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# The Effect of the Iron(III) Chelator, Desferrioxamine, on Iron and Transferrin Uptake by the Human Malignant Melanoma Cell[1]

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## ABSTRACT

**The mechanism of action of the clinically used iron(III) chelator, desferrioxamine (DFO), on preventing iron (Fe) uptake from transferrin (Tf) has been investigated using the human melanoma cell line SK-MEL-28. This investigation was initiated due to the paucity of information on the mechanisms of action of DFO in neoplastic cells and because recent studies have suggested that DFO may be a useful antitumor agent. The effect of DFO was dependent on incubation time. After a 2-h incubation, DFO acted like the extracellular chelators, EDTA and diethylenetriaminepentaacetic acid, because there was little inhibition of $^{59}$Fe uptake from Tf. In contrast, after a 24-h incubation, DFO (0.5 mM) efficiently reduced internalized $^{59}$Fe uptake from Tf to 18% of the control value. These observations suggested the existence of a kinetic block to the entry of the apochelator to intracellular Fe pools and/or to the exit of the DFO-$^{59}$Fe complex. Indeed, cellular fractionation demonstrated that, in contrast to the decrease in the percentage of $^{59}$Fe in the ferritin and membrane fractions, a marked increase in the percentage of $^{59}$Fe present in the ferritin-free cytosol occurred. These observations suggested an accumulation of the DFO-$^{59}$Fe complex within the cell. The highly lipophilic Fe chelator, pyridoxal isonicotinoyl hydrazone, was far more effective than DFO at preventing $^{59}$Fe uptake from Tf, illustrating the importance of membrane permeability for effective Fe chelation. Desferrioxamine at a concentration of 1 mM decreased internalized $^{125}$I-Tf uptake to 70% of the control. However, the decrease in $^{59}$Fe uptake observed could only be partially accounted for by a decrease in Tf uptake, and it appeared that DFO was chelating $^{59}$Fe at an intracellular site consistent with the transit Fe pool. The results are discussed in the context of the use of Fe chelators as effective antineoplastic agents.**

## INTRODUCTION

Iron is an essential element for cell growth and division because iron-containing proteins catalyze key reactions involving energy metabolism, respiration, and DNA synthesis (1). Without iron, cells are unable to proceed from $G_1$ to S of the cell cycle (2). Hence, all cells require iron and neoplastic cells have a high requirement related to their rapid rate of proliferation, and this is reflected by an increase in the expression of the TfR[3] (3).

DFO (Desferal) is a tris-hydroxamate chelator (siderophore) produced by *Streptomyces pilosus*, which has a very high affinity and specificity for Fe(III) (4), and is currently used clinically to treat iron overload diseases such as thalassemia (5). Recently, DFO has been shown to be highly cytotoxic to human NB cells *in vitro* while having little effect on non-NB cell lines (6–8). Indeed, in a recent clinical trial, a single, 5-day course of DFO resulted in 7 of 9 patients having more than a 50% decrease in bone marrow infiltration of NB cells (9). Apart from the cytotoxic effect on NB cells, DFO has also been shown to have a favorable antitumor effect on human hepatoma xenografts in nude mice (10) and hematopoietic tumors (11) as well as a favorable effect in patients suffering from refractory leukemia (12).

However, despite the impressive cytotoxic effect of DFO, very little is known about its mechanism of action in neoplastic cells, particularly its effect on iron uptake from the serum iron transport protein, Tf. Malignant melanoma is a highly aggressive tumor having a long history of resistance to multiple classes of cytotoxic drugs, and once metastasis has occurred, the prognosis is very poor (13). Previous investigations by the authors have demonstrated that the malignant melanoma cell line, SK-MEL-28, has a very high iron uptake rate from Tf related to the presence of two highly efficient mechanisms (14, 15).

Considering this high iron uptake rate and the paucity of information in general on the mechanism of action of DFO in tumor cells, it was of interest to examine the effect of DFO on Fe uptake by melanoma cells and any antiproliferative effect which may be clinically useful. For comparison, the effect of several other chelators were also examined, including the membrane impermeable chelators, EDTA plus DTPA, and the membrane permeable chelator, PIH.

