Differential Cytotoxicity of Iron Chelators on Malaria-Infected Cells Versus Mammalian Cells

By Hava Glickstein, William Breuer, Mark Loyevsky, Abraham M. Konijn, Jacqueline Libman, Abraham Shanzer, and Z. Ioav Cabantchik

Iron chelators of the hydroxamate class arrest in vitro proliferation of malaria parasites and of mammalian cells. The factors determining the biological activity of the chelators have classically been attributed to the chelators’ capacity for binding iron and to their ability to traverse membranes as free chelators and as chelator-iron complexes. We show in this work that the nature of the chelatable pool of cell iron also contributes to the susceptibility of cells to iron chelators. A class of N-terminal (Nt)-derivatives of desferrioxamine (DFO), (Nt-DFO), is shown here to differentially affect growth and replication of intraerythrocytic parasites (Plasmodium falciaparum). Methyl-anthranyllic DFO ( MADFO), the relatively less hydrophilic member of the Nt-DFOs series, reduced parasite proliferation (48 hour test) with an IC50 of 4 ± 1 µmol/L and mammalian cell (K562 and HepG2) proliferation with an IC50 > 100 µmol/L. On the other hand, the more hydrophilic Nt-free DFO, displayed IC50 values of 21 ± 5 µmol/L for parasites and 7 ± 1 µmol/L for mammalian cells. The selective antiparasitic activity of MA-DFO, as reflected in the speed of action and IC50 values on cell proliferation, is attributed primarily to membrane permeation and iron(III) binding properties of the drug. In contrast, the relatively low antiproliferative activity of the more permeant MA-DFO on mammalian cells, resulted from MA-DFO’s reduced capacity for scavenging intracellular iron. This is apparent from MA-DFO reduced effects on: (1) the chelatable iron(III) pool that is associated with the cell cytosol; (2) the cell chelator-extractable iron, and (3) cell ferritin levels. The potent antimalarial efficacy and biological selectivity of MA-DFO relative to the parent DFO, is of importance for improved design of chemotherapeutic agents.

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mance of iron chelators. Moreover, this class of agents can also be of use as biochemical or pharmacological tools for assessing the mechanisms of metal acquisition and handling by cells.

MATERIALS AND METHODS

Materials

DFO and DFO-E were kindly provided by Dr H. Schnebel (Ciba-Geigy, Basel, Switzerland) and were used for X; N-terminal derivatization with either anthranilic (A-) or MA groups to yield A-DFO and MA-DFO, respectively. Nitro-DFO (N-DFO) was kindly provided by Dr G. Gasparini (Ciba-Geigy, Basel). [3H]hypoxanthine, [1H]histidine, and [3H]-thymidine were from the Radiochemical Centre (Amersham, UK). Unless specified otherwise, all other chemicals were from Sigma Chemical Co (St Louis, MO) or best available grade.

Parasite Cultures

Plasmodium falciparum, strain FCR-3 used for all experiments was maintained in Nuncion culture flasks (Nunc, Roskilde, Denmark) in human A- or O- RBCs in RPMI 1640 medium (Biological Industries, Beth Haemek, Israel) supplemented with 25 mmol/L HEPES, 10 mmol/L glucose and 20 mmol/L NaHCO3, and 10% (vol/vol) heat inactivated A- human plasma pooled from different blood donors. The cultivation method used was a modified version of Trager and Jensen’s22 as described elsewhere.15 Parasitemia and growth stage distribution were determined on methanol fixed and Giemsa stained smears. Parasite growth synchronization and assessment of antimalarial activity of iron chelators were done as described previously.15 The compounds were added from concentrated stock solutions in dimethyl sulfoxide (DMSO) to microcultures (24 wells; Costar, Cambridge, MA) containing infected RBCs (2.5% hematocrit and 2% parasitemia). The cultures were synchronized to the ring stage by incubation in 300 mmol/L alanine and 10 mmol/L Tris-HCl, pH 7.418 or by Percoll gradient centrifugation.18 After 18 to 24 hours exposure to the indicated drug, the cells were either supplemented directly with 6 μCi/mL of [3H]hypoxanthine or washed 3 times with large volume of RPMI 1640 medium and replenished with fresh growth medium before addition of radiolabel, incubation for an additional 18 to 24 hours, and lysis by freezing. The freeze-thawed lysate of labeled cells was passed through glass fiber filters (Tamar Inc, Jerusalem, Israel). Incorporation of label into nucleic acid was measured in a Beckman Scintillation Counter (Beckman, Fullerton, CA). All systems were run in hexatuplicates.

