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Heme alkylation by artemisinin and trioxaquines†

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ABSTRACT: Artemisinin is an efficient antimalarial drug containing a 1,2,4-trioxane which is able to alkylate heme both *in vitro* and *in vivo*, giving rise to covalent heme-artemisinin coupling products. The low valent iron(II) protoporphyrin-IX, which is the prosthetic group of hemoglobin, induces the homolysis of the peroxide bond of artemisinin by an electron transfer. The generated alkoxy radical is quickly rearranged to a C-centered radical that efficiently alkylates the heme macrocycle at *meso* positions. Heme is therefore both the activating agent and the target of artemisinin.

Additionally, the iron(II) heme cofactor of carboxyhemoglobin was found to react as efficiently with artemisinin as oxyhemoglobin does, providing a heme-drug covalent adduct in up to 60% yield. This result indicates that the presence of a CO ligand bound to iron does not preclude the reductive activation of the peroxide, thereby confirming the high affinity of artemisinin for iron(II) heme.

On the basis of this mechanism of action, a variety of new peroxide-based antimalarials named trioxaquines® have been synthesized. Trioxaquines are made by the covalent attachment of a trioxane, having alkylating ability, to a quinoline, known to readily penetrate infected erythrocytes. Several trioxaquines are active *in vitro* on chloroquine resistant malaria parasite at nanomolar concentrations, and are efficient to cure infected mice by oral route at 20–50 mg/kg. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: artemisinin; carboxyhemoglobin; heme alkylation; hemoglobin; malaria; trioxane; trioxaquine

INTRODUCTION

At the interface of inorganic chemistry and biology, the discovery of metal-based drugs is a growing field with some impressive large-scale applications (anticancer *cis*-platinum or gadolinium contrast agents for magnetic resonance imaging). In such complexes, the metal center plays a key role in the observed pharmacological effects. Beside this category, some organic drugs need to be activated *in vivo* by a metal center, for example by the iron center of cytochrome P450, in order to generate biologically reactive entities. The antimalarial endoperoxide artemisinin is the only reported example of a drug that interacts *in vivo* with a metal complex acting both as a trigger and a target.

Malaria is a parasitic disease due to the erythrocyte infection by a *Plasmodium* species transmitted to man by the bite of an infected anopheles mosquito. *P. falciparum* is responsible for 95% of the 1–3 million malaria death occurring each year. On the model of quinine, a natural product extracted from the bark of the cinchona tree, several synthetic drugs based on a 4-aminoquinoline (such as chloroquine) or on a quinoline-alcohol (such as mefloquine) have been widely and efficiently used (see Fig. 1 for structures). However, the parasites have become resistant to major antimalarial drugs, rendering the formerly efficient chloroquine useless in many tropical countries, which has resulted in a come-back of a high level of malaria in recent decades.[1–3] Even for the reference drug quinine, there are now assessments of progressive emerging resistance.[4] Vaccine development against *P. falciparum* malaria is ongoing, however, despite several decades of work, a malaria vaccine is not yet round the corner.[5,6]

The sesquiterpene artemisinin (**1**, Fig. 1) is naturally formed in the aerial part of *Artemisia annua*, a plant whose leaves were used in China for centuries as traditional medicine against fever. The trioxane entity, responsible for the antimalarial activity of artemisinin, is a chemical structure completely different from that of the classical antimalarial drugs based on a quinoline residue, and is thus likely to have a different mechanism of action. Artemisinin and its hemisynthetic derivatives artemether and artesunate are nowadays the faster acting antimalarial drugs and the only family for which no clinically relevant parasite resistance has been reported up to now.[7] Actually, the WHO recommends a drug therapy based on a formulation containing an artemisinin derivative as policy standard. Unfortunately, the need to treat 500 million *P. falciparum* malaria attacks per year results in a global shortage of artemisinin, and a short-term quadrupling in