## MATERIALS AND METHODS

All materials and methods including the labeling of human Tf with $^{59}$Fe and $^{125}$I and the culture of the human melanoma cell line, SK-MEL-28 (American Type Culture Collection, Rockville, MD), were the same as described previously (14). The chelator PIH was synthesized as described by Johnson *et al.* (16). DFO was obtained from Ciba-Geigy Pharmaceutical Co.

To measure $^{59}$Fe-$^{125}$I-Tf uptake in the presence of chelators, these compounds were dissolved in Eagle's minimum essential medium containing 1% (v/v) nonessential amino acids, bovine serum albumin (5 mg/ml), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (20 mM; pH 7.4), and $^{59}$Fe-$^{125}$I-Tf (1.25 μM) and incubated with the cells for 2 or 24 h. The medium was then removed, and the cell monolayer was washed four times with ice-cold BSS. The amount of radioactivity bound to the plasma membrane or internalized by the cells was then measured after incubation with Pronase (1 mg/ml) for 30 min at 4°C (14). The distribution of internalized $^{59}$Fe into ferritin, stromal-mitochondrial membranes, and ferritin-free cytosol was determined as described previously (17). It should be noted that the iron pools defined by these methods are functional and may not correspond to actual cell locations.

The effect of the chelators on [$^3$H]thymidine uptake was measured by incubating melanoma cells with DFO (0.01–5 mM) for 24 h. The uptake of [$^3$H]thymidine was then measured after washing the monolayer twice with BSS prior to incubation at 37°C for 1 h in Eagle's minimum essential medium containing [$^3$H]thymidine (1 μCi/plate). The radioactive medium was then removed by aspiration, and the cell monolayer was washed four times with ice-cold BSS. Acid-insoluble material was precipitated by incubation for 1 h at 4°C with 20% trichloroacetic acid followed by washing twice with ice-cold 10% trichloroacetic acid. The cells were removed from the plates in 1 ml of 1 M NaOH and transferred to scintillation vials. The [$^3$H]thymidine incorporation was determined by liquid scintillation counting.

The results were calculated as moles of iron or Tf per g of protein (gPR) and as a percentage of the control and are expressed as the mean or mean ± SEM. Each determination was derived from 1 confluent petri dish containing approximately $5 \times 10^6$ cells, and this was equivalent to about 0.45 mg protein via

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[3] The abbreviations used are: TfR, transferrin receptor; Tf, transferrin; DFO, desferrioxamine; NB, neuroblastoma; DTPA, diethylenetriaminepentaacetic acid; PIH, pyridoxal isonicotinoyl hydrazone; BSS, balanced salt solution; gPR, gram of protein; EDTA, ethylenediaminetetraacetic acid.

the bicinchoninic acid protein assay procedure (14). Data were compared using the Student t-test. Results were considered statistically significant when $P < 0.05$.

## RESULTS

### Iron Uptake

**Total and Internalized Iron Uptake.** Preliminary experiments with melanoma cells demonstrated that DFO (0.5 mM) had a similar effect on $^{59}$Fe and $^{125}$I-Tf uptake as the membrane impermeable chelators, EDTA and DTPA, reducing total $^{59}$Fe uptake from Tf to 60–80% of the control value after a 2-h incubation (Table 1). In contrast, the membrane permeable chelator PIH was far more effective and reduced total $^{59}$Fe uptake to 10% of the control. All of the chelators also slightly reduced $^{125}$I-Tf uptake to 70–90% of the control value (Table 1).

The low activity of DFO described above after a 2-h incubation with melanoma cells was in contrast to its marked effect on other cell types (17). This was investigated further using a range of DFO concentrations (0.01–5 mM) and also a longer incubation period of 24 h.

In contrast to the effect of DFO after a short-term incubation, internalized $^{59}$Fe uptake decreased markedly over a 24-h incubation, and at a DFO concentration of 1 mM, $^{59}$Fe uptake was reduced to 16% of the control value (Fig. 1). To investigate the relationship of membrane permeability to the efficacy of iron chelation, a similar experiment was done with the membrane impermeable chelator, EDTA (1 mM). Interestingly, EDTA was far less effective than DFO, and after a 24-h incubation, decreased internalized $^{59}$Fe uptake to 55% of the control without affecting $^{125}$I-Tf uptake (not shown).

