Trioxaquinines and Heme-Artemisinin Adducts Inhibit the In Vitro Formation of Hemozoin Better than Chloroquine

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Received 16 February 2007/Returned for modification 16 April 2007/Accepted 1 August 2007

Trioxaquinines, potential antimalarial agents, and heme-artemisinin adducts, resulting from the alkylation of heme by artemisinin, were evaluated as inhibitors of β-hematin formation in 10 M acetate medium at pH 5.

During hemoglobin proteolysis, inside the malarial parasite digestive vacuole, free heme is released. Owing to its capacity to create oxidative damage to cell membranes and parasite proteins, redox-active free heme (ferritroporphyrin IX (PPIXFe)) is toxic. To avoid this toxicity, Plasmodium spp. convert free heme entities in an inert microcrystal made by the aggregation of heme dimers called hemozoin or malaria pigment. Because the hemozoin pathway is unique to the malarial parasite, it offers an attractive target for the design of new antimalarials. Chloroquine, a blood schizonticide, is considered an efficient inhibitor of hemozoin formation (21). Chloroquine stacks with heme to form a stable π-π complex, PPIXFe-chloroquine, which is not incorporated into hemozoin and which could kill the parasite via a redox process. In vitro, β-hematin, a crystal structurally and chemically identical to hemozoin (14), could be synthesized from hemin (PPIXFe-CI) under acidic conditions with or without parasitic material (4, 6, 8, 19, 21). Thus, drugs acting as inhibitors of hemozoin formation, such as chloroquine, can be evaluated in vitro by monitoring their ability to prevent the formation of β-hematin from hemin.

Potential antimalarial trioxaquinines, effective in vitro and in vivo against both chloroquine-sensitive and chloroquine-resistant strains of Plasmodium, have been prepared in our laboratory by the covalent attachment of a trioxane entity to N-(7-chloro-4-quinolinyl)-1,2-ethanediamine (DU1301) or to primaquine (DU2303) (Fig. 1) (1, 2). Recently, we showed that the trioxane motif of these hybrid molecules was responsible for the artemisinin-like activity, since a heme-trioxane adduct has been characterized which is similar to that obtained with artemisinin derivatives (11, 16, 17). To further document the mode of action of these new antimalarial compounds, we wanted to determine if these hybrid trioxaquinines might have a dual activity (heme alkylation and hemozoin inhibition) by evaluating their capacity to inhibit β-hematin formation in vitro, using chloroquine for comparison.

Different methods have been proposed to study the inhibition of β-hematin formation by drugs (3, 12). One of these methods is particularly well adapted to evaluate compounds with low water solubility like trioxaquinines (15). A 16 mM solution of hemin (312 μl) dissolved in dimethyl sulfoxide (DMSO) was added to a suitable molar equivalent of the tested compound dissolved in 312 μl of DMSO. In a control test, hemin was added to 312 μl of DMSO. H2O (624 μl; Milli-Q-quality) was added before the initiation of β-hematin formation with 1.25 ml of 10 M acetate buffer (pH 5). The final ratio of DMSO was kept constant at 25% (vol/vol). The solution was stirred at 37°C for 18 h, cooled on ice for 5 min, and then filtered on Ultrafree-CL centrifugal filter units (4,000 μm for 10 min). The pellets were washed eight times with 2 ml of warm Milli-Q-quality H2O (at 30°C) in order to remove acetate salts (4,000 × g for 10 min). The solid was dried under vacuum at 90°C for 2 h. Infrared (IR) spectra were obtained from discs of the solids (1.5 mg) in KBr pellets (200 mg). β-Hematin has two characteristically sharp bands at 1,666 cm⁻¹ and at 1,210 cm⁻¹ that are attributed to the carboxylate groups coordinated to the iron atom of ferriprotochlorin (5). These two bands were missing from the IR spectra when the inhibition occurred. The major advantage of this infrared assay method for β-hematin inhibition is that it allows unequivocal identification of the reaction products. A disadvantage is that it does not allow the quantification of the inhibition process. However, we prefer this method to solubilization-based methods because of its ability to provide a clear answer concerning the formation of carboxylato-iron bonds that are essential in the dimerization process that is at the origin of the hemozoin formation. The inhibiting capacities of the trioxaquinines DU1301 and DU2303 were compared to those of their own precursors [N-(7-chloro-4-quinolinyl)-1,2-ethanediame and primaquine (HCl salt), respectively] and their trioxane precursors, namely the trioxane entity with a keto group replacing the aminooquinoline moiety of these hybrid molecules and to those of chloroquine (base form), artemisinin, arteether, and artesunate. The results are summarized in Table 1.