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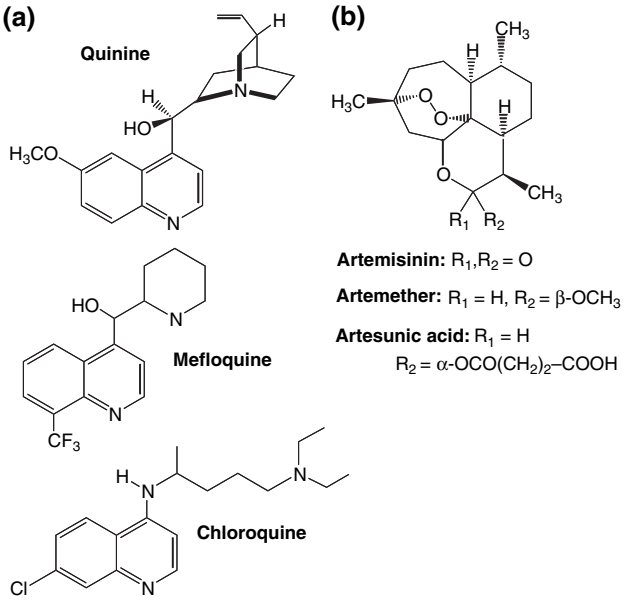

Figure 1. Structure of (a) some quinoline-based antimalarial drugs, and (b) artemisinin **1** and some hemisynthetic derivatives

its cost.[2,8] To overcome these difficulties, considerable progress in the agricultural and chemical technologies of artemisinin production and extraction have been recently reported:[9] in the Indo-gangetic plains of India, the development of new cultivars allow harvests as big as 75 kg/ha, in contrast to a maximum yield of artemisinin at 25 kg/ha reported from Brazil, and 15 kg/ha from China, Vietnam, and Africa. In spite of such efforts, artemisinin is actually in short supply.

A recent study suggests that uncontrolled use of artemisinin derivatives in some geographic areas may lend a reduced *in vitro* susceptibility to *P. falciparum* field isolates, and this feature may be the first step of the development of artemisinin resistance.[10] As an additional drawback, the poor pharmacokinetic parameters of artemisinin derivatives make necessary the development of new, cheap, and easily affordable synthetic endoperoxide-based antimalarial drugs. For this purpose, understanding the essential features of the mechanism of action of artemisinin is necessary to design synthetic antimalarial peroxides.

ARTEMISININ, AN ALKYLATING ANTIMALARIAL DRUG

Iron in malaria

After a mosquito bite, the malaria parasites invade the liver and, then, colonize erythrocytes where parasite multiplication is achieved. The symptomatology of malaria is due to this erythrocytic phase. The synchronous maturation of parasites leads to the erythrocyte burst together with an attack of fever and deep anemia. At each red blood cell burst (every 48 h for *P. falciparum*), parasites are released for further erythrocytic reinvasion.

Within infected erythrocytes, up to 70% of the host hemoglobin is imported into a specialized acid compartment of the parasite (food vacuole), and broken down by specific proteases.[11,12] The amino acids released by this catabolic process are used by the parasite to build its own proteins. Of the four equivalents of heme by hemoglobin molecule, only a minor amount is degraded and used as source of iron for the parasite. Upon degradation of globin and release of heme, the iron(II) protoporphyrin-IX is able to reduce molecular oxygen thus inducing the cascade of reduced oxygen species. This should result in a lethal oxidative stress within the parasite which lacks the heme oxygenase that vertebrates use for heme catabolism. To avoid the heme-mediated oxidative damages, the released heme is converted into a redox inactive iron(III)-heme microcrystalline aggregate called hemozoin.[13,14]

Any perturbation of this heme detoxification process that is unique to *Plasmodium* is expected to have drastic consequences for the parasite survival, due to the accumulation of soluble redox active heme residues. The control of malaria largely depends on drugs that disrupt this heme aggregation process which is assisted by the parasitic histidine-rich protein (HRP). For instance, chloroquine has a high affinity for ferric heme ($K_d = 3.5$ nM)[15] as a result of a strong π-stacking between the quinoline ring of the drug and the porphyrin macrocycle.[16–18] Other quinoline-based antimalarials are supposed to interact with heme in a similar way.

Reactivity of artemisinin in the presence of heme

The mechanism of action of artemisinin has been the matter of a lot of work and intense debates for the last decade.[19–23] In a pioneering work by Meshnick's group, radiolabeled artemisinin was found to alkylate *in vitro* heme proteins such as catalase, cytochrome *c* and hemoglobin.[24] When incubated in Plasmodium cultures, artemisinin alkylates heme and some unidentified, but specific parasitic proteins.[25,26] More recently, the artemisinin-induced inhibition of two parasitic proteins, a TCTP homolog[27] and the calcium-dependent ATPase PfATP6,[28] was clearly reported as being iron-dependent.

