Potentiation of Artemisinin Activity against Chloroquine-Resistant Plasmodium falciparum Strains by Using Heme Models

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The influence of different metalloporphyrin derivatives on the antimalarial activity of artemisinin was studied with two chloroquine-resistant strains of Plasmodium falciparum (FcB1-Colombia and FeM29-Cameroon) cultured in human erythrocytes. This potentiation study indicates that the manganese complex of meso-tetakis(4-sulfonatophenyl)porphyrin has a significant synergistic effect on the activity of artemisinin against both Plasmodium strains.

Artemisinin (Fig. 1) and its hemisynthetic derivatives artesunate and artemether are currently used to treat severe and multidrug-resistant malaria, including cerebral malaria (9). As artemisinin is active during the intraerythrocyte stage of infection and contains an endoperoxide function that is essential for its activity, a reasonable hypothesis for the mechanism of action of artemisinin is the reductive activation of its endoperoxide bridge by Fe(II) heme resulting from the digestion of hemoglobin by Plasmodium species (8, 13). This reaction might lead to dioxygen-derived radicals responsible for oxidative stress (10) within infected erythrocytes or to C-centered radicals (1, 3, 6, 7, 12, 14). We recently illustrated the alkylating properties of one of these C-centered radicals by isolating a covalent adduct between artemisinin or other antimalarial trioxanes and a heme model based on meso-tetraphenylporphyrin (TPPS) and tetramesitylporphyrin (TMPS) were used in the present study (Fig. 2).

meso-Tetramesitylporphyrin (5) and the corresponding octasulfonated derivative H$_2$-TMPS (18) were prepared as previously described. The tetrasulfonated derivative H$_2$-TPPS was prepared by modification of the classical synthesis procedure (19), and metallization (18) resulted in the complexes Fe-TPPS, Fe-TMPS, Mn-TPPS, and Mn-TMPS.

Culture of two chloroquine-resistant strains of Plasmodium falciparum, FcB1-Colombia and FeM29-Cameroon, and estimations of parasite growth inhibition in the presence of drugs were carried out according to procedures published in references 2 and references therein. Each drug was diluted in dimethyl sulfoxide and then with RPMI 1640. Parasite growth was estimated by $[^{3}H]$hypoxanthine incorporation (20). Concentrations inhibiting 50% of parasite growth (IC$_{50}$) were determined for artemisinin and for each metalloporphyrin after 32 or 72 h of incubation, $[^{3}H]$hypoxanthine being added to the medium at 8 or 16 h, respectively, before the end of incubation. The IC$_{50}$ at 32 h, close to the end of the trophozoite stage, measures the influence of the drug on parasite maturation. Incubation for 72 h, 1.5 times longer than the parasitic erythrocytic life cycle, indicates a possible cumulative effect on the main metabolic pathways of the parasite and also a putative effect on erythrocyte reinvasion. Toxicity was estimated on human fibroblast cells (HeLa). The cell line was cultured under the same conditions as P. falciparum except that human serum was replaced by fetal calf serum.

To determine the effects of the different metalloporphyrins on the IC$_{50}$ of artemisinin, potentiation experiments were carried out with combinations of artemisinin and a metalloporphyrin. The IC$_{50}$ obtained for each combination were compared to those of each isolated drug after the same incubation time, and isobolograms were plotted according to previously described methods (4, 11). An isobologram as a straight diagonal line indicates an additive effect; curves above or below the diagonal indicate antagonistic or synergistic effects, respectively. Results close to the diagonal (see the hatched regions of Fig. 3) are considered additive.

Table 1 reports concentrations inhibiting 50% of parasite growth measured separately for artemisinin and for each of the four metalloporphyrins. The IC$_{50}$ of the different metalloporphyrin derivatives depend on the central metal, the porphyrin ligand, and the parasite strain. The iron complexes exhibited significantly lower values than the corresponding manganese complexes: 30 and 65 μM with Fe-TPPS and Fe-TMPS, respectively, compared to 113 and 166 μM with the manganese analogues Mn-TPPS and Mn-TMPS against FeM29 after 32 h.

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Mn-TMPS presented higher IC_{50} than the other metalloporphyrins for both strains and, therefore, a less selective activity against *P. falciparum*. The main feature is that the antimalarial activity of metalloporphyrins tested alone (IC_{50} from 22 to 412 μM at 32 h) is weak compared to that of artemisinin (0.35 to 0.89 nM), but this activity is delayed, the IC_{50} being in almost all cases significantly lower after 72 h than after 32 h. For example, the IC_{50} of Fe-TPPS for FcB1 at 72 h is 43 μM, compared to 412 μM at 32 h, suggesting that this molecule is probably more active in the reinvasion step than in the maturation step of the parasite.

The toxicity of metalloporphyrins for HeLa human fibroblast cells is reported in the last row of Table 1. A significant toxicity was observed with TMPS complexes, the IC_{50} being 30 μM for Fe-TMPS and 59 μM for Mn-TMPS. On the other hand, both complexes of TPPS, Fe-TPPS and Mn-TPPS, had no significant activity for this cell line (IC_{50} > 450 μM, corresponding to 500 μg/ml at 32 and 72 h). Such a low toxicity was described previously for Mn-TPPS on human MT-4 lymphocyte cells (IC_{50} > 200 μg/ml) (17). Under these conditions, the activities of the iron and manganese complexes of TPPS, Fe-TMPS and Mn-TMPS, against the tested *Plasmodium* strains can be considered selective. All results have been confirmed by the Bartlett and Cochran tests for homogeneity of variance. Using a single-factor (metalloporphyrin) analysis of variance, we carried out global comparisons of different groups. Then, the multiple comparison procedure of the Neuman-Keuls test permitted us to examine the differences between all possible pairs of means (PC-pcsm software, version 6.0 [1992]; Deltasoft, Meylan, France).