Iron Extraction Capacity of DFO Derivatives

K562 cells were preloaded for 1 hour with 35Fe-diferric Transferrin (6.25 × 106 dpnmol Fe, 0.05 μg/mL transferrin), extensively washed with complete growth medium and buffered saline solutions and incubated for 24 hours with the respective chelators in 24 well plates. The suspensions were separated into cell supernates (S1) and pellets by centrifugation. The pellets were extensively washed with full growth medium and buffered saline solutions, treated with 0.2% Triton X-100 (in 150 mmol/L NaCl, 20 μmol/L Tris-HCl, pH 7.4) supplemented with an antiprotease mixture.24 An aliquot of the detergent-dispersed cells was taken for protein determination (BCA method, Pierce Chemical Co) and the remainder was centrifuged at 15,000 × g for 5 minutes. Radioactivity was counted in both cell supernate (S1) and soluble (ie, supernate) fraction of detergent extract (supernate TS2) after decolorization with H2O2 (5%).

Chelation of Intracellular Iron: Assessment by a Fluorescent Method

Human erythroleukemia K562 cells were treated overnight with chelators in full growth medium, washed extensively and loaded with 0.125 μmol/L calcein-acetoxyxymethyl ester (calcien-AM) for 5 minutes at 37°C in HEPES-buffered, bicarbonate-free α-MEM medium containing 20 mmol/L HEPES, pH 7.3 (α-MEM-HEP) containing 1 mg/mL bovine serum albumin, washed from excess calcien-AM and resuspended in α-MEM-HEP medium.25 Just before measurements, 1 mL of calcien loaded cell suspension (5 × 106 cells) was centrifuged in a microcentrifuge and the cells were resuspended in 2 mL of prewarmed 150 mmol/L NaCl, 10 mmol/L
HEPES-Tris, pH 7.3 (HBS buffer). The cell suspension was transferred to a stirred, thermostated (37°C) cuvette, and fluorescence measurements were initiated. Fluorescence measurements (Exc. 488 nm, Em. 517 nm) were done in a PTI Spectrometry Station (Oscar configuration) (PhotoMed, Wedel, Germany), equipped with a temperature controlled cuvette holder and magnetic stirrer. The magnitude of the baseline fluorescent signal remained relatively stable throughout the measurements, the estimated leakage of the probe was lower than 10% per hour at 37°C. A fluorescence-quenching, anticalcein antibody (10 μL/cuvette) was added to eliminate all extracellular fluorescence, which amounted to ~2% of the total fluorescence. The baseline signal under this condition remained stable for ~20 minutes, indicating minimal leakage of the probe during the experimental period. At the indicated time, salicylaldehyde-isonicotinoyl hydrazone (SIH) was added (100 pmol/L final concentration), to obtain the maximal level of chelatable iron, as shown elsewhere.20,21

RESULTS

The repertoire of DFOs used in this study comprises the hydrophilic DFO and its nitrilo derivative N-DFO and the less hydrophilic methylanthranilic derivative MA-DFO and the cyclic DFO-E (Fig 1). Although these agents differ primarily in the nature of the substituent group at the terminal amino group, they display comparable binding affinities for iron(III) (pKa 29-31) (measured as in ref 20).

Effect of DFOs on Macromolecule Synthesis in Parasitized Cells

P. falciparum infected cells were exposed to various concentrations of DFOs starting at mid-rings. After 24 hours treatment, they were assessed for nucleic acid synthesis in terms of [3H]hypoxanthine incorporation into nucleic acids. The dose-response curves for the various DFOs are given in Fig 2. Two groups of agents can be identified in terms of their growth inhibitory profiles. The first is composed of the N-derivatized and relatively less hydrophilic MA-DFO and DFO-E, which gave IC50 values of 4 ± 1 and 7 ± 2 μmol/L, respectively. The second is composed of the more hydrophilic DFO and N-DFO, which gave IC50 values of 21 ± 5 μmol/L and 17 ± 4 μmol/L, respectively. A fifth DFO derivative, anthranilic DFO (A-DFO) (not shown), performed comparably to MA-DFO (IC50 = 6 ± 2 μmol/L). None of the structural congeners of DFO had any detectable effect on parasite cultures when administered as iron(III) complexes (not shown). Having previously shown that iron(III)-DFO complexes and even more so iron(III)-MA-DFO complexes, were demonstrably permeant to malaria infected cells,20 it can be implied that DFO effects on malaria are primarily caused by iron deprivation.