When a peroxide is activated by a low-valent transition metal complex, the main reaction pathway is the homolytic cleavage of the weak peroxide bond.[29,30] The reductive activation of artemisinin or related peroxides by iron(II) heme or iron(II) salts have been reviewed.[31–36]

Covalent heme-artemisinin adducts

Alkylating species generated by ferrous iron-mediated homolytic cleavage of the endoperoxide function of artemisinin, in particular the alkyl radical centered at

position C4 of artemisinin (or related 1,2,4-trioxanes), were early proposed to be important.[37] However, the concentration of free iron ions in living cells is close to zero, whereas heme concentration is 20 mM in red blood cells. After preliminary experiments with synthetic metalloporphyrins,[38] we have reported that iron(III)-protoporphyrin(IX) incubated with artemisinin, in the presence of a reducing agent able to generate iron(II)-heme, was readily converted in high yield to heme-artemisinin covalent adducts. These adducts result from alkylation of the four *meso* positions of the macrocyclic ligand by the C4-alkyl radical derived from artemisinin (adducts **2**, Fig. 2).[33] The reductive cleavage of artemisinin is initiated by an electron transfer from the iron(II)-heme to the antibonding σ^* LUMO orbital of the peroxide bond. This reductive activation generates a short-lived alkoxy radical which quickly rearranges, via β-fragmentation, to a C4-centered primary radical thermodynamically facilitated by concomitant formation of an ester functionality. Intramolecular addition of this alkylating species occurs without regioselectivity on the four *meso* carbons of the protoporphyrin ligand. After demetallation of the heme moiety, complete NMR characterization of these adducts has been obtained.[39]

In addition, artemisinin is able to alkylate heme in non-denaturated human iron(II) hemoglobin in the absence of any added protease, or even in the presence of high amounts of non-heme proteins when artemisinin was added to human hemolyzed whole blood.[40] These results clearly indicate the high reactivity of this drug toward hemoglobin under very mild conditions. In this regard, the selective toxicity of artemisinin to malarial parasites is probably due to the selective accumulation of the drug into the parasite within infected erythrocytes compared to non-infected erythrocytes.[41,42]

The question, thus, arises as to whether the formation of heme-artemisinin adducts is just a laboratory 'curiosity' or is biologically relevant and related to the mechanism of action of the drug *in vivo*. We addressed this question upon treatment of *Plasmodium vinckei* infected mice with artemisinin at pharmacological doses by intra-peritoneal or oral routes. The covalent adducts heme-artemisinin **2** were identified in the spleen of all treated mice. The hydroxylated and glucuroconjugated derivatives of heme-artemisinin adducts, clearly indicative of a hepatic metabolism, were identified in the urine of treated mice. On the opposite, no heme-artemisinin adduct could be detected in healthy mice treated under the same conditions. This result confirms that the alkylation ability of artemisinin exists in mammals, and that this effect is triggered by the presence of the parasite.[43]

The exact role of heme-artemisinin adducts in the *in vivo* parasiticidal activity may remain for a long time a matter of debate.[44] 'Nevertheless, **1** alkylates free heme or hemoglobin heme by way of the primary carbon-centered radical, *possibly the only malaria-parasite-relevant fully characterized alkylation reactions reported so far for* ***1.***'[45]

REACTIVITY OF CARBOXYHEMOGLOGIN TOWARD ARTEMISININ

Let us remind that the role of iron heme for artemisinin antimalarial activity has been debated. A recently published article indicates that artemisinin is active on *P. falciparum* cultured under different O_2/CO atmosphere ratios.[46] Making the *hypothesis* that, in carboxyhemoglobin, 'iron(II) is no longer available for the reductive scission of the trioxane pharmacophore,' the authors

