Cytotoxic effects of the lipophilic iron chelator omadine

George J. Kontoghiorghes, Andrea Piga* and A. Victor Hoffbrand

Department of Haematology, Royal Free Hospital School of Medicine, Pond Street, Hampstead, London NW3 2QG, England

Received 5 June 1986

Cytotoxic effects were observed following 4 h incubation of human leukaemic cells with the iron chelator 1-hydroxypyridine-2-thione (omadine). Its cytotoxic activity was comparable to that of the cytotoxic drug doxorubicin. At the same concentration two other effective iron chelators, desferrioxamine and 1,2-dimethyl-3-hydroxypyrid-4-one, were not cytotoxic. Addition of iron augmented the effect of omadine. It is suggested that the lipophilic properties of omadine and of its iron complex cause their intracellular accumulation and potent cytotoxic activity.

Omadine *Chelator* *Iron* *Cytotoxicity* *Thymidine uptake*

1. INTRODUCTION

Many iron requiring enzymes are involved in metabolic pathways essential for the growth and development of normal and malignant cells [1,2]. Under normal conditions iron is well confined and controlled by proteins and other molecules but if it is not strongly chelated it can be toxic [3]. Iron chelators by affecting intracellular and extracellular iron transport and storage may inhibit DNA synthesis and other metabolic processes dependent on iron [2,4,5]. A direct effect on the iron-dependent enzyme ribonucleotide reductase has been shown in a cell free system [6], and has been postulated in the intact cell [7,8]. Effective iron chelators like desferrioxamine may inhibit the toxicity of iron in vitro, but less effective ones like EDTA may exacerbate its toxic effects [9,10].

Desferrioxamine has been reported to inhibit DNA synthesis in a variety of cell culture systems, although the effect has only been seen at high drug concentrations or with prolonged exposure of the cells to the drug [11–13]. Other potential iron chelating drugs may be more effective in inhibiting DNA synthesis [7,11], and some have been reported to be cytotoxic [11,14,15].

Here we have assessed inhibition of DNA synthesis and cytotoxicity for human myeloid leukaemic cells of three iron chelators, namely desferrioxamine, 1,2-dimethyl-3-hydroxypyrid-4-one [2], 1-hydroxypyridine-2-thione (omadine) alone or with added iron and compared their effectiveness to that of the cytotoxic drug doxorubicin.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Desferrioxamine was obtained from Ciba-Geigy, England, 1-hydroxypyridine-2-thione from Sigma, England, doxorubicin from Farmitalia Carlo Erba and 1,2-dimethyl-3-hydroxypyrid-4-one was prepared as described [2] based on a method of preparation of other pyridone derivatives [16]. Chelators were dissolved in RPMI

* On study leave from Oncologia Clinica, University of Ancona, Italy

Abbreviations: K_{par}, partition coefficient; IC_{50}, the chelator concentration causing 50% inhibition; PBS, phosphate buffered saline; [^{3}H]TdR, [*methyl*-^{3}H]thymidine; FCS, foetal calf serum

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

1640 medium (Flow Labs, England) at 2×10^{-3} M; and appropriate dilutions were dispensed in 20 μl triplicate aliquots in the wells of 96-well microtitre plates and the plates kept at 4°C and used within one month. Doxorubicin was freshly dissolved or stored at −20°C. In the cytotoxicity experiments in which $FeCl_3$ (Sigma, London) was used, the iron was dissolved in RPMI and preincubated with the chelators for 2 h at room temperature and subsequently stored at 4°C for up to 5 days.

2.2. *Partition coefficients*

The *n*-octanol/water partition coefficients of the chelators were determined as follows: 4 ml drug (10^{-5} M) dissolved in PBS mixed for 5 min in the Whirley mixer with 4 ml *n*-octanol, the two layers allowed to separate and the mixture centrifuged for 5 min at 2200 × *g*. The absorbances of both the aqueous and *n*-octanol layers were measured at selected wavelengths of the UV region, in the vicinity of or at the λ_{max} of each chelator, by using an SP 1700 Unicam spectrophotometer.