**Subcellular Distribution of $^{59}$Fe.** DFO at concentrations greater than 0.5 mM decreased $^{59}$Fe uptake into the stromal-mitochondrial membranes and ferritin fractions to a greater extent than total cell $^{59}$Fe uptake (Table 2). However, $^{59}$Fe uptake into the ferritin-free cytosol decreased less markedly than either the stromal-mitochondrial membrane or ferritin fractions (Table 2). As a proportion of the total amount of $^{59}$Fe internalized by the cell, the stromal-mitochondrial membrane $^{59}$Fe decreased with increasing DFO concentration, while $^{59}$Fe uptake into ferritin was also markedly reduced (Fig. 2). This indicated that in melanoma cells, ferritin, or more likely an iron pool in direct equilibrium with it, was the main site of action of DFO. In contrast to the decrease in $^{59}$Fe uptake into both the ferritin and stromal-mitochondrial membrane, a pronounced increase in the percentage of internalized $^{59}$Fe in the ferritin-free cytosol occurred (Fig. 2).

Table 1 *The effect of PIH, DFO, EDTA, and DTPA at a concentration of 0.5 mM on total $^{59}$Fe and $^{125}$I-Tf uptake by melanoma cells after a 2-h incubation at a Tf concentration of 1.25 μM*

| Chelator | $^{59}$Fe uptake (nmoles iron/g protein) | $^{125}$I-Transferrin Uptake (nmoles Tf/g protein) |
|---|---|---|
| Control | 220 ± 2[a] (4) (100%)[b] | 10.5 ± 0.4 (4) (100%) |
| PIH | 22 ± 2 (3) (10%) | 8.3 ± 0.3 (3) (79%) |
| DFO | 163 ± 4 (4) (74%) | 9.2 ± 0.4 (4) (88%) |
| EDTA | 172 ± 4 (4) (78%) | 8.7 ± 0.4 (4) (83%) |
| DTPA | 132 ± 4 (4) (60%) | 9.4 ± 0.4 (4) (90%) |

[a] Results are expressed as mean ± SEM (3–4 experiments; 10–17 determinations).
[b] Results in parentheses are expressed as a percentage of the control.

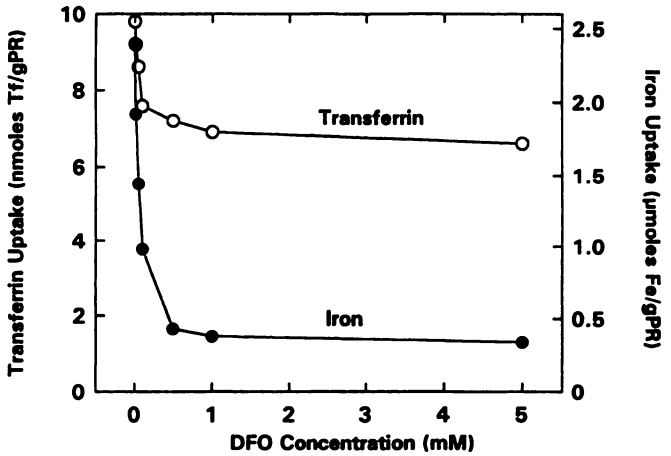


Fig. 1. The effect of DFO concentration (0.01–5 mM) on $^{125}$I-transferrin and $^{59}$Fe internalization over a 24-h incubation with $^{59}$Fe-$^{125}$I-Tf (1.25 μM). The cells were then washed four times and incubated with Pronase (1 mg/ml) for 30 min at 4°C. Results are means of 2 experiments (8–12 determinations).

**$^{59}$Fe Uptake into the Plasma Cell Membrane.** DFO and EDTA did not affect membrane Tf uptake after a 24-h incubation (not shown) but did substantially reduce the uptake of non-Tf-bound $^{59}$Fe into the plasma membrane-bound compartment (Fig. 3). Non-Tf-bound membrane $^{59}$Fe uptake was calculated by assuming that each Tf molecule in the membrane compartment had two iron atoms bound. This was then subtracted from the total membrane iron (14). At concentrations from 0.1–1 mM, DFO was significantly ($P < 0.0005$) more effective than EDTA at reducing the uptake of non-Tf-bound membrane $^{59}$Fe. Previous work by the authors demonstrated that membrane non-Tf-bound $^{59}$Fe uptake by melanoma cells was consistent with the presence of a membrane iron-binding molecule (14). The present results suggest that both DFO and EDTA have access to $^{59}$Fe released from Tf en route to the membrane iron-binding component, reducing iron uptake by this molecule. In addition, as the cell membrane is impermeable to EDTA, it appears possible that at least part of the $^{59}$Fe in the membrane compartment may be derived from $^{59}$Fe released from Tf at a site in contact with the extracellular medium.

