Biochemical Pharmacology, Vol. 36, No. 14, pp. 2343–2347, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

IMPAIRMENT OF NUCLEOTIDE METABOLISM BY IRON-CHELATING DEFEROXAMINE*

JERZY BARANKIEWICZ† and AMOS COHEN
Division of Immunology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

(*Received* 23 *May* 1986; *accepted* 23 *December* 1986)

Abstract—The effect of deferoxamine on nucleotide metabolism in HL-60 leukemic cells was studied to explore the mechanism of its antiproliferation activity. It was found that in intact cells deferoxamine markedly inhibited the ribonucleotide reduction and incorporation of bases (adenine, hypoxanthine), ribonucleosides (inosine, guanosine) and deoxyribonucleosides (thymidine, deoxyadenosine, deoxyguanosine) into nucleic acids. Although deferoxamine did not inhibit thymidine and uridine incorporation into free nucleotides, inhibition of hypoxanthine and adenine incorporation into nucleotides as well as inhibition of nucleotide biosynthesis *de novo* was found. Nucleotide catabolism, protein synthesis, and intracellular levels of ribonucleotides were not affected significantly by deferoxamine. These results showed that deferoxamine selectively affects several specific reactions of nucleotide metabolism. Inhibition of ribonucleotide reduction, inhibition of ribonucleotide and deoxyribonucleotide incorporation into nucleic acids, as well as inhibition of purine biosynthesis, may alter significantly cellular physiology and, therefore, contribute significantly to the antiproliferative activity of deferoxamine.

Deferoxamine (Desferal) is a well-known chelator of ferric ion, used therapeutically to decrease the amount of iron in overload diseases [1, 2]. Since iron plays an important role in cellular physiology, the ability of deferoxamine to bind iron could have a number of effects on cellular events. Indeed, it was suggested that deferoxamine inhibits iron uptake and arrests DNA synthesis in HeLa cells [3, 4]. Although non-dividing cells are resistant to iron deprivation, decrease of extracellular iron levels in the mitotic stage of HeLa cells rapidly inhibits DNA synthesis and cell proliferation [4]. Also, in PHA-stimulated peripheral blood lymphocytes, deferoxamine markedly decreases thymidine incorporation [5–7]. Recent studies have shown that this hydroxylamine exhibits strong anti-proliferative action for both B and T human lymphocytes [3]. It was shown that deferoxamine equally inhibits DNA synthesis in both B and T cells and decreases intracellular levels of deoxyribonucleotides but not ribonucleotides. Detailed study on the effect of deferoxamine on the cell cycle showed that it is a reversible S phase blocking inhibitor [8]. Since similar findings were demonstrated with hydroxyurea, a known inhibitor of ribonucleotide reductase, this enzyme was considered as a possible target for deferoxamine action [5, 8], especially because ribonucleotide reductase requires iron for its catabolic activity [9]. Since it has been suggested that deferoxamine could be used as an antileukemic drug [8], in this study the effect of deferoxamine on nucleotide and nucleic acid metabolism was studied in a leukemic cell line (HL-60) to explain its mechanism of inhibitory action.

MATERIALS AND METHODS

Chemicals. Radioactive [8-^{3}H]adenine and [G-^{3}H]hypoxanthine were purchased from Amersham International (Amersham, U.K.). Radioactive adenosine, uridine, thymidine, cytidine, deoxyadenosine, deoxyguanosine, guanosine and glycine were obtained from ICN (Irvine, CA, U.S.A.). Radioactive [G-^{3}H]-L-threonine, [^{3}H-*methyl*]thymidine, and [G-^{3}H]valine were purchased from New England Nuclear (Boston, MA, U.S.A.). Non-radioactive purine and pyrimidine bases, nucleotides and nucleosides were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Deferoxamine (Desferal) was obtained from CIBA GEIGY (Canada). 13255 Cellulose TLC sheets were obtained from the Eastman Kodak Co. (Rochester, NY, U.S.A.). Polyethyleneimine-cellulose TLC sheets were purchased from the Fisher Scientific Co. (Don Mills, Ontario, Canada). Alamine-336 was a gift from the Henkel Corp. (Mississauga, Ontario, Canada). RPMI medium was obtained from the Ontario Cancer Institute (Toronto, Ontario, Canada) and fetal calf serum (FCS) from Gibco (Grand Island, NY, U.S.A.).

Incubation of cells with deferoxamine. HL-60 leukemic cells were cultured in RPMI medium containing 10% heat-inactivated FCS. Cells were collected by centrifugation and resuspended in RPMI medium containing 10% FCS to obtain 2×10^6 cells/ml. Samples (1 ml) of cell suspension containing increasing concentrations of deferoxamine from 0 to 500 μM were incubated for 24 hr. After incubation, cells were collected and resuspended in 0.1 ml of

* This study was supported by grants from CIBA GEIGY Canada.