2.3. *Red blood cell membrane permeability*

Fresh heparinised blood was collected from a normal donor; packed red cells were prepared by spinning for 5 min at 2200 × *g* and by removing the supernatant, white cells and the top layer of red cells containing the reticulocytes. The packed red cells were washed three times with PBS and spun for 5 min at 2200 × *g* each time. Chelator-iron mixtures were prepared by adding to each chelator (4×10^{-4} M final concentration) a solution of iron (10^{-5} M final concentration) containing traces of ^{59}Fe ($^{59}FeCl_3$, Amersham, England). Equal volumes (750 μl) of packed red cells were added to the chelator-iron mixture (0.2 μCi ^{59}Fe) in PBS and 200 μl aliquots were withdrawn at 15 min and 1 h after mixing, spun for 3 min through 100 μl silicon oil (d = 1.05, Aldrich, Gillingham, England) in a micro-tube using a Beckman B microfuge, frozen in liquid nitrogen, cut and separated into a cell part (bottom) and a supernatant/oil part (top). The ^{59}Fe activity in the cell and the supernatant/oil parts of each sample were counted separately using an LKB-Wallac 1280 ultragamma counter.

2.4. *Cell cultures*

The human leukaemic myeloid cell lines U937, HL60 and ML2 were grown at 37°C in humidified atmosphere containing 7.5% CO_2 and fed three times a week with RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell concentration was kept between 0.8 and 2.5 × 10^6 cells/ml and the cultures were always fed the day before each experiment to ensure exponential growth. Cell viability at the beginning of experiments was always >95% by the trypan blue exclusion method. Before each experiment cells were washed twice with RPMI and resuspended in the same medium (with no FCS added) at a concentration 0.25–1 × 10^6 cells/ml. The cell suspension was dispensed in 180 μl aliquots into the wells of 96-well microtitre plates (flat bottom) containing 20 μl of drug solution in RPMI and incubated for 4 h at 37°C in humidified atmosphere + 7.5% CO_2. For DNA synthesis studies 0.4 μCi of [^{3}H]TdR (5 Ci/mmol, Amersham, TRA 120) were added to each well for the last 1 h incubation. At the end of the incubation period the plates were washed once with cold PBS and the radioactivity incorporated in the cells was measured by harvesting the cultures with a cell harvester (Skatron, Norway) and washing with distilled water and methanol. Only [^{3}H]TdR which was incorporated into DNA was measured.

The effect of chelators on cell growth was also evaluated by washing the cells twice after 4 h of incubation with the drugs and prolonging the incubation in RPMI + 10% FCS + antibiotics; counts of viable cells were performed daily by the trypan blue exclusion method. Inhibition of [^{3}H]leucine incorporation into cellular proteins was also studied as described [24] using U937 and HL60 cells at 0.5 × 10^6/ml, incubation for 4 h with omadine or omadine iron and addition of [^{3}H]leucine (1 μCi per well) 20 h later.

3. RESULTS AND DISCUSSION

3.1. *Partition coefficients and red cell membrane permeability*

The *n*-octanol/water partition coefficients of the chelators varied, omadine being more lipophilic (K_{par} = 0.4) than the other two chelators (table 1). It is known that small neutral molecules or metal complexes of $K_{par} > 0.3$ can diffuse into matured red cells [2] which are not metabolically active on iron uptake and also possibly through other cell

membranes. In this study 74% of ^{59}Fe from the omadine iron complex mixture diffused into the red cells (assessed by labelled iron entry) after 15 min and 76% after 1 h of incubation but less than 6% ^{59}Fe from the desferrioxamine or 1,2-dimethyl-3-hydroxypyrid-4-one iron complex mixtures entered (table 1). These data suggest that omadine is a lipophilic chelator which has the ability to rapidly transport substantial amounts of iron across the red cell membrane. In contrast the other two chelators, namely desferrioxamine which is charged and 1,2-dimethyl-3-hydroxypyrid-4-one which is neutral but hydrophilic, form iron complexes which do not facilitate iron transport across the red cell membrane even after 1 h of incubation (table 1) [2].

