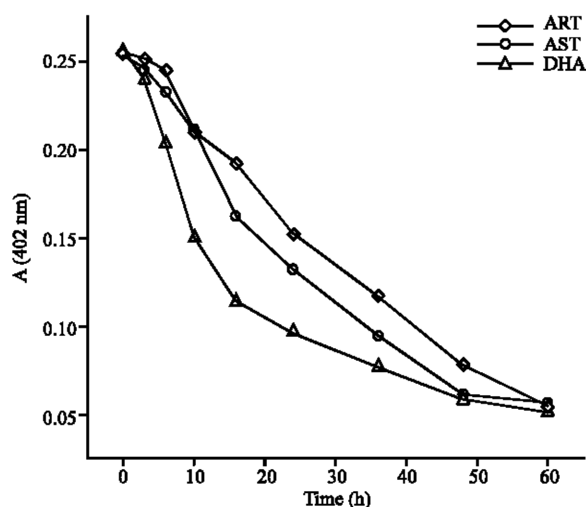

Fig. 2: Variation of absorbance at 402 nm observed when hemin is titrated with artemisinin derivatives (24 µmol L^{-1}) within 60 h of observation in 0.1 mol L^{-1} Tris-HCl buffer (pH 9, 37°C)

Figure indicates that the reaction between hemin and artemisinin compounds takes very long time for reaching equilibrium. Consequently, the evaluation of the equilibrium constant is useless.

Some typical titration curves demonstrating the changes in hemin-artemisinin interactions as function both of time and the total concentration of artemisinin compounds (Fig. 3). It can be seen from experimental curves that the extinction of the hemin solution decreases with increasing in total drug concentration. Generally, at drug concentrations smaller than 50 µmol L^{-1} (molar ratio hemin/drug = 1:17), there is a significantly and progressive decay in Soret absorbance, more perceptible in the case of DHA. At highest concentrations of drug (molar ratio hemin/drug >1: 25), this decrease is less significant, indicating the saturation of drug binding to heme.

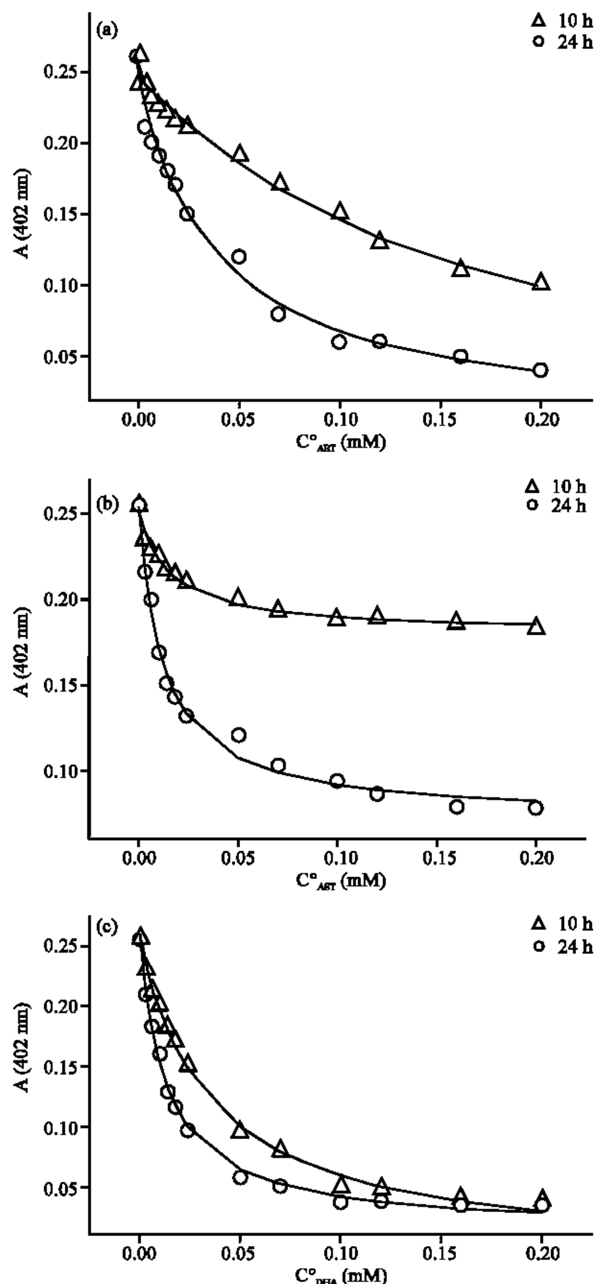

Fig. 3: Variation in absorbance of hemin (3 µmol L^{-1}) at 402 nm as function of total concentration of artemisinin-based drugs after 10 and 24 h of incubation at 37°C (pH 9)