### Transferrin Uptake

DFO had much less effect on $^{125}$I-Tf uptake than $^{59}$Fe uptake, suggesting that its main site of action was distal to Tf cycling. DFO had little effect on total or membrane $^{125}$I-Tf uptake at concentrations less than 5 mM. In contrast, after an incubation period of 24 h, DFO (1 mM) decreased the internalization of Tf to 70% of the control (Fig. 1). As a proportion of total Tf uptake, internalized Tf uptake decreased from the control value of 29 ± 1% (12 determinations) to 23 ± 1% (12 determinations) at a DFO concentration of 1 mM.

### Effect of Desferrioxamine Concentration on [$^3$H]Thymidine Incorporation

The impressive ability of DFO to reduce $^{59}$Fe uptake from Tf after a 24-h incubation suggested that this chelator may have some cytotoxic effects on these cells and may be able to reduce [$^3$H]thymidine incorporation into DNA. In addition, this effect may be particularly marked considering the high iron requirement of melanoma cells (14, 15). Indeed, after a 24-h incubation, DFO markedly decreased [$^3$H]-thymidine uptake, and at a concentration of 0.5 mM or greater, DFO almost completely inhibited [$^3$H]thymidine incorporation (Table 2).

Table 2 *The effect of DFO concentration (0.01–5 mM) on the incorporation of [$^3$H]thymidine and the uptake and distribution of $^{59}$Fe by melanoma cells after a 24-h incubation*

| DFO mM | [$^3$H]Thymidine incorporation (% control) | Iron uptake (μmoles Fe/gPR) | | | |
|---|---|---|---|---|---|
| | | Total internalized iron | Ferritin | Ferritin-free cytosol | Stromal-mitochondrial membrane |
| Control | 100 ± 3 | 2.28 ± 0.05[a] (100%)[b] | 1.35 ± 0.05 (59%) | 0.59 ± 0.03 (26%) | 0.34 ± 0.02 (15%) |
| 0.01 | 71 ± 7 | 1.89 ± 0.05 (100%) | 1.19 ± 0.04 (63%) | 0.50 ± 0.04 (26%) | 0.26 ± 0.01 (14%) |
| 0.05 | 62 ± 4 | 1.39 ± 0.05 (100%) | 0.78 ± 0.04 (56%) | 0.40 ± 0.02 (29%) | 0.23 ± 0.01 (17%) |
| 0.1 | 20 ± 3 | 0.93 ± 0.02 (100%) | 0.47 ± 0.01 (51%) | 0.33 ± 0.02 (35%) | 0.15 ± 0.01 (16%) |
| 0.5 | 1 | 0.41 ± 0.02 (100%) | 0.16 ± 0.01 (39%) | 0.21 ± 0.01 (51%) | 0.05 ± 0.01 (21%) |
| 1 | <1 | 0.36 ± 0.02 (100%) | 0.12 ± 0.01 (33%) | 0.21 ± 0.01 (58%) | 0.04 ± 0.01 (11%) |
| 5 | <1 | 0.27 ± 0.04 (100%) | 0.05 ± 0.01 (19%) | 0.20 ± 0.01 (74%) | 0.01 ± 0.01 (4%) |

[a] Results are expressed as means ± SEM (6–12 determinations in 1 experiment).
[b] Results in parentheses represent the relative distribution of iron between ferritin, ferritin-free cytosol, and stromal-mitochondrial membrane expressed as a percentage of the total internalized iron at each DFO concentration.

## DISCUSSION

The iron chelation efficacy of DFO in melanoma cells was dependent on incubation time. After a short incubation of 2 h, DFO (0.5 mM) had little effect on $^{59}$Fe uptake from Tf, acting in a similar way to the membrane impermeable chelators, DTPA and EDTA. However, after 24 h of incubation, DFO (0.5 mM) was significantly more effective than EDTA and decreased $^{59}$Fe uptake to 18% of the control. These observations suggested a kinetic block to the entry of the apochelator into the cell and/or to the exit of the $^{59}$Fe complex from the cell. Indeed, after a 24-h incubation with DFO, it is of interest to note that in contrast to the decrease in the percentage of $^{59}$Fe in the ferritin and stromal-mitochondrial fractions, a marked increase in the percentage of $^{59}$Fe in the ferritin-free cytosol occurred (Fig. 2). This may suggest that an intracellular accumulation of the DFO-$^{59}$Fe chelate (ferrioxamine) was occurring. These data are in accordance with previous studies demonstrating that ferrioxamine is far more hydrophilic and hence far less membrane permeable than the apochelator, which may explain the accumulation of the $^{59}$Fe chelate within the cell (18).