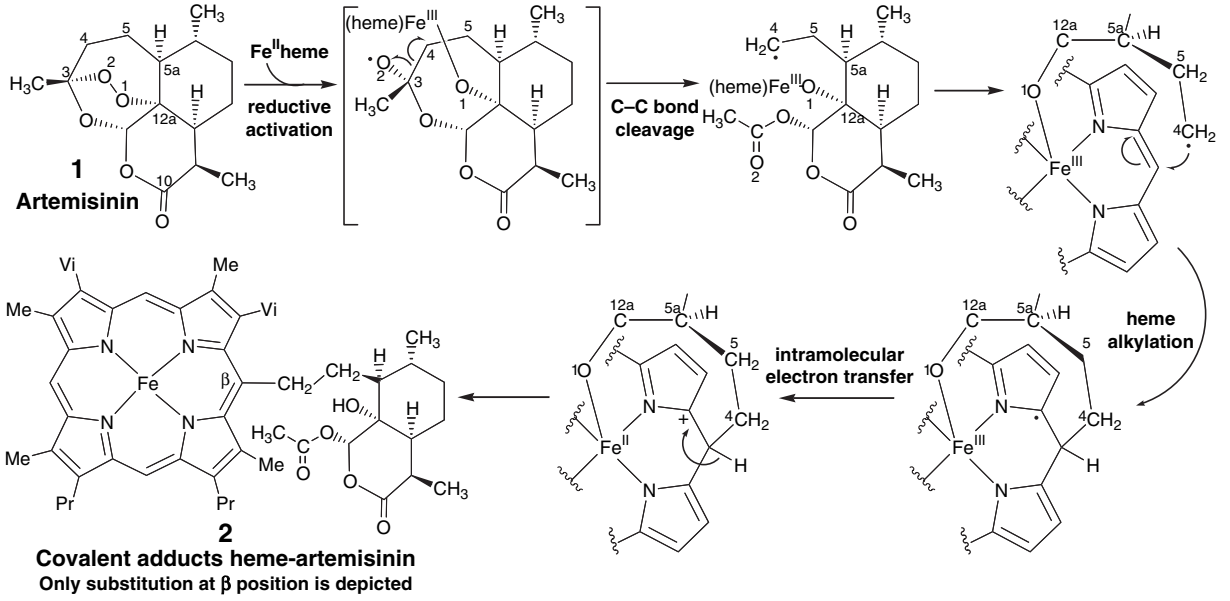

Figure 2. Alkylation of heme by artemisinin. Alkylation occurred at the four *meso* positions of the protoporphyrin-IX ligand. For simplification, only alkylation at β-position is depicted

concluded that 'iron(II) heme is not necessary for the antimalarial activity of artemisinin.'

We considered this hypothesis. To address this question, we compared the alkylating ability of artemisinin in the presence of iron(II) oxyhemoglobin (HbO_2) and in the presence of iron(II) carboxyhemoglobin (HbCO). The results and experimental conditions are reported here.

Preparation of HbO_2 and HbCO solutions

The UV-visible spectra of HbO_2 and HbCO have been reported for many years. They are significantly different in the Q-band region, allowing calculation of the proportion of each species in a HbO_2/HbCO mixture. Pure HbO_2 solutions in water were obtained by dissolving human ferrous oxyhemoglobin A_0 in argon saturated water $[(H_2O)_{Ar}]$. Pure HbCO solutions in water were obtained by dissolving human ferrous oxyhemoglobin A_0 in carbon monoxide saturated water $[H_2O)_{CO}]$, and then keeping the solution under a CO pressure (1.5 bar) for 15 min. For preparation of the solutions containing different HbO_2/HbCO molar ratios, oxyhemoglobin was dissolved in mixtures of $(H_2O)_{CO}$ and $(H_2O)_{Ar}$ in variable proportions.

Quantification of HbCO was performed using the absorbance ratio at 540 and 579 nm, according to Eqn (1):

$$\mathrm{HbCO}(\%) = \frac{\mathrm{abs}_{540}/\mathrm{abs}_{579} - A}{B - A} \times 100 \qquad (1)$$

where abs_{540} and abs_{579} stand for the absorbance intensities of the solution at 540 and 579 nm, respectively, $A = abs_{540}/abs_{579}$ for pure $HbO_2 = 1.02$, and $B = abs_{540}/abs_{579}$ for pure HbCO = 1.53.[47,48] An example of this titration is given in Fig. 3.