We found that 10 eq of chloroquine or N-(7-chloro-4-quinolinyl)-1,2-ethanediamine was necessary to completely inhibit the formation of β-hematin, as previously reported (7). It is
reasonable to assume that the 4-aminoquinoline entity of trioxaquines inhibits the formation of β-hematin via a noncovalent interaction with heme (12). In contrast, the addition of a large excess of primaquine dihydrochloride (up to 15 eq) did not inhibit the formation of β-hematin. With a few exceptions (20), it is known that 8-aminoquinoline forms a weak complex with heme that cannot interfere with β-hematin formation (15). Moreover, primaquine is an antimalarial drug active against the liver stage but not the blood stage of Plasmodium.

The four trioxane-containing molecules tested (artemisinin, artemesunate, artemether, and the trioxane precursor) in amounts of up to 15 eq did not interfere with β-hematin synthesis. These results are not surprising (9), given that these trioxane compounds have a mechanism of action that is distinct from that of simple aminoquinolines (13). Moreover, we found that the addition of 3 eq of DU2303 resulted in complete inhibition of β-hematin formation, whereas the addition of up to 15 eq of its precursors, either the trioxane precursor or primaquine, had no such effect. In parallel, only 2 eq of DU1301 prevented the formation of β-hematin. Both trioxaquines are more potent inhibitors at low charge than their quinoline precursors. The data from this IR study of heme crystallization and the preceding results with heme alkylation (11, 16, 17) suggest that these trioxaquines (hybrid molecules) could have a dual mode of action: one chloroquine-like mechanism whereby hemozoin formation is prevented and one artemisinin-like mechanism whereby heme is alkylated.

Artemisinin alkylates heme both in vitro and in vivo and forms a well-defined heme-artemisinin adduct mixture (Fig. 2 and Fig. 3A) (16, 17). The infrared spectra from an equimolar mixture of heme and artemisinin were compared (Fig. 3B). These adducts should not be able to crystallize into hemozoin and could induce oxidative damages to parasite biomolecules, as is reported for free heme (21). We first determined if these heme-artemisinin adducts alone were able to dimerize under the conditions that were used for the formation of β-hematin in the control test. We found that the IR spectra of these adducts treated alone under heme crystallization conditions with acetate buffer for 18 h at 37°C did not exhibit bands at 1,660 cm⁻¹ and 1,210 cm⁻¹, as shown in Fig. 3B. Moreover, the powder recovered was soluble in DMSO and methanol, solvents that do not solubilize β-hematin (15). We propose that the bulky artemisinin residue linked to the porphyrin ring prevented the coordination of the propionate side chain on the iron atom (thus preventing the initial dimerization, a required step in the hemozoin formation). It should be noted that this result does not support the theoretical studies suggesting that the dimerization of heme-artemisinin adducts is possible (18).

We then checked the hypothesis that heme-artemisinin adducts act as inhibitors of β-hematin formation (18). The inhibition was evaluated with adduct/hemin ratios ranging from 1 to 6 under the conditions described above. The powder recovered after aqueous washing was washed again with 5 ml of methanol in order to remove the excess of adducts. IR spectra were recorded after drying the resulting powder under vacuum conditions. We found that 5 eq of heme-artemisinin adducts completely blocked the conversion of hemin to β-hematin. A recent study (10) suggested that heme-artemisinin adducts act as inhibitors of β-hematin formation. The inhibition was evaluated with adduct/hemin ratios ranging from 1 to 6 under the conditions described above. The powder recovered after aqueous washing was washed again with 5 ml of methanol in order to remove the excess of adducts. IR spectra were recorded after drying the resulting powder under vacuum conditions. We found that 5 eq of heme-artemisinin adducts completely blocked the conversion of hemin to β-hematin.

In conclusion, the reported results indicate that trioxaquines, like chloroquine, are able to inhibit β-hematin formation. This study also shows that (i) trioxane-containing antimalarials like artemisinin are unable to act as inhibitors of β-hematin formation, (ii) heme-artemisinin adducts are not dimerizable, and (iii) a small amount of these adducts inhibits
We thank CNRS, Palumed, and the AntiMal EU program for financial support.

We thank Jean Bernaud and Anne Robert (both from LCC-CNRS) for fruitful discussions.

REFERENCES

FIG. 3. (A) Infrared spectra of heme-artemisinin adducts. (B) Infrared spectra of a mixture (1:1) of heme and artemisinin. (C) Infrared spectra of a β-hematin (light line) and adduct polymerization assay (bold line). The characteristic peaks for β-hematin at 1,660 cm⁻¹ and 1,210 cm⁻¹ are marked with arrows.

the formation of β-hematin. Trioxaquines with heme-alkylating and hemozoin-inhibiting capacities have a dual mode of action that might be an advantage in avoiding the development of resistant strains of parasites.