The results of the potentiation study of artemisinin by metalloporphyrins were essentially the same after 32 or 72 h of incubation. The results at 72 h are reported in Fig. 3. Figures 3a and b clearly indicate a significant synergistic effect by Mn-TPPS, the activity of artemisinin being increased by a factor of 11 (Fig. 3a) or 4 (Fig. 3b) over artemisinin used alone against strain FcM29 or FcB1, respectively. On the other hand, Mn-TMPS did not significantly potentiate the activity of artemisinin (Fig. 3a and b). These data indicate a specific activity of the metal complexes due to the nature of the porphyrin ligand, TPPS or TMPS. The iron complexes, Fe-TMPS against strain FcB1 (Fig. 3d) and Fe-TMPS against both strains (Fig. 3c and d), had no significant influence on the antimalarial activity of artemisinin (additive effect). Fe-TPPS exhibited an antagonist effect on the activity of artemisinin against strain FcM29 (Fig. 3c).

These results indicate that metal-TMPS complex (M-TMPS) derivatives have their own toxicities against *Plasmodium*, which are dependent on the metal and the parasite strain but independent of the presence of artemisinin. In the case of M-TMPS complexes, the methyl substituents create an important steric hindrance around the central metal that is not accessible for a close interaction with the peroxide bridge of artemisinin. In this case, the reductive activation of the drug by the metal(II) porphyrin is not possible, leading to a lack of influence of TMPS derivatives on the activity of artemisinin. On the other hand, TPPS derivatives, as rather flat molecules, are able to interact with artemisinin to activate the peroxide function. This activity of M-TMPS can be attributed to the penetration of these heme models within infected erythrocytes, leading to an increased concentration of reducible heme derivatives. Consequently, both M-TPPS and Fe(II) heme perform reductive

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**TABLE 1.** IC_{50} of artemisinin and metalloporphyrins tested independently against two *P. falciparum* strains and HeLa cells

<table>
<thead>
<tr>
<th>Strain or cell line and time (h)</th>
<th>IC_{50}a</th>
<th>Fe-TPPS</th>
<th>Mn-TPPS</th>
<th>Fe-TMPS</th>
<th>Mn-TMPS</th>
<th>Artemisinin</th>
<th>Chloroquineb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/ml</td>
<td>μM</td>
<td>μg/ml</td>
<td>μM</td>
<td>μg/ml</td>
<td>μM</td>
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<tr>
<td>FcB1-Colombia</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>458 (80)</td>
<td>412</td>
<td>377 (121)</td>
<td>340</td>
<td>465 (135)</td>
<td>625 (147)</td>
<td>100 × 10^{-6}</td>
</tr>
<tr>
<td>72</td>
<td>48 (8)</td>
<td>43</td>
<td>94 (31)</td>
<td>85</td>
<td>38 (4)</td>
<td>252 (46)</td>
<td>100 × 10^{-6}</td>
</tr>
<tr>
<td>FcM29-Cameroon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>33 (5)</td>
<td>30</td>
<td>125 (12)</td>
<td>113</td>
<td>110 (21)</td>
<td>280 (19)</td>
<td>250 × 10^{-6}</td>
</tr>
<tr>
<td>72</td>
<td>24 (4)</td>
<td>22</td>
<td>30 (16)</td>
<td>27</td>
<td>10 (8)</td>
<td>50 (16)</td>
<td>250 × 10^{-6}</td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>32, 72</td>
<td>&gt;500</td>
<td>&gt;450</td>
<td>&gt;500</td>
<td>&gt;450</td>
<td>&gt;50 (11)</td>
<td>&gt;50 (13)</td>
<td>&gt;50 &gt;0.18</td>
</tr>
</tbody>
</table>

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a Values in parentheses are standard deviations (n = 10).

b For comparison.
activation of the endoperoxide function of artemisinin. In the case of Fe-TPPS associated with artemisinin against FcM29, the product of reductive activation of artemisinin may be not highly toxic for the parasite. The net effect is then a lowering of the artemisinin concentration, giving rise to an antagonist effect. On the contrary, the action of Mn-TPPS against both strains increases the concentration of alkyl radicals derived from artemisinin. These radicals are able to alkylate parasitic proteins, leading to the death of the parasite. The artemisinin-derived C-centered radicals are also able to alkylate heme itself or a heme model. An alternative hypothesis is that the alkylated heme and heme model are both unable to be incorporated in hemozoin, contributing to the formation of oxygen-reduced species and consequently to a lethal oxidative stress within the infected erythrocytes.

In summary, a significant synergistic effect of the manganese complex Mn-TPPS, which can increase the activity of artemisinin up to 11-fold, was observed. This result is of particular interest owing to the low toxicity of this metalloporphyrin for different human cell lines. Furthermore, this potentiation of artemisinin efficiency was obtained with two different chloroquine-resistant P. falciparum strains. We are currently extending these investigations in vivo to the murine strain Plasmodium vinckei petteri in order to confirm whether this potentiation of artemisinin by Mn-TPPS can be considered a general feature.

Finally, this potentiation effect of Mn-TPPS on the activity of artemisinin confirms that the reductive activation of the endoperoxide function of artemisinin is a key feature of the antimalarial activity of these trioxane-containing drugs.

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REFERENCES