† Address correspondence to: Dr. Jerzy Barankiewicz, Division of Immunology/Rheumatology, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8.

medium containing the appropriate concentration of deferoxamine and then incubated with respective radioactive precursors. The presence or absence of deferoxamine during labeling did not affect the results. Increased density of cells (2×10^6 cells/0.1 ml) for incubation with radioactive precursors did not affect cellular metabolism during 1–3 hr of incubation (ATP/ADP ratio > 8). Although 24-hr incubation with deferoxamine was provided for all the experiments presented, 6-hr and 5-hr incubations were sufficient to inhibit 20% of ribonucleotide reductase activity and 30% of the incorporation of thymidine into nucleic acids. At 1500 μM deferoxamine, 50% inhibition of thymidine or adenine incorporation into nucleic acid was reached after 3 and 20 hr respectively.

Purine and pyrimidine metabolism. Cells (2×10^6) were incubated in 0.1 ml with 1 μCi of radioactive precursors for 1–3 hr. Samples were then centrifuged, and cell pellets were extracted with 50 μl of 0.4 perchloric acid (PCA). After 10 min in ice, extracts were centrifuged. Pellets containing insoluble fractions were washed five times with 2 ml of 0.4 M PCA, and its radioactivity was measured. Supernatant fractions containing free nucleotides, nucleosides and bases were neutralized with a mixture of Alamine 336-Freon [10], and separation of all metabolites was done using TLC chromatography. Separation of ribonucleotides was done using modified PEI-TLC chromatography and separation of nucleosides and bases was performed using Cellulose-TLC [11]. However, in most cases incorporation of radioactive precursors into total nucleotide fraction was measured. When radioactive ribonucleotides were separated from deoxyribonucleotides, modified PEI-cellulose TLC was used according to Ref. 12.

For evaluation of ATP catabolism, samples containing 2×10^6 cells in 1 ml were incubated with 2 μCi of radioactive adenine for 1 hr. Unincorporated adenine was then washed out, and cells were incubated in 0.1 ml for 30 min with different concentrations of deferoxamine. Analyses of radioactive nucleotides in cell extracts and nucleosides and bases in medium were carried out.

Measurements of intracellular nucleotide concentrations. Cells (10×10^6) preincubated for 24 hr in 5 ml with or without deferoxamine were collected by centrifugation and extracted with 100 μl of 0.4 M PCA for 10 min at 0°. Then extracts were centrifuged down and neutralized with Alamine 336-Freon [6]. Ribonucleotides were separated and quantitated using HPLC on a Partisil-10SAX exchange column in 0.5 M ammonium phosphate (pH 3.4) [13]. Quantitation of ribonucleotides was performed by automatic integration of absorption at 254 nm.

All experiments presented in this paper were performed in duplicate and are typical of at least three experiments.

RESULTS

Effect of deferoxamine on thymidine metabolism. Incorporation of radioactive thymidine into DNA and free nucleotides in HL-60 leukemic cells was measured after 24-hr incubation of cells with deferoxamine. It was found that deferoxamine markedly inhibited incorporation of thymidine into DNA (Fig. 1A) but not incorporation of thymidine into free nucleotides (Fig. 1B). Only a slight increase of radioactive thymidine incorporation into nucleotides was observed.

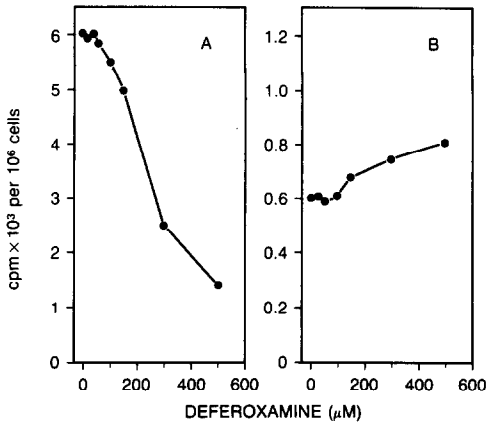

Fig. 1. Effect of deferoxamine on thymidine metabolism. Cells (2×10^6) were incubated for 24 hr with a range of deferoxamine concentrations. Cells were washed and incubated with 2 μCi of radioactive thymidine for 3 hr. Incorporation of radioactivity into DNA (A) and nucleotides (B) was analysed.

Effect of deferoxamine on uridine metabolism. Incorporation of radioactive uridine into RNA and free nucleotides was studied in HL-60 cells after 24-hr incubation with increased concentrations of deferoxamine. It was found that deferoxamine markedly inhibited uridine incorporation into RNA (Fig. 2A), but incorporation of uridine into free nucleotides was affected only slightly. Only a small increase of radioactive uridine incorporation into nucleotides was observed (Fig. 2B).

