The antimalarial effect of iron chelators is attributed to their interaction with a labile iron pool within parasitised erythrocytes, and it was postulated that increased affinity to iron as well as increased lipophilicity may improve antimalarial activity. In the present study we have examined the antimalarial effect of 3-hydroxypyridin-4-one (3-OHP) compounds modified by altering the length of the R$_2$ substituent on the ring nitrogen. A significant dose-related suppression of Plasmodium falciparum cultures was observed with all drugs tested in vitro at concentrations of 5 μmol/L or higher. In contrast, there was a clear segregation of the in vivo effect on Plasmodiumberghei in rats (300 mg/kg/d subcutaneous) into two categories: compounds CP20, 38, and 40 failed to suppress the antimalarial activity of an iron chelator. The present study, including positive (DF) and negative (untreated) controls, indicates that 3-hydroxypyridin-4-ones are able to suppress malaria in vivo and in vitro. Because lipid solubility is an important determinant of antimalarial activity, our study provides useful information regarding the selection of orally effective iron-chelating compounds that may be suitable for clinical application as antimalarial agents.

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of freshly drawn blood in sterile normal saline. Parasitemia was estimated by counting 500 cells in stained blood films. Parasite counts in all animals were performed on days 6, 8, 10, 11, and 13 of infection.

Antimalarial effect of new chelators. The design of these in vivo studies was similar to our previous studies on the antimalarial effects of DF. Rats inoculated with P. berghei were treated by subcutaneous injections or gavage every 8 hours of the following iron chelators: DF, CP20, CP38, CP40, CP51, CP94, and CP96 according to the dose schedule indicated. The chemical structure of the various CP compounds is shown in Table 1. To control for possible spontaneous variations in parasite virulence, each study included its own control group of untreated animals, and all groups described in the various tables and figures were studied simultaneously using the same batch of parasites.

Statistics. Differences in parasitic counts were evaluated by Student's t-test. In some animal studies where parasite counts varied widely a nonparametric (Wilcoxon-rank) test was used to confirm differences at the peak day of infection.

Table 1. Chemical Structure and Partition Coefficient of CP Compounds

<table>
<thead>
<tr>
<th>Chelator</th>
<th>R₁</th>
<th>R₂</th>
<th>Free Ligand</th>
<th>Iron Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP 20</td>
<td>CH₃</td>
<td>CH₃</td>
<td>0.21</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>CP 38</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₂CO₂H</td>
<td>NA</td>
</tr>
<tr>
<td>CP 40</td>
<td>CH₃</td>
<td>CH₂</td>
<td>CH₂OH</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>CP 51</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₂OCH₃</td>
<td>0.3</td>
</tr>
<tr>
<td>CP 94</td>
<td>C₂H₅</td>
<td>C₂H₅</td>
<td>0.85</td>
<td>0.07</td>
</tr>
<tr>
<td>CP 96</td>
<td>C₂H₅</td>
<td>C₂H₅</td>
<td>CH₂CH₂OCH₃</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

The effect of in vitro incubation of P. falciparum cultures with iron-chelating compounds at concentrations ranging from 5 to 45 μmol/L is shown in Table 2. With all compounds tested, there was a significant suppression of parasite counts starting at a concentration of 5 μmol/L, with maximal effects observed at concentrations of 20 to 45 μmol/L at 3 days of culture. At comparable molar concentrations of the various compounds studied, only minor differences in the suppression of parasite cultures have been found.

Figures 1A and B show the in vivo effect of the same compounds used in rats infected with P. berghei. Groups of six animals were inoculated on day 0 and then either left untreated (control) or given 300 mg/kg per day of an iron chelator (DF, CP20, CP38, CP40, CP51, CP94, or CP96) administered subcutaneously in three divided doses at 8-hour intervals for 13 consecutive days. There was no mortality encountered during the 13 days of study. In contrast to the in vitro studies showing a similar antimalarial effect of all compound tested, there was a clear segregation in vivo into two categories: compounds CP20, CP38, and CP40 failed to suppress malaria in rats. In contrast, CP51, CP94, and CP96 had a strong antimalarial effect in vivo similar to or better than DF.