The decrease in $^{59}$Fe uptake observed using DFO could only be partially accounted for by a decrease in Tf uptake. Under similar experimental conditions, DFO has been shown by dialysis experiments to release 17–21% of Tf-bound $^{59}$Fe (19). Hence, in the presence of DFO, there may be a significant generation of apoTf or monoferric Tf whose affinity for the TfR is far less than diferric Tf (20), and this may account for the decrease in Tf internalization observed as the DFO concentration was increased (Fig. 1). In contrast, EDTA, DTPA, and PIH cannot remove significant amounts of $^{59}$Fe from Tf (19, 21), and the decrease in total $^{125}$I-Tf uptake seen after a 2-h exposure to these chelators (Table 1) may be due to the chelation of calcium(II), which is required for the binding of Tf to the TfR (22).

The long incubation time (24 h) required for effective chelation by DFO, the greater efficacy of DFO compared to the impermeable

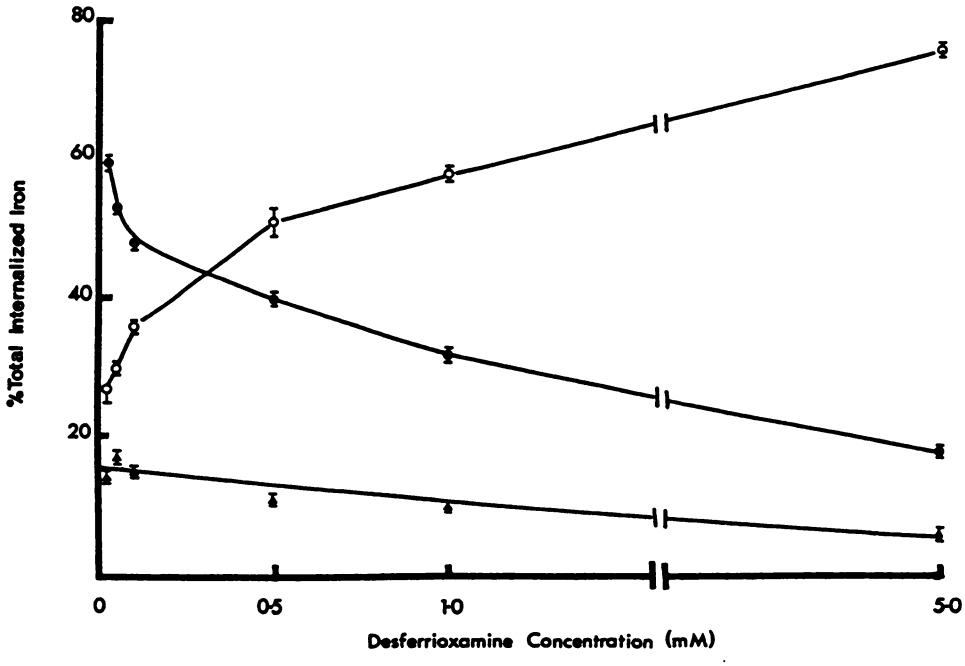


Fig. 2. The effect of DFO concentration (0.01–5 mM) on the distribution of internalized iron between ferritin (●), ferritin-free cytosol (○), and stromal-mitochondrial membrane (▲) over a 24-h incubation with $^{59}$Fe-$^{125}$I-Tf (1.25 μM). The cells were then washed four times and incubated with Pronase (1 mg/ml) for 30 min at 4°C. Results are mean ± SEM (2 experiments; 8–12 determinations).

