

Comparison of Activity of Deferoxamine with That of Oral Iron Chelators against Human Neuroblastoma Cell Lines¹

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ABSTRACT

The iron chelator, deferoxamine, has demonstrated cytotoxicity against neuroblastoma cells. In this study we examined the *in vitro* antineuroblastoma activity of several potentially less expensive oral chelating agents. On a mole for mole basis, 1-hydroxypyridine-2-thionine (omadine) had 100 times the cytotoxicity of deferoxamine. 1,2-Dimethyl-3-hydroxypyrid-4-one also caused demonstrable cell death but at considerably higher molar concentrations than those required for deferoxamine. 2,3-Dihydroxybenzoic acid had no effect on neuroblastoma cell viability over a range of concentrations. In contrast to the effect of both deferoxamine and 1,2-Dimethyl-3-hydroxypyrid-4-one, those due to omadine were permanent within 24 hours of incubation, were not significantly altered by the presence of ionic iron, and correlated with an increase in the percentage of cells in the S-G₂-M phases of the cell cycle. On the basis of these *in vitro* studies, we believe that the use of omadine in particular and iron chelators in general, by themselves or as cell cycle-recruiting agents together with standard cell cycle specific drugs, is an approach to the treatment of cancer worth further investigation.

INTRODUCTION

We and others have shown previously that deferoxamine is capable of killing neuroblastoma cells *in vitro* (1, 2). This cytotoxicity, which is dose dependent, appears to be a result of chelation of iron. Ferrioxamine, the iron-saturated analogue of deferoxamine, is inactive, and inhibitory effects of deferoxamine on growth and on DNA synthesis can be prevented by coinubation with various iron salts or iron-saturated transferrin (1, 3). Moreover, these effects of deferoxamine on neuroblastoma are relatively specific in that a number of nonneuroblastoma cell lines derived from malignant or normal tissue were not as sensitive.

Deferoxamine has been used extensively to treat patients with transfusion-related iron overload. However, in addition to being costly, it is given parenterally and therefore is inconvenient. A number of oral chelators, some of which already have been tested in patients, have been developed as possible alternatives to deferoxamine (4-8). In this study we compared the *in vitro* antineuroblastoma cytotoxicity of several of these agents with that of deferoxamine.

MATERIALS AND METHODS

Two neuroblastoma cell lines, CHP 126 and CHP 100 (gift from G. Johnson, Pediatric Oncology Branch, National Cancer Institute, Bethesda, MD, and H. W. Hann, M.D., Fox Chase Cancer Center, Philadelphia, PA), were maintained in tissue culture RPMI 1640 (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin ("complete medium"). Final concentration of iron in this medium was 0.23 µg/ml (Clinical Chemistry Laboratories, University of Pittsburgh School of Medicine). Cells from one or the other of the cell lines were

plated into 16-mm tissue culture dishes (5 × 10⁴/dish) in 2 ml complete medium.

Because 60 µM deferoxamine (CIBA-Geigy, Summit, NJ) has been shown to reproducibly cause significant cell death with exposure for 72 h (1, 2), duplicate wells were incubated with this amount of drug to serve as "positive controls." Test wells, also in duplicate, contained one of three oral iron chelators: L₁³ (9); 2,3-dihydroxybenzoic acid (gift of Dr. R. Grady, Cornell University School of Medicine, New York, New York); or 1-hydroxypyridine-2-thione (omadine) (Sigma Chemical Co., St. Louis, MO). A range of concentrations for each of these compounds was selected so as to include amounts equal in both molarity and weight to the 60 µM deferoxamine. At serial time points, cultures were examined using an inverted microscope to obtain a qualitative estimate of cell number and to ensure that cell attachment had taken place. Because cells grew loosely adherent to plates, they were removable by pipeting without prior trypsin digestion. That all cells had been harvested by this technique was confirmed by again viewing plates under an inverted microscope. Viable cells were counted in a hemocytometer using trypan blue exclusion.

In contrast to its effects on other chelators, addition of iron to cultures of leukemia cell lines has been found to enhance rather than abrogate the cytotoxic effects of omadine (7, 8). To study the effects of supplemental iron on antineuroblastoma activity of these various chelators, equimolar or greater amounts of ferrous sulfate or ferric chloride were preincubated with each of the above compounds for 2 h prior to plating tumor cells, which were then processed as noted above. Control wells contained either the ferrous sulfate or chelator by itself.