Figure 2 shows the peak parasite counts in groups of six animals treated by the six hydroxypyridinones, compared with DF and untreated controls. The mean peak parasite count in untreated controls was 14.7% compared with 12.7% with CP20 (P = .409); 12.0% with CP38 (P = .350); and 14.1% with CP40 (P = .197). In contrast, mean peak parasite counts with CP51, CP94, and CP96, and DF were 0.2% (P = .001), 2.4% (P = .001), 2.8% (P = .002), and 2.2% (P = .001), respectively.

Table 3 shows the effect of iron-chelating therapy on total body weight, liver weight, and iron status. Comparison

Table 2. Effect of In Vitro Iron Chelation on P. falciparum Cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μmol/L)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.27 ± 0.14*</td>
<td>1.27 ± 0.04</td>
<td>13.35 ± 0.21</td>
</tr>
<tr>
<td>DF</td>
<td>5</td>
<td>1.25 ± 0.18</td>
<td>1.41 ± 0.07</td>
<td>6.16 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.69 ± 0.14</td>
<td>1.05 ± 0.10</td>
<td>5.13 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.18 ± 0.05</td>
<td>1.10 ± 0.04</td>
<td>0.44 ± 0.10</td>
</tr>
<tr>
<td>CP 20</td>
<td>5</td>
<td>1.66 ± 0.13</td>
<td>1.46 ± 0.14</td>
<td>8.02 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.14 ± 0.07</td>
<td>1.41 ± 0.13</td>
<td>8.26 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.56 ± 0.04</td>
<td>0.55 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>CP 40</td>
<td>5</td>
<td>1.73 ± 0.14</td>
<td>1.22 ± 0.10</td>
<td>8.22 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.41 ± 0.05</td>
<td>1.08 ± 0.07</td>
<td>7.85 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.87 ± 0.10</td>
<td>1.02 ± 0.08</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>CP 51</td>
<td>5</td>
<td>1.41 ± 0.07</td>
<td>1.19 ± 0.10</td>
<td>8.24 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.06 ± 0.06</td>
<td>1.03 ± 0.10</td>
<td>6.41 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.79 ± 0.10</td>
<td>0.55 ± 0.10</td>
<td>0</td>
</tr>
<tr>
<td>CP 94</td>
<td>5</td>
<td>1.55 ± 0.06</td>
<td>1.19 ± 0.11</td>
<td>7.05 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.02 ± 0.06</td>
<td>1.19 ± 0.14</td>
<td>6.30 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.34 ± 0.04</td>
<td>0.33 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td>CP 96</td>
<td>5</td>
<td>1.24 ± 0.07</td>
<td>1.16 ± 0.08</td>
<td>7.76 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.97 ± 0.10</td>
<td>1.21 ± 0.14</td>
<td>5.88 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.74 ± 0.07</td>
<td>0.58 ± 0.07</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD.
of hepatic nonheme iron content shows a sharp distinction between compounds with and without an antimalarial effect. Hepatic iron stores in animals treated by CP20, CP38, and CP40 that were unable to suppress malaria were identical with those in untreated controls. In contrast, there was a sharp reduction in hepatic iron stores in all groups treated with the effective antimalarial compounds CP51, CP94, CP96, and DF. CP51, which was the most effective
antimalarial, was also the most effective in reducing hepatic iron stores. Unlike hepatic iron stores, the effect of treatment on serum iron concentrations was marginal, with wide variations in individual animals and no consistent correlation between mean values and antimalarial effect.

Further comparison of the various groups in Table 3 shows that total body weight in untreated controls and in the groups treated with CP20, CP38, and CP40 increased by about 30 to 40 g during the 13 days of study. A similar growth rate was found with DF. However, animals treated with CP51, CP94, and CP96 failed to gain weight and with CP051 there was actually a weight loss of 28 g. Similarly, liver weights in animals treated with CP51, CP94, and CP96 were about 2 g less than in controls or in the groups treated with the ineffective antimalarial compounds.

