Antimalarial Effect of HBED and Other Phenolic and Catecholic Iron Chelators

By A.M. Yinnon, E.N. Theanacho, R.W. Grady, D.T. Spira, and C. Hershko

Previous studies showed that deferoxamine inhibits malaria by interacting with a labile iron pool within parasitized erythrocytes. Consequently, we studied the antimalarial properties of other iron-chelating drugs such as 2,3-dihydroxybenzoic acid (2,3-DHB) and its methyl ester as well as two polyanionic amines, N,N'-bis(o-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED) and N,N'-ethylenediamine(o-hydroxyphenylglycine) (EHPG) in rats infected with Plasmodium berghei. All drugs were delivered by subcutaneous injection at 8-hour intervals, 40 mg per animal per day. All animals receiving N,N'-ethylenediamine(o-hydroxyphenylglycine) died of drug toxicity between days 6 and 11. Peak parasitemia on day 11 of infection was 32.8% in control animals; 25.3% with methyl 2,3-DHB, 15.5% with 2,3-DHB, 8.0% with deferoxamine, and 0.9% with HBED. Subsequent studies of HBED and deferoxamine in P falciparum cultures in human erythrocytes showed a marked suppression of parasite counts by both drugs at concentrations of 0.9 μmol/L. At all concentrations tested, HBED was four to five times more effective than deferoxamine in suppressing parasite counts. The superior antimalarial activity of HBED is attributed to its increased lipophilicity and higher affinity to ferric iron. These findings indicate that selective iron deprivation by interaction with an intracellular chelator may represent a novel approach to antimalarial drug development, and that systematic screening of available iron-chelating drugs may result in identification of potentially useful antimalarial compounds.

MALARIA IS STILL one of the most serious health hazards in the world. Several factors have been responsible for the failure of coordinated international efforts to eradicate malaria. One of these has been the emergence of Plasmodium falciparum strains resistant to existing antimalarial drugs. Hence, continuing efforts to develop new therapeutic agents for control of drug-resistant malaria are of considerable current interest.

Deferoxamine (DF), a selective iron-chelating agent, was shown to suppress malaria in vitro and in several species in vivo.4-8 Unfortunately, DF has several shortcomings as a potential antimalarial agent: its absorption from the gastrointestinal tract is poor; its half-life in the circulation is very short, requiring continuous parenteral administration for optimal clinical effectiveness; and finally, its rate of penetration into erythrocytes is very slow.9 These limitations of DF underscore the need to explore, in a systematic fashion, the antimalarial properties of other iron chelators to identify orally effective compounds of possible clinical usefulness.

In the text that follows we report on the antimalarial properties of 2,3-dihydroxybenzoic acid (2,3-DHB) and its methyl ester as well as two polyanionic amines, N,N'-bis(o-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED), and N,N'-ethylenediamine(o-hydroxyphenylglycine) (EHPG). These iron chelators were previously shown to be effective in vivo orally.9-11

MATERIALS AND METHODS

Drugs

Chemicals of greater than 97% purity were obtained from the following sources and used without further purification: DF (Ciba Pharmaceuticals, Summit, NJ), 2,3-DHB (Aldrich Chemical, Cedar Knolls, NJ), EHPG (Sigma Chemical, St Louis, MO); and HBED (Strem Chemicals, Newburyport, MA). The methyl ester of 2,3-DHB was prepared as described previously.12 DF and 2,3-DHB were dissolved in normal saline immediately before use. Methyl 2,3-DHB, EHPG, and HBED were suspended in normal saline containing 0.25% methylcellulose and used within 24 to 48 hours. The final volume of drug injected was 0.5 to 1.0 mL.

Iron Assays

Serum iron and total iron binding capacity (TIBC) were determined by the ICSH iron panel method.13 Total nonheme iron was measured by the method of Torrance and Bothwell.14

In Vitro Studies

The ability of iron chelators to inhibit proliferation of P falciparum was studied in vitro. Parasites were grown in 9-cm Petri dishes and six-well tissue culture plates using the candle jar method of Jensen and Trager.15 They were cultured in group A+ or O red blood cells (RBCs) in RPMI 1640 medium containing 25 mmol/L HEPES, 2 g/L NaHCO3, 100 μg/L gentamicin (Sigma), and 10% human serum type A. Parasites were synchronized by the gelatin sedimentation method.16 After synchronisation, parasites were diluted with fresh human RBCs to 0.4% parasitemia and 4% hematocrit. Stock solutions (1 mmol/L) of HBED and DF were prepared in double-distilled water and diluted serially in RPMI 1640 medium to final concentrations ranging from 2 to 40 μmol/L. Parasite growth was determined by microscope slide counts; a minimum of 500 cells was counted on each slide, and each point represented the mean of three cultures. Variations between triplicate counts were less than 10%.