3.2. *Inhibition of cell growth*

Growth of U937 was substantially inhibited by 4 h incubation with omadine which, at a concentration of 2×10^{-5} M, caused complete killing of the leukaemic cells within the first 24 h (fig.1). Similarly the incorporation of [^{3}H]leucine into U937 and HL60 was also inhibited at the same concentration (2×10^{-5} M), and at lower concentrations (table 3). The addition of equimolar iron to omadine (2×10^{-6} M) enhanced its cytotoxicity at each time point (fig.1). Iron or desferrioxamine alone at the same concentrations and the addition of equimolar iron to desferrioxamine did not increase inhibition of DNA synthesis or cytotoxicity under the same experimental conditions (not shown).

3.3. *Inhibition of [^{3}H]TdR uptake*

The effect of the chelators, at concentrations of 2×10^{-5} M or lower, on [^{3}H]TdR uptake in short-term culture varied between the three different cell lines studied (table 2). Inhibition of [^{3}H]TdR uptake by omadine was higher in the HL60 and ML2 cell lines than in U937. The degree of inhibition was comparable to that of the cytotoxic drug doxorubicin which was more effective than omadine in U937, similar on HL60 and less effective on ML2. Desferrioxamine and 1,2-dimethyl-3-hydroxypyrid-4-one had no effect at the concentrations studied.

The cytotoxic and [^{3}H]TdR cell uptake inhibiting effects obtained with short-term exposure of the lipophilic chelator omadine or its iron complex suggests that its mechanism of action is a rapid accumulation and irreversible inhibition of an intracellular or membrane-associated component to which the chelator or a metal complex of the chelator can bind and react. A different mechanism of action can be postulated for the hydrophilic chelators since no significant cytotoxicity or DNA inhibition was obtained after short-term incubations (4 h) with desferrioxamine or with 1,2-dimethyl-3-hydroxypyrid-4-one. The inhibitory effects previously observed with desferrioxamine at high concentrations [11] or prolonged (16 h) incubations [12] may have been mediated through interactions with the cell membrane or extracellularly through iron starvation. Alternatively these effects [11,12] may be due to a slow in-

Table 1

Physicochemical properties of the chelators

Chelator	M_r	Charge at pH 7.3	K^a_{par} *n*-octanol/water	%Fe[b] incorporation	
				15 min	1 h
Desferrioxamine	561	positive	0.02	4.0	5.0
1,2-Dimethyl-3-hydroxypyrid-4-one	139	neutral	0.18	3.0	6.0
Omadine	127	neutral zwitterionic	0.40	74.0	76.0

[a] K_{par} is the partition coefficient of the chelators estimated in *n*-octanol/water at pH 7.3

[b] The percentage incorporation of ^{59}Fe into red blood cells in the presence of the chelators was estimated from the total ^{59}Fe count of red blood cells plus supernatant/oil (100% ^{59}Fe, 1.4×10^3 cpm)