Effect of DFO Derivatives on Mammalian Cells

Nucleic acid and protein synthesis. The two human cell lines K562 and HepG2 (exponential growth phase) were
exposed for 43 hours to the various DFOs and pulsed for 5 additional hours with the \([^{3}H]\)-labeled precursors hypoxanthine or thymidine. Figure 3 depicts the dose-response curves of the various agents on nucleic acid synthesis and Table 1 summarizes the calculated IC\(_{50}\) values for chelator effects on DNA, protein, and nucleic acid synthesis. Although quantitatively different, the rank order of inhibitory potencies was essentially similar for the three parameters: DFO was considerably more potent than all its derivatives on either mammalian cell line, whereas MA-DFO was the least potent of the four. The relative potencies of the two other DFO derivatives were intermediate to those of DFO and MA-DFO. We have found the IC\(_{50}\) values for drug effects on \([^{3}H]\)-thymidine incorporation into DNA particularly variable for HepG2 cells. Factors contributing to that phenomenon were found to be associated with the state of the culture, such as culture passage number, phase of cell growth and the time of exposure to drug. Most of the variability could be minimized by extending the drug exposure to 48 hours. As shown above for antimalarial activity, none of the structural congeners of DFO had any detectable effect on mammalian cells proliferation when administered as iron(III) complexes (not shown).

Table 1. IC\(_{50}\) Constants of Chelator Effects on HepG2 and K562 Cells

<table>
<thead>
<tr>
<th>Chelator</th>
<th>HepG2 Cells</th>
<th>K562 Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>H</td>
</tr>
<tr>
<td>DFO</td>
<td>7 ± 1</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>MA-DFO</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>N-DFO</td>
<td>45 ± 4</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>DFO-E</td>
<td>26 ± 2</td>
<td>19 ± 4</td>
</tr>
</tbody>
</table>

Cells were exposed to various concentrations of chelators (3 to 100 \(\mu\)mol/L) for 48 hours and analyzed for DNA synthesis by \([^{3}H]\)-thymidine (T) incorporation, nucleic acid synthesis by \([^{3}H]\)hypoxanthine (H) incorporation and total protein (P) by the sulforhodamine method, as described in the text. Data were analyzed as described in Fig 2, the IC\(_{50}\) values (50% inhibitory concentrations) (± SEM, n = 7) given in \(\mu\)mol/L.

Because the various DFOs differ in the lipophilic nature of the N-terminal group and therefore, in their putative permeation properties, we assessed whether any correlation existed between these drug properties and the onset of growth inhibition, ie, the drugs' speed of action (Fig 4). For that purpose we followed the kinetics of nucleic acid synthesis in the presence of drugs' in both K562 and HepG2 cells. Cells exposed to a single concentration of 100 \(\mu\)mol/L DFO derivative were time monitored for \([^{3}H]\)-hypoxanthine incorporation into nucleic acid (Fig 4). The onset of inhibition for all four DFO derivatives was between 4 to 6 hours, suggesting that permeation into cells per se did not apparently limit the rate of chelator action. This was found to be the case for the least potent of the drugs tested, MA-DFO. This fluorescent derivative accumulated in the cells within 4 to 6 hours, as visualized by fluorescence microscopy image analysis (not shown).

Cytosolic-chelatable iron (i) and ferritin levels (ii). The effect of DFOs on mammalian cells was also assessed in terms of cytosolic chelatable iron and cell ferritin levels. Both cell parameters provide a measure for the iron status in the cytosol. The first gives a direct measure of the chelatable metal and the second indicates how changes in cytosolic iron affected ferritin levels.

(1) The cytosolic chelatable iron pool that might be affected by the various DFOs, was assessed with the recently developed fluorescence-quenching method based on the metal-sensor calcein. K562 cells treated with DFOs were loaded with the fluorescent calcein. The initial level of fluorescence attained represents the amount of free calcein loaded into cells. The increase in fluorescence resulting from addition of the highly permeant chelator SIH provides an in situ measure for the chelatable iron pool in the cell. A typical experiment is depicted in Fig 5. Control cells showed a 0.25 \(\mu\)mol/L level of intracellular chelatable iron, as shown previously. After pretreatment with chelators, the basal level of cell fluorescence increased relative to control, as relatively less chelatable iron is present in cells. Consequently, the amount of fluorescence restored by SIH decreased. For all concentrations used (20 to 100 \(\mu\)mol/L), DFO was considerably more potent than MA-DFO. The cell chelatable iron was reduced from 0.25 \(\mu\)mol/L to 0.013 to...
Fig 4. Time-dependence of DFOs' action on nucleic acid synthesis in mammalian cells: K562 cells were exposed to a single concentration of drug, supplemented with [3H]-hypoxanthine and processed at the indicated times. Incorporation of radiolabel into nucleic acids is given as a function of time of incubation.