In Vivo Studies

Female Wistar rats of the Hadassah strain weighing 110 to 120 g have been used throughout. Malarial infection was produced by the intraperitoneal injection of a standard inoculum of 3 × 107 parasites of the ANKA strain of P berghei (donated by Dr M. Jarra, Mill Hill, London, UK). These parasites were maintained in our laboratory by biweekly passage in rats of the same strain. Material for inoculation

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was prepared by dilution of freshly drawn blood in sterile normal saline. Parasitemia was estimated by counting 500 cells in stained blood films.

**Antimalarial effect of new chelators.** The design of these in vivo studies was similar to our previous studies of the antimalarial effects of DF. In each group of experiments, rats in the control and experimental groups were inoculated simultaneously with *P. berghei*. The experimental groups were treated by 8-hourly injections of the following iron chelators: DF, 2,3-DHB, methyl 2,3-DHB, EHPG, and HBED administered subcutaneously according to the dose schedule indicated.

**Statistics**

Differences in parasite counts between control and experimental groups were evaluated by Mann-Whitney U test. Differences between hepatic iron stores were evaluated by Student's t test.

**RESULTS**

Groups of six animals were inoculated with *P. berghei* on day 0 and then either left untreated (control) or administered 40 mg per day of an iron chelator (DF, 2,3-DHB, methyl 2,3-DHB, HBED, or EHPG) subcutaneously in three divided doses at 8-hour intervals for 15 consecutive days. The mortality on day 15 among the various groups of rats was 2 of 18 (11%) in untreated controls, 1 of 6 (17%) in animals treated with 2,3-DHB, and 2 of 6 (33%) in those treated with methyl 2,3-DHB. None of the animals treated with DF or HBED died. In contrast, all animals receiving EHPG died between days 6 and 11, not of infection but of drug toxicity manifested in anorexia and severe weight loss with parasite counts not greater than 10%.

The mean (±1 SE) parasite counts in untreated controls and in all treated groups that completed 15 days of therapy are shown in Fig 1. A peak parasitemia of 32.8% ± 4.9% was reached in untreated controls on day 11 of infection. In comparison, mean parasite counts on day 11 in treated rats were methyl 2,3-DHB, 25.3% ± 6.0% (NS); 2,3-DHB, 15.5% ± 3.5% (*p < .01*); DF, 8.0% ± 3.9% (*p < .004*); and HBED, 0.9% ± 0.4% (*p < .001*). A delayed escape of parasitemia was observed on day 15 with both 2,3-DHB and methyl 2,3-DHB. Thus, of the four new compounds studied, EHPG was abandoned because of its apparent toxicity and both 2,3-DHB and methyl 2,3-DHB were abandoned because of limited effectiveness. HBED, on the other hand, showed both an excellent antimalarial effect and low toxicity.

The effect of varying the timing schedule in groups of six animals treated with DF or HBED is shown in Fig 2. In all groups, a constant daily dose of 40 mg per animal was maintained throughout the study. Drugs were administered by subcutaneous injection at 8- or 12-hour intervals. Similar to the first study, parasitemia in untreated controls peaked at 32.4% ± 7.0% on day 12 after inoculation. In contrast to the effective control of parasitemia in animals receiving either DF or HBED at 8-hour intervals, the same dose delivered at 12-hour intervals failed to suppress infection. By day 14 and afterward, parasitemia in animals injected only twice daily was equal to or higher than that in untreated controls.

The effect of varying the dose of HBED on parasite counts is shown in Fig 3. Groups of six animals received subcutaneous injections at 8-hour intervals; total daily doses ranged from 5 to 40 mg. In comparison with the mean parasite count in untreated controls on day 11 (32.1% ± 6.9%), mean parasite counts in animals receiving HBED were as follows: 5 mg/day, 31.9% ± 5.7%; 10 mg/day, 18.1% ± 2.5%; 20 mg/day, 9.0% ± 2.6%; and 40 mg/day, 1.2% ± 0.9%.