Figure 3 shows the relation between antimalarial effect and hepatic iron stores. There was a close linear correlation \( r = 0.9837 \) between mean peak parasite counts and hepatic nonheme iron content in the various groups studied.

Because CP51, the most effective antimalarial compound identified in the present study, had an adverse effect on growth rates, it was decided to explore the dose-response relation to reduce toxicity. In contrast to the daily dose of 300 mg/kg resulting in a 30-g weight loss, reducing the dose to 150 and 40 mg/kg/d resulted in a weight gain of 11 and 43 g, respectively. However, as shown in Fig 4, this reduction in toxicity was also associated with a marked reduction in antimalarial activity.

**DISCUSSION**

In our previous studies we have shown that iron-chelating therapy with DF or with HBED administered parenterally at 8-hour intervals is capable of suppressing malaria in rats.
infected by *P. berghei.* We have also shown that the antimalarial effect of iron chelating therapy is independent of host iron status, as effective doses of DF and HBED did not result in a reduction of transferrin iron saturation or interference with erythropoiesis. Conversely, it was found that DF is able to penetrate infected erythrocytes both in vivo and in vitro, and that it interacts with a labile intracellular iron pool.

Because the ability of DF to penetrate the RBC membrane is limited, it was postulated that other iron chelators with a higher affinity for iron and/or increased lipophilicity would show improved antimalarial activity. The present studies were undertaken to explore the antimalarial effect of a series of bidentate hydroxypyridinone iron chelators, which have therapeutic potential as oral iron chelators in the clinical management of transfusional iron overload.

The hydroxypyridin-4-ones shown in Table 1 have an identical stability constant for iron(III) of log \( \beta \) 36. However, they may be made more or less lipophilic by increasing or reducing the length of the R1 substituent on the ring nitrogen. This change is expressed in the partition coefficient between the lipid and aqueous phases of the free ligand and the iron complex. Increasing lipophilicity is expressed by an increasing value of partition coefficient. Previous studies of hydroxypyridin-4-ones using radioiron-labeled hepatocytes have shown that the partition coefficient is critical in determining iron release from liver cells. Highly hydrophilic ligands had no appreciable effect on iron release. Conversely, iron release increased with increasing lipophilicity, with optimal effects observed when the partition coefficient was close to 1. With a further increase in the partition coefficient the ligands acquire appreciable toxicity. These findings imply that membrane permeability, determined by lipophilicity, is an important factor in determining the ability of an iron chelator to mobilize iron from cells, and that hydrophilic iron chelators, which are largely restricted to the extracellular space, are unable to enhance iron efflux from the hepatocyte.

Of the hydroxypyridin-4-ones investigated in this work, CP40 and CP20 had the lowest partition coefficients. Not surprisingly, these proved to be the least-efficient antimalarial compounds, and the least effective in reducing hepatic iron stores. Increased lipophilicity results in appreciable iron mobilization from cultured hepatocytes in vitro as well as from hepatic iron stores in vivo. Both of these effects are apparently associated with improved antimalarial activity. Thus, in line with our previous studies of DF and HBED, the in vivo antimalarial effect of CP51, CP94, and CP96 was associated with a marked reduction in hepatic iron stores.

We have shown previously that the antimalarial effect of DF cannot be explained by the induction of a general iron-deficient state in the host. The association of antimalarial effect with hepatic iron depletion is therefore not a cause-and-effect relation, but rather an indirect indicator that a given compound is able to penetrate cells and deplete chelatable iron directly. Indeed, the ability of DF, HBED, and the hydroxypyridin-4-ones to inhibit *P. falciparum* cultures demonstrates that the antimalarial action of the iron chelators is the result of a direct interaction of these compounds with the parasitized erythrocyte.