HPLC/MS analysis of hemin-artemisinin based drugs interaction: As already reported by some researchers, artemisinin reacts with heme (Fe^2) but do not react with metal (3) porphyrins (like hemin (Fe^3), (Mn^3) tetraphenylporphyrin (Hien and White, 1993; Cazelles *et al*., 2001; Robert *et al*., 2002)

Recently, Bilia *et al*. (2002) reported formation of two isomers artemisinin-hemin covalent adduct in DMSO solutions. Considering the fact that and most of the reported investigations so far have focused on artemisinin and very few have related to artesunate and dihydroartemisinin, we have attempted to identify possible formation of supramolecular adducts between artemisinin derivatives like artesunate and dihydroartemisinin by using HPLC/DAD/MS spectrometry techniques (Fig. 4).

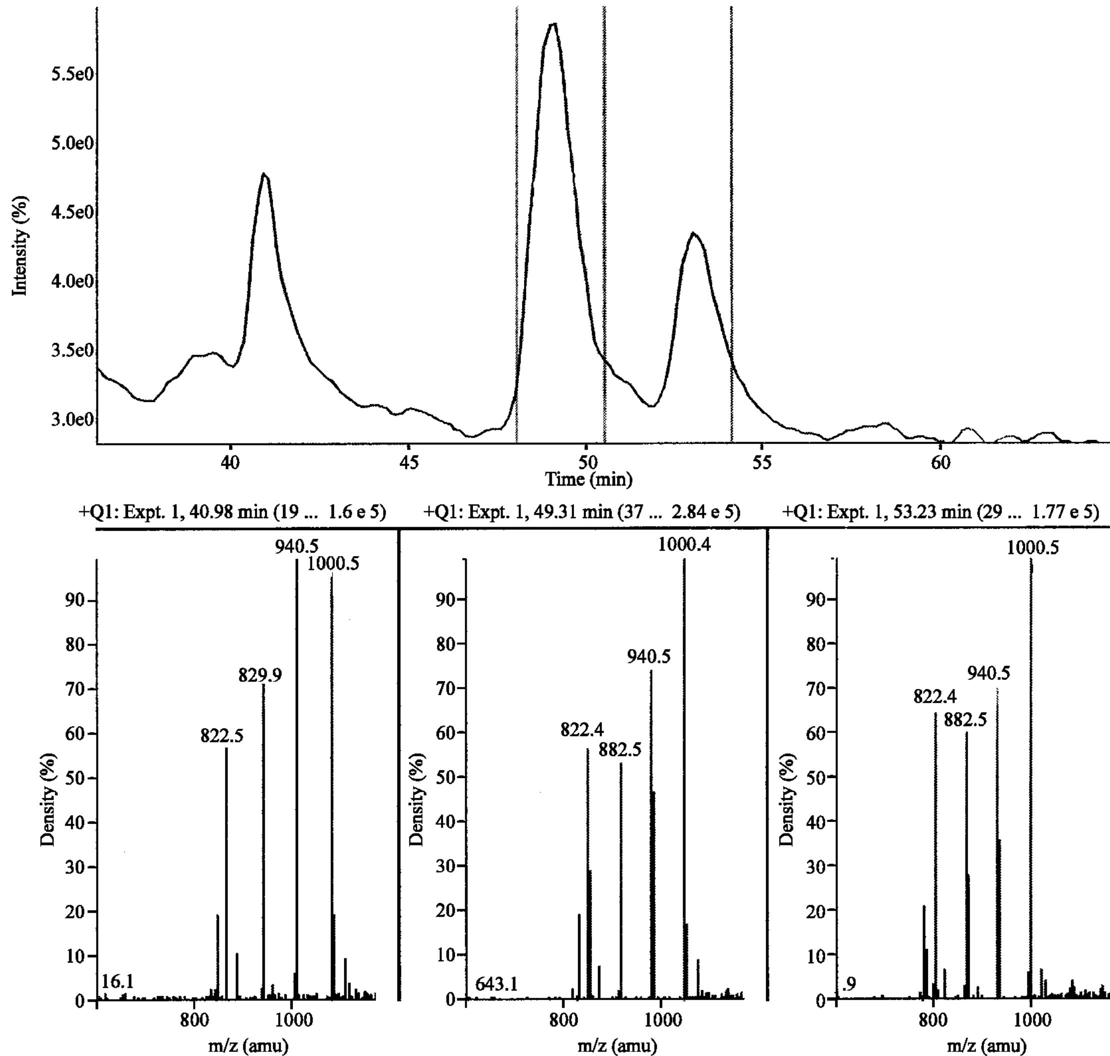

Fig. 4: HPLC/MS spectrum of AST-hemin complex. Above is presented the reacting mixture chromatogram and below, MS spectra corresponding to mixture components

DISCUSSION

The spectral changes resulting from artemisinin drug-hemin interaction show a substantial hypochromic effect of the hemin soret band at 402 nm, accompanied with a red shift of 2-5 nm. The new peak around 407 nm may belong to the complex.

About the decrease in absorbance of the Soret band of hemin, two possible processes can be envisaged which would lead to these changes. Either addition of micromolar concentrations of drug induces aggregation of hemin or the changes reflect drug association with hemin. While a large decrease in the absorbance of the Soret band is often an indication of aggregation, equally large decreases are caused by formation of p-p (donor-acceptor) complexe (Posner *et al.*, 1995). Generally, spectral changes of iron porphyrins in the visible region vary depending on the conditions of solvents and pH and the nature of interacting species. The decrease of hemin absorbance is dependent on the drug concentration. A dilution experiment showed that Beer's law is strictly adhered to in the presence of 306 μmol L^{-1} drug and in the concentration range 0.99 to 48 μmol L^{-1} Fe(3) PPIX thus providing no evidence of hemin aggregation in this concentration range. Thus, the most reasonable explanation for these spectral changes is the presence of drug-hemin association. There is another feature on the titration curves of artemisinin and derivatives, i.e. a quite regular spectral change over time with a well-defined isosbetic point around 325 nm. These spectral