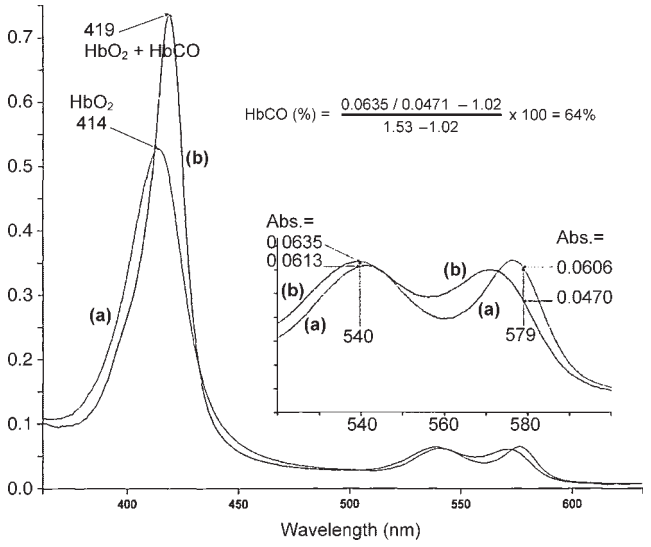

Figure 3. (a) UV-visible spectrum of HbO_2. (b) UV-visible spectrum of a mixture HbO_2 + HbCO containing 64 mol% of HbCO

Alkylation of heme in HbO_2 or HbCO by artemisinin

Pure HbO_2 was reacted with artemisinin for 1 h at 37 °C in a water/dimethylsulfoxide medium (H_2O/DMSO = 9/1, v/v; heme/artemisinin molar ratio = 1/2.2). The formation of heme-artemisinin adducts, resulting from the *meso* positions of the porphyrin macrocycle by an artemisinin derived C-centered radical, was evidenced by HPLC and LC-MS after dilution. The reaction selectively provided four heme derivatives with $R_t = 30.0$, 30.3, 32.8 and 33.5 min, respectively. The consistency of these products with the already characterized four regioisomeric heme-artemisinin adducts was checked by HPLC comigration, UV-visible spectroscopy and LC-MS (UV-visible at 406 nm: $\epsilon_{heme}/\epsilon_{adducts} = 4.7$; ES^+-MS: $m/z =$ 898.4, 838.4). After 1 h of reaction, the yield of heme-artemisinin adducts was 71% from the starting amount of heme. For comparison, similar alkylation reactions were made after incorporation of carbon monoxide on heme higher than 94 mol%, that is, $abs_{540}/abs_{579} > 1.50$ ($n = 4$). After 1 h of reaction, the yield of alkylation of heme in carboxyhemoglobin was as high as 69% ± 5% with respect to the starting amount of heme (namely, 74%, 74%, 64%, and 67%, $n = 4$) (Fig. 4). This result unambiguously shows that artemisinin efficiently alkylates carboxyhemoglobin as well as oxyhemoglobin.

In addition, mixtures containing different HbO_2/HbCO ratios were reacted with artemisinin for 1 h at 37 °C in a water/dimethylsulfoxide medium (H_2O/DMSO = 9/1, v/v; heme/artemisinin molar ratio = 3/1). Under these conditions, the maximum theoretical yield of heme-artemisinin adducts was 33 mol% with respect to the starting amount of heme. From 0 to 95% HbCO, the alkylation efficacy of heme by artemisinin, calculated as the yield of heme-artemisinin adducts divided by the maximum theoretical yield, was found to fall within the range 66–100%, whatever the proportion of HbCO was, thereby confirming that the reactivity of HbCO toward artemisinin peroxide is similar to that of HbO_2.

This result unambiguously makes invalid the hypothesis by S. Parapini *et al.* that stated the inhibition of iron(II) heme reactivity by carbon monoxide. In addition, the role of heme or unidentified iron species has been shown to be required for the artemisinin-mediated alkylation of *Pf*TCTP[25] or *Pf*ATP6[26] *in vitro*. In conclusion, the possible role of iron(II) species for the antimalarial activity of artemisinin *in vivo* cannot be ruled out. On the opposite, artemisinin may be triggered not only by iron(II) heme, but also by any other biological iron(II) species, so giving rise to the alkylation of different biomolecules in the vicinity. By the way, the absence of artemisinin resistance in malaria parasite might be related to multiple biological activators and targets inherent in the endoperoxide reactivity.[49]