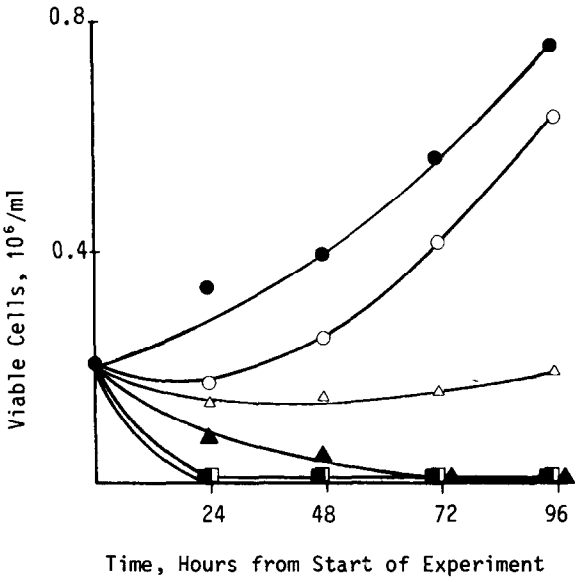

Fig.1. The effect of chelators on cell growth. U937 cells (0.2×10^6 cells/ml) were incubated for 4 h with the drugs, washed twice, and the incubation was prolonged in RPMI 1640 + 10% FCS. Viable cells were counted daily by the trypan blue exclusion method. (●—●) Control, (○—○) 2×10^{-5} M desferrioxamine, (□—□) 2×10^{-5} M omadine, (■—■) 2×10^{-5} M omadine + 2×10^{-5} M $FeCl_3$, (△—△) 2×10^{-6} M omadine, (▲—▲) 2×10^{-6} M omadine + 2×10^{-6} M $FeCl_3$.

tracellular accumulation of this chelator, its iron complex, or its metabolites.

The incubation of omadine with iron markedly increased its cytotoxic effects in contrast to the lipophilic spermidine catecholamide chelators which were also shown to be cytotoxic in culture but for which the effects are reversed by iron [14]. The difference may be related to the inability of the spermidine catecholamide chelator-iron complexes to diffuse across the cell membrane due to their larger size.

The cytotoxic effects of iron chelators may be related to the subtraction of iron from proteins (e.g. ribonucleotide reductase) or from metabolic pathways involving iron. Our data, however, show that in contrast to the above belief, in the case of omadine the addition of iron, rather than decreasing, enhances its toxicity substantially. Furthermore, in the case of desferrioxamine and of 1,2-dimethyl-3-hydroxypyrid-4-one which was shown to remove iron from transferrin [17,18] and ferritin [19] in vitro and also from animals to levels similar to those caused by desferrioxamine [2,10], no cytotoxic or [^{3}H]TdR cell uptake effects were caused. Albert [20] suggested that the antibacterial and antifungal activity of omadine may be due to the oxidative damage caused by its iron complex. This mechanism of toxic activity was also suggested to occur with doxorubicin [21] and bleomycin [22] and there is further evidence from fluorimetric studies on lymphocytes that direct DNA damage occurs within 2 h incubation with omadine alone or plus iron (in preparation). Our data are in agreement with this mechanism for the direct cytotoxic effect of omadine but we also suggest that the enhancement of its toxicity in the presence of iron is due to the increased membrane permeability of the complex which is neutral at physiological pH in contrast to the chelator alone

Table 2

The IC_{50}[a] of chelators on the [^{3}H]thymidine uptake by U937, HL60 and ML2 cell lines

Chelator	U937	HL60	ML2
Desferrioxamine	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M
1,2-Dimethyl-3-hydroxypyrid-4-one	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M	$>2 \times 10^{-5}$ M
Omadine	$>2 \times 10^{-5}$ M	5×10^{-6} M	10^{-7} M
Doxorubicin	10^{-6} M	10^{-6} M	$>2 \times 10^{-5}$ M

[a] IC_{50} is the chelator concentration causing 50% inhibition of [^{3}H]thymidine incorporation in the cells following 4 h incubation, in comparison to non-chelator treated cells (100% [^{3}H]thymidine uptake: U937, 15×10^3 cpm/10^6 cells; HL60, 37.1×10^3 cpm/10^6 cells; ML2,75×10^3 cpm/10^6 cells)

Table 3

Inhibition of [^{3}H]leucine incorporation into U937 and HL60 by omadine and omadine iron

Chelator concentration (M)	[^{3}H]Leucine incorporation, %[a]			
	U937		HL60	
	No added iron	Added iron	No added iron	Added iron
2×10^{-5}	7.7	1.3	1.7	0.5
2×10^{-6}	58.3	50.9	3.1	1.2
2×10^{-7}	80.5	87.9	–	–

[a] Percent of control (U937, 22.3×10^3 cpm/10^6 cells; HLGO, 26.7×10^3 cpm/10^6 cells)

The results are the mean of three experiments. In the incubations with iron both omadine and iron were added at the same concentrations

which is charged (zwitterionic).