modifications suggest a progressive disruption of delocalised π-electron system of the hemin tetrapyrrole ring. This behavior is suggestive of equilibrium between two species and indicates that ART, DHA and AST interact also with hemin, a Fe^{3+} porphyrin, contrarely to previous works (Berman and Adams ,1997; Cazelles *et al*., 2001; Robert *et al*., 2002; Yang *et al*., 1994) which excluded this possibility of interaction between hemin and artemisinin derivatives and agreed only heme (Fe^{2+} porphyrin) interaction with these drugs.

At 37°C, the curves of absorbance-time relationship for the complexation showed that the time taken to reach absorbance minimum is shorter for DHA. It was found that the reaction rate is faster with dihydroartemsinin, followed by artesunate and artemisinin.

For example, for 24 μmol L^{-1} of drug (at pH 9), the residual intensity of the soret band, compared to the control (hemin, 100%), is:

- After 3 h, 98% (ART), 96% (AST), 94% (DHA)
- After 6 h, 96% (ART), 91% (AST), 89% (DHA)
- After 10 h, 84% (ART), 83%(AST), 59% (DHA)
- After 24 h, 66% (ART), 57% (AST), 37% (DHA)
- After 60 h, 21% (ART), 20% (AST), 20% (DHA)

In the light of above results, we observed that fixing the concentration of hemin at 3 μmol L^{-1} and varying drug from 0 to 200 μmol L^{-1}, the Soret band of hemin decreased. Along with the increase in incubation time and drug concentration, it arises a new peak centered at 290 nm, indicating that the peak belongs to artemisinin compounds. The origin of which is unclear atthistime but we suggested that it is a decomposed product of artemisinin as a result of reaction between hemin and drugs. The spectral propriety of this product with strong absorbance is similar to that observed by Green *et al*. (2000) caused by alkali decomposition of ART. They called it ARTS 290 and have described this compound as an enolate/carboxylate. Enolates readily couple to electrophilic dyes such as diazonium salts (Zollinger, 1991), therefore it is possible that the alkali decomposition product of ART is similar in structure. In order to verify this assertion, wemixed 0.001 M of AST with 1 M of NaOH at room temperature for 20 min. We observed exactly a peak at 290 nm, which has the same characteristic of ART 290.