The effect of iron-chelation therapy on host iron status is shown in Table 1. Serum iron, transferrin saturation, and total hepatic nonheme iron stores were measured in groups of animals killed on day 15. Iron-chelation therapy had no
effect on serum iron or transferrin saturation. In contrast, a significant reduction in hepatic iron stores was observed with DF ($p < .025$) and with HBED ($p < .001$).

Finally, we examined the ability of the most effective compounds, DF and HBED, to inhibit the growth of *P. falciparum* cultured in human erythrocytes. Fig 4 shows the effect of continuous exposure to these chelators on the growth of cultured nonsynchronized malarial parasites at concentrations ranging from 2 to 20 μmol/L. Both DF and HBED caused a marked reduction in parasite counts at concentrations of ≥5 μmol/L, with maximal inhibition observed on day 3. At all concentrations tested, HBED was more effective than DF in inhibiting parasite growth; there was a four- to fivefold difference in parasite counts on day 3 of culture.

To examine the effect of pulse treatment on parasite growth, parasites synchronized at the schizont stage were mixed with fresh RBCs to a parasitemia of 0.4% and a hematocrit of 4% after which they were incubated with DF or HBED at either 20 or 40 μmol/L for 2 hours at 37°C. After this pulse treatment, the parasites were washed three times and then cultured in normal growth medium. At 3 days of culture, there was no significant difference in parasite counts between untreated controls (15%) and cultures treated with the two drugs at either molar concentration (range 13% to 17%).
ANIMALARIAL EFFECT OF HBED

Table 1. Effect of Iron Chelators on Host Iron Status

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Nonheme Iron Stores (µg/L/mg)</th>
<th>Serum Iron (µg/dL)</th>
<th>Transferrin Saturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1,203 ± 318</td>
<td>316 ± 168</td>
<td>59 ± 32</td>
</tr>
<tr>
<td>HBED</td>
<td>6</td>
<td>547 ± 144†</td>
<td>281 ± 81</td>
<td>47 ± 15</td>
</tr>
<tr>
<td>2,3-DHB</td>
<td>6</td>
<td>1,114 ± 214</td>
<td>346 ± 134</td>
<td>77 ± 11</td>
</tr>
<tr>
<td>Methyl 2,3-DHB</td>
<td>4</td>
<td>1,127 ± 280</td>
<td>293 ± 100</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>DF</td>
<td>6</td>
<td>830 ± 111†</td>
<td>314 ± 156</td>
<td>48 ± 22</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD.
†P < .001.
‡P < .025.

DISCUSSION

In a previous study we showed that parenteral administration of DF at 8-hour intervals is capable of suppressing malaria in rats infected by *P. berghei.* We also showed that the antimalarial effect of DF is independent of host iron status, as effective doses of DF did not result in a reduction of transferrin iron saturation or interference with erythropoiesis. Conversely, DF penetrated infected erythrocytes both in vivo and in vitro and interacted with a labile intracellular iron pool.

Because the ability of DF to penetrate the RBC membrane is limited, we postulated that other iron chelators with a higher affinity for iron and/or increased lipophilicity would show improved antimalarial activity. The present study explored the antimalarial effect of several iron-chelating agents previously considered to be of potential usefulness in clinical management of transfusional iron overload.

The compounds selected for evaluation cover a range of in vivo chelating efficacy. 2,3-DHB is a weak, orally effective iron chelator which is probably converted into a more active species by the liver. Its methyl ester was designed to improve its intestinal absorption. EHPG and HBED are phenolic ethylenediamine derivatives. The affinity of these compounds for ferric iron is greater than that of DF by 3 and 9 orders of magnitude, respectively. In previous studies, we showed that EHPG and HBED mobilize iron from hepatocellular stores with an efficiency 8 to 14 times higher than that of an equivalent amount of DF. Like DF, all four compounds promote mainly biliary excretion of iron in the rat, attesting to their ability to enter cells in vivo. The lipophilicities of the various compounds, however, are markedly different: DF and 2,3-DHB are relatively water soluble whereas methyl 2,3-DHB, EHPG, and HBED are not. Thus, we anticipated that the latter compounds would show an improved antimalarial effect.