Omadine is a known fungicide commonly used in the cosmetic industry as a component of shampoos and deodorants and has considerable low toxicity in animals [23]. The subcutaneous or intragastric administration of omadine (270 mg/kg) to 15 and 5 mice, respectively, for 3 days caused no apparent ill-effects (unpublished). Its low animal toxicity and its high in vitro cytotoxic activity which is comparable to doxorubicin demand its further investigation as an anticancer agent.

ACKNOWLEDGEMENTS

We thank the UK Thalassaemia Society for financial assistance and Miss J. Martin for the typing.

REFERENCES

[1] Barnes, D. and Sato, G. (1980) Cell 22, 649–655.
[2] Kontoghiorghes, G.J. (1982) PhD Thesis, Essex University, Br. Library Microfilm D66194/86.
[3] Editorial (1985) Lancet i, 143–145.
[4] Landschulz, W., Thesleff, I. and Ekblom, P.J. (1984) J. Cell Biol. 98, 596–601.
[5] Neckers, L.M. and Cossman, J. (1983) Proc. Natl. Acad. Sci. USA 80, 3494–3498.
[6] Thelander, L. and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133–158.
[7] Brockman, R.W. (1974) Cancer Chemother. Rep. 4, 115–129.
[8] Hoffbrand, A.V., Ganeshaguru, K., Hooton, J.W.L. and Tattersall, M.H.N. (1976) Br. J. Haematol. 33, 517–526.
[9] Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1979) Biochem. J. 184, 469–472.
[10] Kontoghiorghes, G.J. (1985) Lancet i, 817.
[11] Ganeshaguru, K., Hoffbrand, A.V., Grady, R.W. and Cerami, A. (1980) Biochem. Pharm. 29, 1275–1279.
[12] Lederman, H.M., Cohen, A., Lee, J.W.W., Freedman, M.H. and Gelfand, E.W. (1984) Blood 64, 748–753.
[13] Robbins, E. and Peterson, T. (1970) Proc. Natl. Acad. Sci. USA 66, 1244–1251.
[14] Bergeron, R.J., Cavanaugh, P.F., Kline, S.J., Hughes, R.G., Elliot, G.T. and Porter, C.W. (1984) Biochem. Biophys. Res. Commun. 121, 848–854.
[15] Hellman, R.M., Glass, J. and Nunej, M.T. (1983) in: Structure and Function of Iron Storage and Transport Proteins (Urushizaki, I. et al. eds) pp.479–480, Elsevier, Amsterdam, New York.
[16] Harris, R.L.N. (1976) Aust. J. Chem. 29, 1329–1339.
[17] Kontoghiorghes, G.J. (1986) Biochim. Biophys. Acta 869, 141–146.
[18] Kontoghiorghes, G.J. and Evans, R.W. (1985) FEBS Lett. 189, 141–144.
[19] Kontoghiorghes, G.J. (1986) Biochem. J. 233, 299–302.
[20] Albert, A. (1981) Selective Toxicity. The Physicochemical Basis of Therapy, 6th edn, Chapman and Hall, London.
[21] Eliat, H., Gianni, L. and Myers, C. (1984) Biochemistry 23, 928–936.
[22] Sansville, E., Pelsach, J. and Horwitz, S.B. (1976) Biochemistry 17, 2740–2776.
[23] Moe, R.A., Kipran, J. and Linegar, C.R. (1960) Toxicol. Appl. Pharmacol. 2, 156–170.
[24] Thorpe, P.E., Brown, A.N.F., Ross, W.C.J., Cumber, A.J., Detre, S.I., Edwards, E.C., Davies, A.J.S. and Stripe, C. (1981) Eur. J. Biochem. 116, 447–454.