It has been postulated that the interaction of artemisininn with the target heme or hemin *in vivo* proceeds through the complexes, in which the peroxide bridge of trioxanes coordinates with the iron of hemin, leading to the formation of drug-hemin adduct. This long-lived intermediate has the spectral characteristics of a heme peroxide compound analogous, in which the hemin is covalently bound to artemisinin via a iron-oxygen-carbon bond, formed by attack of the iron center on the endoperoxide group of drug (Berman and Adams, 1997). This metastable species gradually decays over incubation (24 h for drug highest concentration or 48 h for drug lowest concentration), changing to final product devoid of Soret absorbance, indicative of disruption of the heme tetrapyrrole ring system.

The interaction of hemin and artemisinin compounds is supported too by molecular mechanic calculations. In fact, Tonmunphen and co-workers reported automated molecular docking of artemisinin to heme. The docking between artemisinin and heme indicated that artemisinin approaches heme by pointing O1 (Fig. 5 and 6) at the peroxide linkage toward the iron center, a mechanism that is controlled by steric hindrance with a lowest binding energy of -33.13 kcal mol^{-1}, this can be the same for hemin (Tonmumphean and Parasuk, 2001; Taranto *et al*., 2002).

The HPLC/DAD/MS results confirmed the hemin-artemisinin derivatives complexation and showed that hemin and artemisinin compounds reacted slowly to give rise to molecular adducts. Without drugs, hemin solution showed a peak with a retention time (Rt) of 38.95 min. The corresponding MS spectra is characterized by the molecular mass/charge ratio (m/z) of 616.5 m/z (M^+-35.5) corresponding to fragment with loss of chloride atom. With the presence of artesunate, three more peaks with Rt 41.26, 49.37 and 53.23 min were seen in the HPLC spectrum. Their MS spectra were similar and characterized by four fragments at 1000.5, 940.5, 882.5 and 822.4 m/z as shown at Fig. 4. The first corresponding exactly to a hemin-AST complex, explained by the addition of the molecular masses of artesunate (384.4) and hemin (616.3). The other three correspond to the covalent adducts between a modified artemisinin and hemin without extensive degradation of both partners. [M^+-60 = 940.5] corresponds to an adduct with likely loss of CH_3COOH and [M^+-118 = 882.5] to an adduct with loss of $HO_2C(CH_2)_2CO_2H$ (succinic acid) as expected from succinate-containing molecule. [M^+-178 = 882.5] corresponds to an adduct with both loss of CH_3COOH and $HO_2C(CH_2)_2CO_2H$. These data evidenced the presence of three supramolecular complexes represented by three isomers.

Similar results have been observed with dihydroartemisinin. The HPLC/DAD/MS spectra analysis of DHA showed two groups of peaks: one with Rt 33.5 (m/z = 616.5) corresponding to hemin spectra and another peaks with Rt 42.1, 46.6, 51.2, 54.8, 61.1, 64.1, 69.8 and 75.7 min. These latter eight latter have all similar MS spectra and exhibited one intense peak at m/z = 840 m/z.

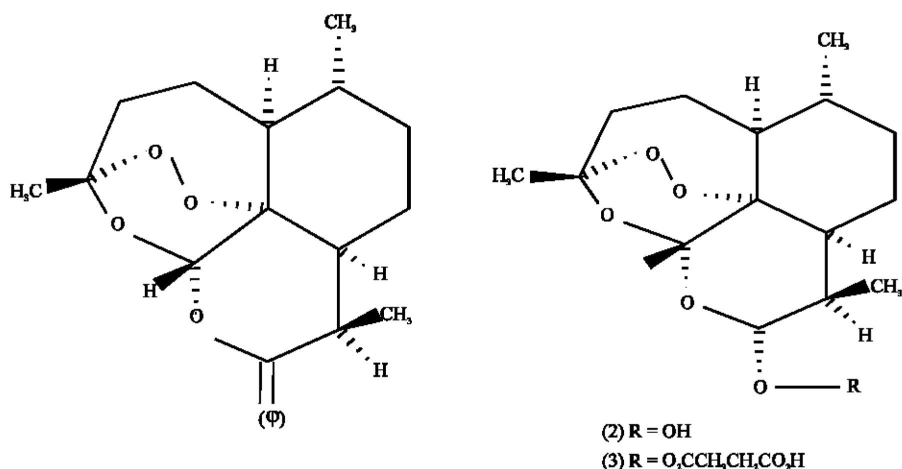

Fig. 5: Structures of three artemisinin-based drugs, artemisinin (1), dihydroartemisinin (2) and artesunate (3)