This synergism between iron and omadine against leukemic cells suggested to us that the mechanism by which this drug was acting might be different from that of deferoxamine, for which iron protects against cytotoxicity. To indirectly examine this possibility we looked at DNA quantitation and cell cycle analysis of CHP 126 cells as described previously (3), at the time of plating and following 24 h incubation with chelators with/without iron. In order to assure adequate cell numbers, 10⁵ cells were plated into each well for these experiments. Aliquots of cells were deposited by cyto centrifugation on slides (Cell Analysis Systems, Inc., Lombard, IL) containing predeposited normal cells. The slides were air-dried and fixed in 10% buffered formalin for 30 min. The fixed preparations were hydrolyzed in 5 N HCl before Feulgen staining, with azure A as the chromophore. Analysis was performed on the CAS 100 System with the Quantitative DNA Analysis software package. The instrument was calibrated independently for each slide by using the previously deposited control cells. On each slide approximately 300 neuroblastoma cells were analyzed, and histograms of cell count versus DNA content were generated. Two preparations from each culture condition were analyzed.

RESULTS

The percentage of viable cells remaining after 72 h incubation of CHP 126 with different chelating agents are shown in Table 1. Results were qualitatively identical with the other neuroblastoma cell line (results not shown). On a mole for mole basis, omadine had almost 100-fold the toxicity of deferoxamine, with 0.6 µM causing cytotoxicity similar to that of 60 µM deferoxamine. L₁ also caused demonstrable cell death but at considerably higher concentrations than those required for deferoxamine. Because L₁ has been given to patients in 1-3-g

³ The abbreviation used is: L₁, 1,2-dimethyl-3-hydroxypyrid-4-one.

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Table 1 Effect of chelators on viability of CHP 126 neuroblastoma cells

Chelator	Molecular wt.	Concentration		% viability ^a
		μM	(mg/100 ml)	
Deferoxamine	657	60	3.9	10
L ₁	139	280	3.9	10
		120	1.6	30
		60	0.8	90
		20	0.3	100
2,3-Dihydroxybenzoic acid	155	280	3.9	100
		120	1.6	100
		60	0.8	100
Omadine	149	120	1.6	0
		60	0.8	0
		30	0.4	0
		10	0.1	0
		5	0.05	0
		2.4	0.03	0
		1.2	0.02	0
		0.6	0.01	20

^a Viability assessed at 72 h and compared with that of untreated control cells.

Table 2 Effects of iron on chelator-mediated cell death

Chelator	Concentration (μM)	Ferrous sulfate (μM)	% viability (72 h)
Deferoxamine	60		10
	60	60	95
L ₁	120		30
	120	60	100
Omadine	0.6		20
	0.6	15 ^a	20
		15 ^a	100
		60 ^a	100

^a Identical results with ferric chloride.

doses (5), similar to what is conventionally used for deferoxamine, the effects of equivalent weights of these agents also was studied. On a weight for weight basis, L₁ produced results comparable to those of deferoxamine. 2,3-Dihydroxybenzoic acid, which is a less powerful iron chelator than L₁ or deferoxamine, had no effect on neuroblastoma cell viability at any of the doses tested.

The time course of L₁- and omadine-induced cell death was further explored. Greater than 90% of cells were killed by omadine within 24 h, even at concentrations as low as 1.2 μM . When omadine-containing medium was aspirated from wells after only 3 h incubation and replaced with fresh complete medium, there was no difference compared with wells which had remained exposed for the entire 24 h. At omadine concentrations of 0.6 μM , cytotoxicity was not obvious until 72 h. The effect of L₁ (120 μM) on cell death was more gradual and similar to what was seen with 60 μM deferoxamine (1).

As described previously, deferoxamine-related cytotoxicity could be abolished by coincubation with ferrous sulfate (Table 2). Similar abrogation could be demonstrated for L₁ but not for omadine. At high concentrations of omadine, the extent of cell death was almost 100% at 24 h so that no deleterious effect of iron on viability could be demonstrated. Addition of iron (as either ferrous sulfate or ferric chloride) in the presence of 0.6 μM omadine did not clearly enhance or abrogate cytotoxicity of the chelator alone. Iron alone had no effect on cell growth. This lack of effect of iron on presumed chelator-related cytotoxicity suggested that omadine might have a mechanism of action different from that of deferoxamine and L₁. In a prior study we had shown that deferoxamine acts as an early S-phase inhibitor (3). The effects of each of these chelators with and without iron on cell cycle analysis are summarized in Table 3. Following 24

Table 3 Effect of 24 h exposure to iron chelators on cell cycling by CHP 126 neuroblastoma cells

Chelator	Ferrous sulfate (15 μM)	%G ₀ -G ₁	%S-G ₂ -M
0	0	49	51
0	+	49.8	50.2
Deferoxamine (60 μM)	0	66.2	33.8
L ₁ (120 μM)	0	73	26.8
Omadine (1.2 μM)	0	25.5	74.5
Omadine (1.2 μM)	+	46	54

h incubation with CHP 126 cells the effects of deferoxamine and L₁ were qualitatively similar. Both drugs caused a decrease in the percentage of cells in the S-G₂-M phases of the cell cycle, an effect which was prevented by coincubation with iron. In contrast omadine caused a significant dose-dependent increase in the percentage of cells in S-G₂-M. Despite our observation that iron had neither an enhancing nor an inhibitory influence on omadine cytotoxicity, by cell cycle analysis omadine-induced changes were in part prevented by iron. Thus in the presence of a combination, the percentage of cells in S-G₂-M was similar to that seen in untreated control cells.