0.04 μmol/L (85% to 95% reduction) after treatment with 20 μmol/L DFO and to undetectable levels (~100% reduction) by treatment with 100 μmol/L DFO. However, by equivalent treatments with MA-DFO, the chelatable iron pool of cells was considerably less affected: 42% and 74% reduction with 20 and 100 μmol/L, respectively. This clearly indicates that the relatively more permeant MA-DFO, was less efficient in intracellular chelation of iron than the parent, more hydrophilic DFO. Measurements of chelator extractable iron were also assessed in cells loaded with 55Fe-transferrin and analyzed for radioactivity in cells and medium (Fig 6). Despite the relatively higher partition coefficient and permeation capacity of MA-DFO versus DFO, it was considerably more efficient than MA-DFO in extracting iron from cells. This was particularly reflected in the parameters obtained following a 24 hour treatment with 20 μmol/L chelator: the iron appearing in the medium was 1.7 ± 0.2 and 0.5 pmol/mg protein after DFO and MA-DFO, respectively. The iron retained in the cells after 24 hour treatment was 26 ± 1, 21 ± 1, and 23 ± 1 pmol/mg protein, in control, DFO and MA-DFO treated cells, respectively.

(2) A similar pattern of chelator activities was observed with K562 and HepG2 cells analyzed for ferritin levels after a 24 hour incubation in the presence of chelators (Table 2).

Table 2. Ferritin Levels in HepG2 and K562 Cells Treated With Chelators

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HepG2 Cells</th>
<th>K562 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFO 100</td>
<td>25 ± 5 (17)</td>
<td>10 ± 2 (6)</td>
</tr>
<tr>
<td>DFO 20</td>
<td>33 ± 4 (33)</td>
<td>23 ± 4 (25)</td>
</tr>
<tr>
<td>MA-DFO 100</td>
<td>73 ± 7 (58)</td>
<td>40 ± 5 (25)</td>
</tr>
<tr>
<td>MA-DFO 20</td>
<td>91 ± 4 (88)</td>
<td>85 ± 6 (95)</td>
</tr>
<tr>
<td>CHX 5</td>
<td>70 ± 7 (75)</td>
<td>65 ± 8 (25)</td>
</tr>
<tr>
<td>FAC 10</td>
<td>&gt;500 (&gt;300)</td>
<td>&gt;190 (250)</td>
</tr>
</tbody>
</table>

Cells were treated for 24 hours and analyzed for cytosolic ferritin by ELISA and by immunoblotting (data in parenthesis were based on densitometry tracings based on Fig 7). Data are given as percent of control: HepG2 100% = 42 ng ferritin/mg protein and K562 100% = 200 ng/mg protein.
For the ELSA method, we used rabbit anti-human spleen ferritin antibodies and rabbit anti-human placental ferritin antibodies. For SDS-PAGE (Western) immunoblotting, we used rabbit anti-human spleen ferritin antibodies and rabbit anti-human placental ferritin antibodies. Each slot on the gel was loaded with an aliquot derived from a culture well which contained the same number of cells at the beginning of the treatment. The blots were scanned in a Umax scanner (Umax Data Systems Ltd., Industrial Park, Hsinchu, Taiwan) and densitized with the aid of the ImagePro program (Media Cybernetics, Silver Spring, MD). Data of ferritin levels normalized per milligram cell protein (BCA method) are given in Table 2 (details in Materials and Methods).

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**Fig 7.** DFO effects on ferritin levels in mammalian K562 cells: Cells grown for 24 hours in growth medium alone (control) or in medium supplemented with either DFO (20 or 100 µmol/L), MA-DFO (20 or 100 µmol/L), FAC (10 µmol/L), or cycloheximide (10 µmol/L), were washed with buffered saline and their cytosol extracted and analyzed for ferritin levels by SDS-PAGE immunoblotting. Each slot on the gel was loaded with an aliquot derived from a culture well which contained the same number of cells at the beginning of the treatment. The blots were scanned in a Umax scanner (Umax Data Systems Ltd., Industrial Park, Hsinchu, Taiwan) and densitized with the aid of the ImagePro program (Media Cybernetics, Silver Spring, MD). Data of ferritin levels normalized per milligram cell protein (BCA method) are given in Table 2 (details in Materials and Methods).