Our in vivo studies in rats infected with *P. berghei* have shown a clear distinction between CP51, CP94, and CP96, which had a strong antimalarial action, and CP20, CP38, and CP40, which were unable to suppress malaria. In contrast, all of these compounds were found to be effective in suppressing *P. falciparum* in vitro, thus confirming a previous report by Heppner et al. Because DF is a hexadentate chelator, whereas all CP compounds are bidentate chelators, at equal molar concentrations DF binds three times as much iron as the CP compounds. Thus, a concentration of 5 \( \mu \)mol/L DF (Table 2) is equal to 15 \( \mu \)mol/L of each CP compound. When allowance is given to this threefold difference in iron-binding capacity, the antimalarial effect in vitro of DF and all CP compounds was identical.

The apparent contradiction between the similarity of in vitro antimalarial action and variability of in vivo effect of the various CP compounds is best explained by the difference in the conditions of drug supply. In the present study, in vitro exposure to all CP compounds was uninterrupted and lasted for 3 days. Under these conditions, the rate of...
drug uptake was probably unimportant because sufficient time was available for reaching critical intracellular concentrations even for the less lipophilic compounds. In contrast, with in vivo administration, lipophilicity and other variables affecting the pharmacokinetics of the drug may become the dominant factors determining intracellular concentration and antimalarial effect.

Additional studies were performed to characterize the antimalarial properties of CP51, the most effective iron chelator and antimalarial agent identified in the present series. The results of varying the dose from 40 to 300 mg/kg/d showed that peak parasitemia was inversely proportional to the dose administered, with complete breakthrough at 40 mg/kg/d. Thus, although reducing the dose of CP51 limited the toxic effects of this compound as manifested in severe weight loss at 300 mg/kg/d, it also resulted in limited antimalarial action. At present, CP94 and CP96 appear to represent the optimal combination of limited toxicity and effective antimalarial action. As shown in Fig 5, CP94 is able to suppress malaria in vivo after oral administration. To our best knowledge, this is the first demonstration of oral antimalarial activity by an iron chelator.

Our previous studies with DF and HBED have shown that the most likely mechanism of the antimalarial effect of iron chelators is interaction with a vital labile iron pool within the parasitized erythrocyte. The origin of the chelatable iron pool in parasitized erythrocytes is at present unclear. In reticulocytes, the chelatable iron may be derived from the continued uptake of transferrin iron, or the induction of transferrin-receptor synthesis in mature parasitized RBCs. It is also possible that some of the chelatable iron originates from the catabolism of intracellular hemoglobin. The effectiveness of DF and all other iron chelators studied so far in inhibiting the growth of P. falciparum in mature human erythrocytes indicates that the chelatable iron pool of reticulocytes derived from ferritin or transferrin iron uptake is not a prerequisite for the intracellular chelation of iron by these compounds, and supports the contention that chelatable iron may be derived from hemoglobin catabolism in parasitized mature erythrocytes.

The direct relation between lipid solubility, facilitating the transit of membranes, and in vivo antimalarial action found in the present study lends support to the concept of direct intracellular iron chelation as the most probable mechanism responsible for the antimalarial effect of iron chelators. However, lipid solubility is only one of several variables that may determine in vivo antimalarial action. Other factors affecting the pharmacokinetics of these compounds should also be considered when evaluating the variability in their in vivo effect. The mechanism responsible for the inhibition of parasite proliferation by intracellular iron chelation is unknown. Ribonucleotide reductase, an enzyme containing enzyme that is rate limiting in DNA synthesis has been shown to be inhibited by DF and is a possible 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.

Depriving the parasite of a metabolically important source of iron may represent a novel approach to antimalarial drug development. DF is a relatively ineffective intracellular chelator, and its poor oral absorption and short half-life in plasma limits its usefulness as an antimalarial drug. The present data indicate that other, orally effective iron chelators may have superior antimalarial activity in vivo, and that a systematic screening of available iron-chelating drugs may result in the identification of potentially useful antimalarial compounds. Because lipid solubility appears to be one of the determinants of antimalarial action, the present study provides useful information regarding the selection of orally effective iron-chelating compounds that may be suitable for clinical application as antimalarial agents.

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The skillful technical assistance of Alon Hershko is gratefully acknowledged.

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The effect of N-alkyl modification on the antimalarial activity of 3-hydroxypyridin-4-one oral iron chelators

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