DISCUSSION

Deferoxamine, a derivative of hydroxamic acid, has shown excellent antineuroblastoma activity *in vitro* at concentrations of 50–60 μM (1, 2). Whether such concentrations can be achieved *in vivo* is not clear, since reliable assays for measurement of deferoxamine levels in serum are not generally available. However, one study in which 10 mg/kg i.v. boluses of deferoxamine were given reported peak plasma levels of 80–130 μM , followed by rapid clearance with a half-life of 5–10 min (10). Infusions s.c. over 24 h of 100-mg/kg doses resulted in steady state deferoxamine levels of 8–20 μM by 12 h.

Because i.v. administration may be more effective than s.c. administration, it seems reasonable that by giving higher doses by the former route, one might anticipate cytotoxic deferoxamine levels. It is significant in this regard that one patient with stage IV neuroblastoma who was piloted on a protocol which utilized 10 mg/kg/h for 96 h every other week experienced an objective response with decreases in serum ferritin levels and some transient shrinkage of a supraclavicular mass.⁴

Even at such high doses, deferoxamine has been used extensively and with relative safety for treatment of iron overload in patients, including children, receiving chronic transfusions (11). Nonetheless deferoxamine presents a significant problem in that it is expensive and must be given parenterally. A search for effective oral chelating agents therefore has been ongoing. Several, including 2,3-dihydroxybenzoic acid (6) and L₁ (5), have been used in a limited number of patients with iron overload due to thalassemia. Both drugs in doses which appeared to be safe could induce urinary and fecal iron excretion and at least retard accumulation of iron. L₁ was found to cause iron excretion similar to that achieved with comparable doses of deferoxamine. However, as far as we are aware, formal phase I toxicity testing results have not been published with either of these compounds. Thus, optimal dosing has not yet been established. In addition, although omadine in preliminary experiments in mice has shown no toxicity when given parenterally at doses of 270 mg/kg (7), there is no experience with systemic use of this drug in humans. This chelator, unlike deferoxamine and L₁, is not effective in increasing iron excretion at doses of

⁴ M. Donaldson and S. Travis, personal communication.

up to 200 mg/kg in mice⁵ but differs from the other two in that it is more lipophilic and forms lipophilic iron complexes which easily diffuse through cell membranes (12).

Our work suggests that in addition to their use in transfusion-related iron overload, some oral chelators may have a role as antitumor agents. With continuous exposure to L₁, we observed dose-dependent cell death of two neuroblastoma cell lines. Although this was less than that seen with molar equivalent amounts of deferoxamine, the effect was similar on a weight for weight basis. This difference arises mainly because 3 mol of L₁, in contrast to 1 mol of deferoxamine, are needed to bind one molecule of iron at physiological pH (13).

Of particular interest is the finding that omadine was cytotoxic for neuroblastoma cells in doses approximately 100-fold less than deferoxamine on either a molar or weight basis. In contrast to the effect of deferoxamine or L₁, that of omadine was almost immediate in that even if drug was removed from cell cultures within 3 h of plating, cells continued to die. These results are similar to what has been reported using leukemic cell lines (7) for which brief exposure to 20 μM omadine but not L₁ or deferoxamine caused inhibition of cell growth and DNA synthesis.

Deferoxamine and L₁ act by slowly depleting serum as well as intracellular iron, in turn causing arrest of cells in early S phase. Their effects on growth inhibition and cell cycling are reversed by supplementation of growth medium with iron. In contrast, iron appears to enhance the cytotoxicity of omadine for leukemia cells and had neither a protective nor a deleterious effect on neuroblastoma. Therefore, it has been suggested that the mechanism of omadine toxicity is different from that of these other chelators; its primary mechanism of action in this setting may not be via iron chelation. That omadine appears to arrest cells in G₀-G₁ rather than in S-G₂-M supports this concept. In our hands, cell cycle changes caused by omadine in the presence of the relatively low concentrations of iron in standard tissue culture medium were quite different from those caused by omadine together with supplemental iron, suggesting

that the drug by itself may have a mechanism of action different from that of its iron complex.

Based on these *in vitro* results, we believe that the use of iron chelators, by themselves or as cell cycle-recruiting agents together with standard cell cycle-specific drugs, is an approach to treatment of cancer worth further investigation. Phase II studies of deferoxamine in cancer patients are planned. To determine whether oral chelators offer a clinically viable alternative to deferoxamine, either in the treatment of cancer in transfusional iron overload, further studies to determine the maximal tolerated dose and pharmacokinetics of L₁ (in humans) and omadine (initially in animals) need to be done.

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⁵ Unpublished observations.

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