**DISCUSSION**

Malaria parasites are among the most susceptible organisms to the action of iron chelators. The mechanisms by which these chelators curtail the proliferative capacity of parasites have recently been reviewed for hydroxamate-based chelators. They include primarily properties associated with the chelators, such as iron binding affinity and membrane partition coefficients of the free chelator and its iron complexes, which provide a measure of their membrane permeation properties. These properties can be assumed to be similar in parasite infected RBCs and mammalian cells, as previously shown. However, the mode of action of the chelators on the different cell systems is associated with additional properties. These include: (1) the mechanisms of iron mobilization by the different cells; (2) the dependence of iron mobilization on the developmental stage of the cell or organism and (3) the nature of the chelatable form of bio-available iron that prevails in parasites as compared to mammalian cells. Parasites apparently differ from mammalian cells in all 3 properties, thus making them unique targets for iron chelating agents. The reversed siderophores used previously and the DFOs used in this study, were found to irreversibly affect P. falciparum growth in vitro, whereas all mammalian cells tested (K562, human hepatoma HepG2, human colon carcinoma HT-29, and CHO cells), fully recovered from 24 to 48 hour treatment with up to 100 µmol/L chelators (not shown). We attributed these findings to the differential capacity of mammalian cells for restoring intracellular bio-available iron on drug removal and for removing the accumulated drug either in the free form or as drug-iron complexes. Because none of the drugs used had any effect on either parasite or mammalian cell proliferation when applied as iron complexes, we assume that their anti-proliferative mode of action is most likely associated with their capacity to deprive cells of metabolic iron. However, at present, we cannot reject the possibility of in situ formation of toxic iron-chelator complexes, particularly in the unique parasite environment.

In this work we showed that differential effects of iron chelators on parasites versus mammalian cells can be more profound than previously described with ROSFs. The effects were found with a series of N-derivatives of the DFO family (Figs 2 and 3 and Table 1). First, their biological efficacy as suppressors of cell growth correlated with the putative permeation properties of the DFOs in parasites but not in mammalian cells. Second, the relatively low susceptibility of mammalian cells to MA-DFO (Fig 3 and Table 2), the most potent antimalarial N-DFO tested, was apparently associated with the chemical nature of the iron present in the chelatable pool of the mammalian cell (Figs 5 and 6). In particular, MA-DFO, which has similar iron(III) binding affinity and even higher partition coefficient and membrane permeation capacity than DFO, was less effective than DFO in chelating and extracting intracellular iron from mammalian cells (Figs 5 and 6) and reducing the ferritin stores (Fig 7).

The relatively lower cytostatic potency of MA-DFO in mammalian cells, which correlates with its relatively lower effects on cell iron stores, could be attributed to several factors. Limited drug access into cells was highly unlikely, because MA-DFO was found to be demonstrably permeant to mammalian cells. The possible association of N, DFO properties with blockage of the terminal –NH₃ group or modification of the pKₐ of ca 10⁶ in DFO to values >5 in MA-DFO or A-DFO should be considered. Such a modification might confer on DFO different accessibility properties, which, in turn, might affect its accumulation in particular cell compartments or its interactions with the chelatable form of intracellular iron. The biological effects of the two other N-blocked or N-modified DFO derivatives provide some support for the previously discussed ideas, but do not necessarily prove them. However, alternative explanations should also be considered, such as specific intracellular modifications of the drugs or different interactions with the form of chelatable iron prevailing in cells, ie, iron(II). Although the affinities of DFOs for iron(II) are relatively small (<10⁴) compared to those for iron(III) (ca. 10⁶), in ambient air, even iron(II) might swiftly and avidly bind to DFO following hydroxamate-catalyzed oxidation to iron(III). Thus, the ca-
Selective cytotoxicity of iron chelators

Pevity of DFOs for forming stable complexes with the iron(II) prevailing in cells, might also depend on their capacity to induce oxidation to the iron(III) form. This property might vary not only with the flexibility of the chelator backbone and the physicochemical nature of the N group, but also with additional factors present in cells. Our in vitro studies of the interaction of DFO and MA-DFO with iron(II) bound to the calcein, support only in part, such a notion. They indicate that MADFO’s capacity for chelating cytosolic iron from mammalian cells, most of which is apparently in the iron(II) form, is significantly lower than that of DFO. Although the reasons for the differential behavior of MA-DFO towards mammalian cells as compared to malaria infected cells remain to be fully elucidated, the implications of the results of the present work are highly relevant for future antimalarial drug design.

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References


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