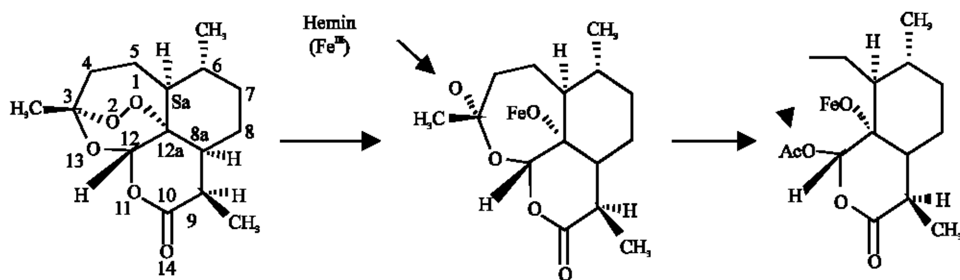

Fig. 6: Proposed mechanism of hemin-artemisinin compounds interaction in the light of MS spectrum analysis. Hemin is simplified as Fe[26]

These peaks are attributed to dihydroartemisinin-hemin adduct, resulting from addition of DHA (m/z = 284.4) with hemin (m/z = 616.3) followed with loss of a fragment of 60 m/z, most likely due to loss of CH_3COOH as expected for anacetate containing molecule. These data evidenced the presence of eight supramolecular complexes represented by eight isomers.

Paradoxically to the findings of Bilia *et al.* (2002), it has been identified seven isomeric hemin-artemisinin covalent adduct with retention time at 37.9, 39.4, 41.2, 42.5, 45.6, 47 and 48.6 min. The complex peak was detected at m/z = 838.8 (M^+), corresponding to the expected mass for an adduct hemin (m/z = 616.5) and artemisinin (m/z = 283.3) with a loss of a fragment of acetic acid molecule within the mass spectrometer.

According to the MS spectra, it has been demonstrated that all complexes between hemin and artemisinin-based compounds lost a mass equivalent to m/z = 60 (acetic acid), except artesunate-complex that lost both m/z = 60 and m/z = 118. This trend allows us to propose the mechanism in which the hemin iron attacks the oxygen in O_1 position, then C3-C4 bond is cleaved to give carbon radical at C4 as shown in Fig. 6. From it, has been showed that the acetate group attacks to artemisinin ring in the hemin-artemisinin complex, allowing loss of acetic acid under appropriate conditions. In contrast, if heme iron attacks the compound at the O_2 position, there will be no presence of acetate group attached to artemisinin as shown in Fig. 6.

This mechanism has been supported by the automated molecular docking of artemisinin to heme (Tonmumphean and Parasuk, 2001). All docking calculations similarly reported that O_1-Fe distance was shorter than the O_2-Fe distance in a heme-artemisinin complex. It was then concluded that iron in heme interacts with O_1 more preferably than O_2. The preference might have arisen from the more negative charge at O_1 and the steric hindrance at O_2. This observation is in agreement with the proposal of Posner *et al.* (1995). The similarity in structures of heme and hemin leads us to suggest that artemisinin compound -hemin interactions follow also the same pathway in terms of mechanism of action. Taranto *et al.* (2002) showed by quantum-mechanical method that C4 radical is a stable species which may be responsible for the activity of the endoperoxide lactone-based drugs.

Based on all these observations we can conclude that both hemin and heme can react with endoperoxide lactone-base drugs. But the structures of these complexes are not well known; this can be the prospective goal. According to previously reported researches, it seems that heme reacts more quickly with artemisnin compounds than hemin does. There has ben even reported that artemisinin didn't react with metal (III) porphyrins (Robert *et al*., 2002). It has been demonstrated in this study that hemin can also form complex with those drugs. It is thus useful to keep this fact in mind in the understanding of the interaction mechanism of artemisinin drugs to either heme or its trivalent metal counterpart.

ACKNOWLEDGMENTS

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