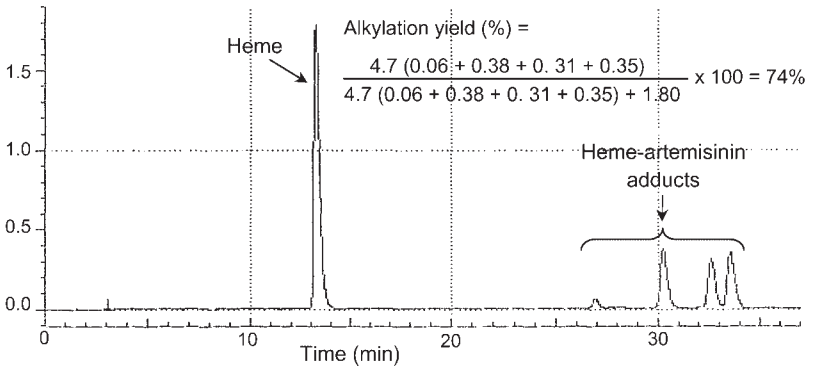

Figure 4. HPLC chromatogram after alkylation of 95 mol% HbCO. Detection at 406 nm. Alkylation yield = $4.7 \times \Sigma abs_{adducts} / [4.7 \times \Sigma abs_{adducts} + abs_{heme}] \times 100$

FROM ARTEMISININ TO TRIOXAQUINES

A lot of efforts have been made in recent years to develop new antimalarial drugs.[50] Amongst them, chloroquine has been modified by covalent link with a ferrocenyl entity. This drug named ferroquine is active against chloroquine-resistant strains of malaria parasite.[51] Ammonium[52] and thiazolium[53] salts were designed to target the parasite choline transporter. Synthetic trioxanes[54] or trioxolanes,[55] simplified analogs of artemisinin, supposed to act in the same way, have also been developed.

The combination of artemisinin derivatives with a second drug having a different mode of action is a good way to increase the efficacy of the treatment, and to prevent the emergence and the spread of drug resistance. As artemisinin and chloroquine both interact with heme, but by two different mechanisms, we designed new chimeric molecules named trioxaquines® (Fig. 5).[56] They combine, in a single molecule, a potentially alkylating trioxane (as in artemisinin), and a 4-aminoquinoline (as in chloroquine) known to easily penetrate within infected red blood cells. It is noteworthy that this strategy has already been developed for the treatment of various diseases including depression, allergy, hypertension, dyslipidemia, and for the development of non-steroidal antiinflammatory drugs.[57] However, it has not yet been used for the design of antiparasitic drugs.

Synthesis of trioxaquines

For obvious reasons, antimalarial drugs must be cheap and easily accessible. Trioxaquines were prepared through a convergent synthesis based on classical reactions. Many simple modulations are possible, leading to a large family of new potentially active molecules. As an example, the synthesis of trioxaquine DU1301 is depicted in Fig. 6 (compound **3**). In trioxaquine DU1301, the amine and the peroxide substituent can be *trans* or *cis* with respect to the cyclohexane ring. The reductive amination reaction, therefore, provided two diastereomeric racemates *trans*-DU1301 and *cis*-DU1301 (50/50).[58] For structure elucidation and biological evaluation, the two diastereomers of DU1301 have been separated and their structures were determined by X-ray diffraction.[58] These stereochemical issues have already been detailed in a previous report.[58]

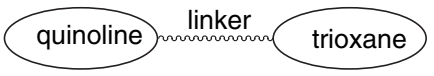

Figure 5. Trioxaquines® are dual antimalarial molecules

Alkylation ability of trioxaquines

The alkylating ability of trioxaquine DU1301 toward iron(II) heme was evaluated under similar conditions as those reported for artemisinin derivatives. At 37 °C in 1 h, 60% of heme was alkylated by two alkyl radicals generated by the reductive activation of the peroxide bond. With artemisinin, no alkylation product arising from a putative radical on O1 has been evidenced (Fig. 7). By the way, a recent report based on the reactivity of trioxolane models of artemisinin suggests a preference for attack of iron(II) on the non-ketal peroxide oxygen atom of artemisinin.[45] By contrast, in the case of DU1301, two different routes produced alkoxy radicals either on O1 or on O2, giving rise to two different heme-drug adducts **4** and **5**.[59]

Antimalarial activity of trioxaquines

The first synthesized trioxaquines were found highly active *in vitro* on laboratory strains (chloroquine-sensitive and chloroquine-resistant ones) of *P. falciparum*. As a matter of example, the concentrations of DU1301 necessary to reduce the parasitemia by 50% (IC_{50} values) ranged from 5 to 20 nM, depending on the parasite strains, whereas IC_{50} values of artemisinin on the same strains range from 5 to 8 nM, and that of chloroquine from 62 to 174 nM.[58] The two diastereomers of DU1301 have been independently tested for antimalarial activity. They exhibit identical activities *in vitro* and *in vivo*, on mice infected by *Plasmodium vinckei*.