Our results show that both lipophilicity and affinity for iron play a role in determining the antimalarial effect of a given chelator. Neither 2,3-DHB nor its methyl ester was an effective antimalarial, and they could not reduce hepatic iron stores (Table I). Indeed, increasing the lipophilicity of 2,3-DHB through formation of the methyl ester had a negative impact on its efficacy. This may have been due to the failure of the liver to convert the ester into an active species. DF and HBED reduced hepatic iron stores by 31% and 55%, respectively, and suppressed the peak parasitemia on day 11 of infection to 24.4% and 2.7% of control values. The overall effectiveness of these two compounds relative to 2,3-DHB can be attributed to their greater affinity to iron. That HBED was the more efficacious is probably due to its higher binding constant, although its increased lipophilicity could also be a factor. EHPG is structurally similar to HBED, yet it proved very toxic. Although in theory it may have antimalarial properties, the very high mortality associated with its use at effective doses precludes further consideration of this compound as a therapeutic agent.

Additional studies were performed to characterize the antimalarial properties of HBED, the most effective iron
chelator and antimalarial agent identified in the present series. The results of varying the dose from 5 to 40 mg/day showed that although all doses appeared to be moderately effective within the first week of infection, peak parasitemia at day 11 was inversely proportional to the dose administered, with complete breakthrough at 5 mg/day. The importance of continuous exposure to the iron chelator is illustrated by the failure of treatment administered at 12-hour intervals as compared with the efficacy of 8-hour administration, the total daily dose being the same (Fig 2). Likewise, pulse therapy with HBED at concentrations of 20 and 40 µmol/L failed to inhibit the in vitro growth of P falciparum schizonts, whereas long-term incubation with 5 µmol/L HBED was very effective. A similar dependence on continuous exposure was also observed with DF, although the antimalarial effect at identical doses was less pronounced than with HBED.

The design of the present study does not permit definitive conclusions to be drawn regarding the mechanism of the antimalarial effect of DF and HBED. However, our previous work with DF showed that its most likely mechanism of inhibition involves interaction with a labile iron pool within the parasitized erythrocyte. DF shows only a limited uptake by normal reticulocytes, and such iron as is chelated by DF intracellularly is unable subsequently to leave the cell. However, parasitized RBCs are, in general, more permeable than healthy erythrocytes, and the uptake of DF by parasitized erythrocytes was directly demonstrated in a recent study using 14C-desferrioxamine.

The manner in which intracellular iron chelation could result in inhibition of parasite proliferation is unknown. Some antimalarial iron chelators such as 8-hydroxyquinoline apparently form extracellular iron complexes which are subsequently internalized to produce a rapidly lethal intracellular reaction. Other compounds, such as rhodotorulic acid, mycobactin, and probably both DF and HBED, may exert their antimalarial effect through deprivation of vital intracellular iron from the parasite. Such iron deprivation would require a critical molar concentration of the drug, below which the metabolic requirements of the parasite may not be seriously impaired. The present data showing an identical minimal inhibitory concentration (5 µmol) for both DF and HBED for inhibition of P falciparum cultures imply that the mechanism of action of both iron chelators is probably identical and that the improved antimalarial action of HBED is most likely due to a higher affinity for iron and/or increased lipophilicity permitting better penetration of the RBC membrane or both.

Ribonucleotide reductase, an iron-containing enzyme that is rate-limiting in DNA synthesis, has been shown to be inhibited by DF and is a very likely target of inhibition by iron chelators within parasitized cells. However, other enzyme systems such as tyrosine hydroxylase as well as enzymes involved in glycolysis and the pentose phosphate shunt may also be inhibited by iron chelation. Although interaction of DF or HBED with other intracellular trace metals such as Zn is possible, this is an unlikely mechanism of parasite inhibition since the affinity of these compounds to iron is much higher than to any other metal and since preincubation of DF with iron completely abolishes its antimalarial action.

Because RBCs contain 1,000 times more iron than plasma, reliance on an intracellular iron pool to meet its metabolic needs would render a parasite independent of fluctuations in host plasma iron concentrations. This type of independence would provide Plasmodium sp with a unique advantage when faced with an increased prevalence of iron deficiency in the population at risk. Conversely, depriving the parasite of such a metabolically important source of iron may represent a novel approach to antimalarial drug development. DF is a relatively ineffective intraerythrocytic chelator, and its poor oral absorption, high price, and short half-life in plasma limit its usefulness as an antimalarial drug. The present data and those published recently by Heppner et al indicate that other orally effective iron chelators may have superior antimalarial activity and that a systematic screening of available iron-chelating drugs may result in identification of clinically useful antimalarial compounds.

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