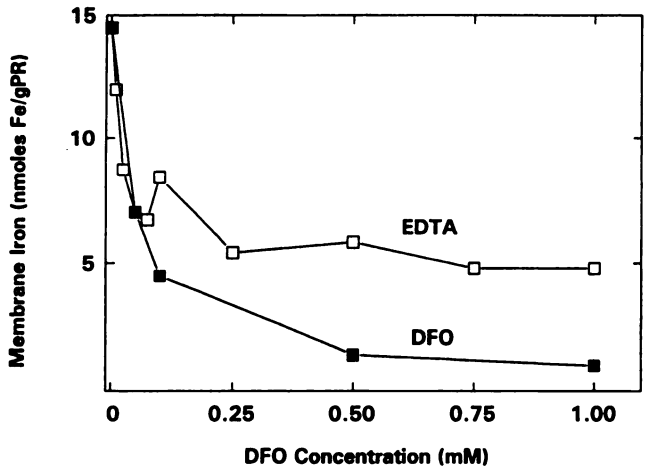


Fig. 3. The effect of DFO and EDTA concentration (0.01–1 mM) on non-Tf-bound membrane $^{59}$Fe uptake over a 24-h incubation with $^{59}$Fe-$^{125}$I-Tf (1.25 μM). The cells were then washed four times and incubated with Pronase (1 mg/ml) for 30 min at 4°C. Non-Tf-bound membrane $^{59}$Fe uptake was calculated by assuming that each Tf molecule in the membrane compartment had two iron atoms bound. This was then subtracted from the total membrane $^{59}$Fe (14). Results for DFO are mean (2 experiments; 8–12 determinations) and for EDTA are mean (1 experiment; 4 determinations).

chelator EDTA, and the small effect of DFO on Tf uptake, suggested diffusion of the chelator to and Fe chelation from an intracellular site. Cellular fractionation suggested that DFO could bind iron from either: (*a*) the intracellular iron storage protein, ferritin, or more likely, (*b*) an iron pool in direct equilibrium with it. Indeed, studies *in vitro* on isolated ferritin have demonstrated that DFO has a very poor ability to remove iron from this protein (23, 24). On the other hand, the intracellular iron pool has been proposed to consist of small molecular weight iron chelates of sugars, amino acids, and nucleotides (25), all of which have a low affinity for Fe(III) and are capable of being chelated by DFO. The intracellular iron pool is of major importance in cellular metabolism and supplies iron to important proteins such as ribonucleotide reductase, the enzyme that catalyses the first step in DNA synthesis (26). Hence, chelation of iron from the transit iron pool is likely to inhibit the activity of this enzyme, which requires a constant supply of iron for activity (27), and may partly explain the decrease in [$^3$H]thymidine incorporation observed in the present study. It is prudent to note that other iron chelators, such as parabactim, have also been shown to decrease [$^3$H]thymidine incorporation by tumor cells (28).

The low activity of EDTA as compared to DFO may be because EDTA is not membrane permeable and is not highly specific for Fe(III) (29–31). The importance of membrane permeability in relation to chelator efficacy is also demonstrated using the Fe chelator PIH (Table 1). Previous studies have suggested that the high activity of PIH is related to its favorable lipophilicity (17) and charge (32), allowing easy access across biological membranes and into intracellular iron pools. Indeed, iron mobilization studies in melanoma cells demonstrated that PIH was far more effective than DFO at increasing the release of $^{59}$Fe from prelabeled cells (33). In addition, investigations using reticulocytes and hepatocytes have suggested that the partition coefficient of the apochelator has a marked influence on intracellular iron mobilization efficacy (34, 35), and it has been demonstrated that iron chelators that are capable of easily diffusing across cell membranes to gain intracellular access are more effective cytotoxic agents than their hydrophilic counterparts (16).

Considering the clinical use of lipophilic iron chelators, the balance between hydrophilicity and hydrophobicity appears to be an important factor in determining the efficacy of a ligand (34, 35). Previous studies with the highly lipophilic iron chelator, cholylhydroxamic acid, have demonstrated that this compound is excreted in the bile as the iron complex and then reabsorbed in the gut, resulting in little net iron excretion (36). On the other hand, PIH appears to have a favorable hydrophilic/hydrophobic balance, and a clinical trial with this chelator showed no evidence of toxicity and produced significant iron excretion (37).

Because there are few therapies to successfully treat the aggressive metastatic form of malignant melanoma (13) and in consideration of the marked iron chelation effect of DFO, and especially PIH in melanoma cells, as well as the substantial effect of DFO on [$^3$H]thymidine incorporation, it appears worthwhile to initiate further studies to examine the effects of these chelators on the growth of human melanoma xenografts in nude mice.

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