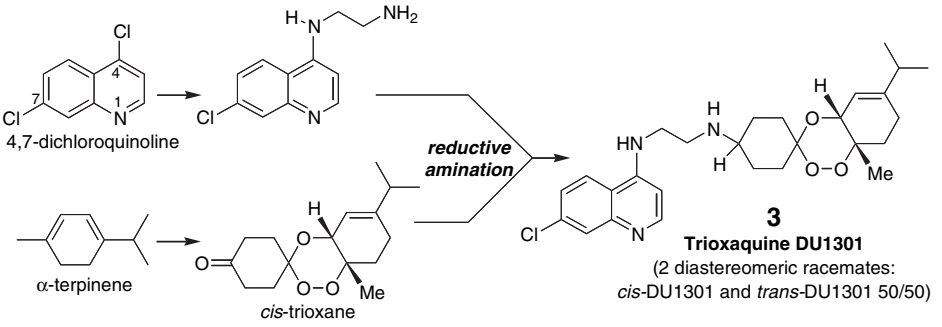

Figure 6. Convergent synthesis of trioxaquine DU1301

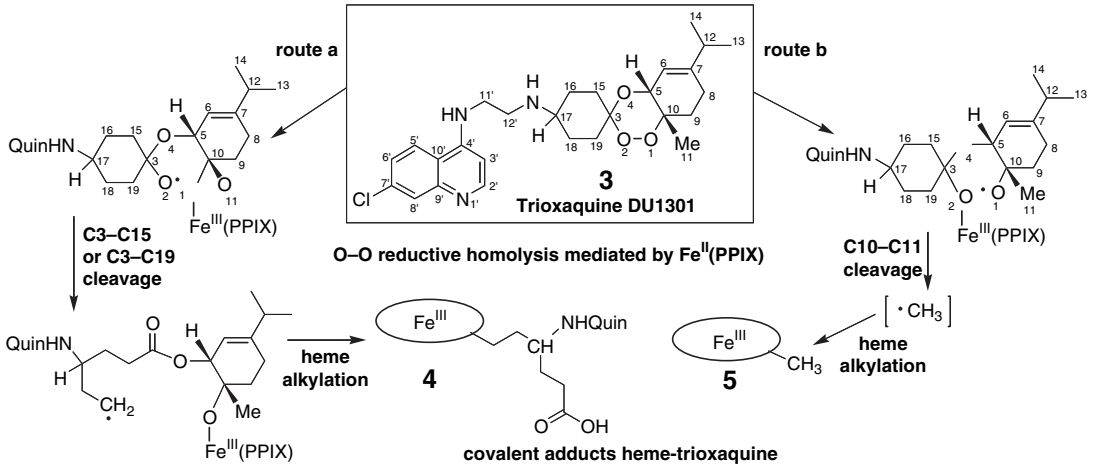

Figure 7. Alkylation of heme by trioxaquine DU1301. Alkylation occurred at the four *meso* positions, only alkylation at β-position is depicted. The ovals stand for the protoporphyrin-IX macrocycle

Moreover, complete cure of parasitemia without recrudescence has been obtained at 20 (ip route) or 50 mg/kg/d (oral route).[58]

The high efficacy of DU1301 *in vitro* and *in vivo*, in particular on chloroquine-resistant strains, its easy synthesis and its chemical stability, its absence of toxicity are making this trioxaquine a promising drug candidate for antimalarial therapy. Trioxaquines are now in pre-clinical development by a young company named Palumed, in collaboration with Sanofi-Aventis.

CONCLUSION

Metal ions are ubiquitous in living organisms. Iron is the most abundant with an average amount of 4 g in an adult human body.

Iron is an important component of many cellular redox reactions and is required for essential enzymes. Heme represents one of the most ubiquitous and stable forms of redox active iron among living organisms. It is required as a prosthetic group for a vast number of proteins or enzymes (e.g., hemoglobin that accounts for ca. 55 wt% of iron in humans, myoglobin, cytochromes, peroxidases, catalases, nitric oxide synthase. . .) involved in various processes such as oxygen and electron transport and metabolism of small molecules. Iron–sulfur clusters Fe_nS_n are also involved in electron transfer proteins.

The ability of iron of heme (or Fe–S clusters) to accept and donate electrons is a key feature which also contributes mostly to its potential toxicity *in vivo*. In normal metabolism, during the sequential reduction of molecular oxygen, superoxide radical intermediates can be formed. Hydroxyl radical can also be generated by the Fenton-catalyzed cleavage of hydrogen peroxide. For this reason, all aerobic organisms have developed sophisticated regulatory systems (superoxide dismutase, peroxidases, catalases) to maintain the concentration of reduced oxygen species as low as possible.

As a unique example, the peroxide of artemisinin interacts with the metal center of heme, which acts at the same time as trigger and target. Artemisinin specifically enters infected red blood cells to reach its activator/target.

EXPERIMENTAL SECTION

Reactivity of carboxyhemoglogin toward artemisinin

Materials. Human ferrous hemoglobin A_0 (HbO_2), and all chemicals were purchased from Sigma-Aldrich.

UV-visible spectra were recorded on a Lambda 35 Perkin–Elmer UV-visible spectrometer.

HPLC analyses were performed using a 5 μm C4-QS-uptisphere 300 column, 250 × 4.6 mm (Interchim, France). Eluents were (*A*) 0.05 vol% trifluoroacetic acid in water, (*B*) 0.05 vol% trifluoroacetic acid in acetonitrile. The gradient was the following: from *A*/*B* = 64/36 to *A*/*B* = 56/44 in 30 min, then to *A*/*B* = 52/48 in 10 min. Flow rate was 1 mL/min, and UV detection at 220 and 406 nm. Under these conditions, hemoglobin is denaturated and heme released from globins. Retention time, min: 13.4 (heme, $\lambda_{max} = 398$ nm), 19.5 (β-globin), 26.0 (α-globin), 30.0, 30.3, 32.8, 33.5 min (four heme-drug adducts, relative ratio 1/3/3/3, $\lambda_{max} = 406$–408 nm).

Water (10 mL) was saturated by bubbling carbon monoxide, at room temperature and atmospheric pressure during 1 h, to provide [$(H_2O)_{CO}$]. Argon saturated water [$(H_2O)_{Ar}$] was prepared in a similar way but bubbling argon instead of CO.

Solution of pure HbCO. Human ferrous oxyhemoglobin A_0 (HbO_2, 1.6 mg, 0.025 μmol, corresponding to 0.100 μmol of heme) was dissolved in $(H_2O)_{CO}$ (100 μL). This solution was stirred under CO pressure (1.5 bar) during 15 min. An aliquot of this solution (10 μL) was diluted with 990 μL of $(H_2O)_{Ar}$ for UV-visible analysis ($\lambda = 419$, 539, 571 nm; $abs_{540}/abs_{579} = 1.53$).

Solution of pure HbO_2. Human ferrous oxyhemoglobin A_0 (HbO_2, 1.6 mg, 0.025 μmol) was dissolved in $(H_2O)_{Ar}$ (100 μL). An aliquot of this solution (10 μL) was diluted with 990 μL of $(H_2O)_{Ar}$ for UV-visible analysis ($\lambda = 414$, 542, 577 nm; $abs_{540}/abs_{579} = 1.02$).

Solutions with different HbO_2/HbCO molar ratios. Human ferrous oxyhemoglobin A_0 (HbO_2, 1.6 mg, 0.025 μmol) was dissolved in variable volumes of $(H_2O)_{CO}$ (20–80 μL). The volume was then adjusted to 100 μL by addition of $(H_2O)_{Ar}$. Aliquots of these solutions (10 μL) were diluted with 990 μL of $(H_2O)_{Ar}$ for UV-visible analysis. For each reaction mixture, the percentage of HbCO was calculated from UV-visible data according to Eqn (1).

Alkylation of hemoglobin by artemisinin. The solution of HbO_2/HbCO in water (90 μL) was mixed with a solution of artemisinin in DMSO (10 μL, H_2O/DMSO = 9/1, v/v, heme/artemisinin molar ratio = 1/2.2 or 3/1). The reaction mixture was allowed to stand at 37 °C for 1 h. It was then diluted with $(H_2O)_{Ar}$ (2.4 mL). Fifty microliters of this diluted solution was then analyzed by HPLC or LC-MS.

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