Effect of deferoxamine on adenine and hypoxanthine metabolism. Incorporation of adenine into

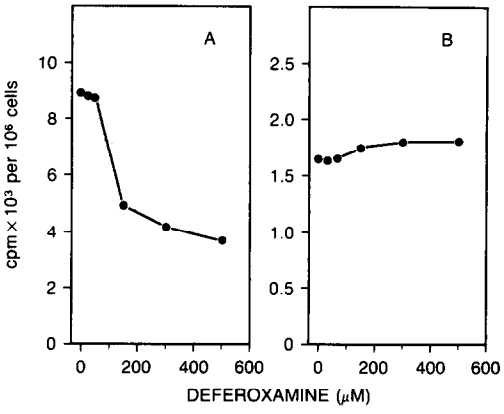

Fig. 2. Effect of deferoxamine on uridine metabolism. Cells (2×10^6) were incubated for 24 hr with a range of deferoxamine concentrations. Cells were washed and incubated with 1 μCi of radioactive uridine for 3 hr. Incorporation of radioactivity into RNA (A) and nucleotides (B) was analysed.

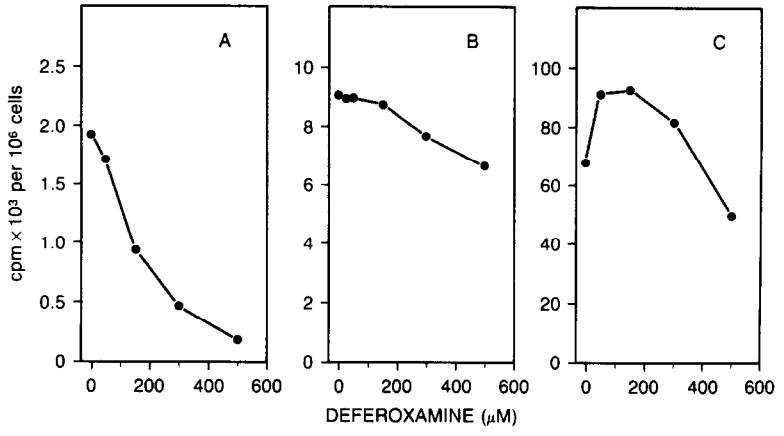

Fig. 3. Effect of deferoxamine on adenine metabolism. Cells (2×10^6) were incubated for 24 hr with a range of deferoxamine concentrations. Cells were washed and incubated with 1 μCi of radioactive adenine for 1 hr. Incorporation of radioactivity into DNA (A), RNA (B), and nucleotides (C) was analyzed.

DNA and RNA by HL-60 cells was also inhibited by deferoxamine (Fig. 3, A and B). However, the effect of deferoxamine on adenine incorporation into DNA was much more pronounced than that into RNA (Fig. 3, A and B). Deferoxamine at 0–150 μM concentrations increased some incorporation of adenine to nucleotides, mainly ATP, but at increased concentrations adenine incorporation into nucleotides was inhibited (Fig. 3C). Deferoxamine also inhibited incorporation of hypoxanthine into nucleic acids and nucleotides; however, inhibition into nucleic acids was more pronounced (Fig. 4, A and B).

Effect of deferoxamine on ribonucleotide reduction. To evaluate the effect of deferoxamine on the ribonucleotide reductase activity in intact cells, radioactive adenine incorporation into both adenine ribonucleotides and deoxyribonucleotides was also measured. It was found that ribonucleotide reduction was inhibited markedly (Table 1) in intact cells by deferoxamine.

Effect of deferoxamine on ATP and GTP intracellular concentrations. Deferoxamine only slightly affected intracellular nucleotide concentrations. Increased deferoxamine concentrations up to 300 μM did not change significantly the intracellular levels of ATP and GTP in HL-60 cells (Table 2).

Effect of deferoxamine on purine biosynthesis de novo. Deferoxamine (0–500 μM) had a small inhibitory effect on glycine incorporation into both nucleic

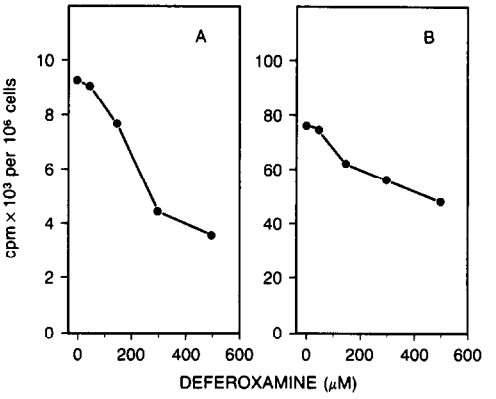

Fig. 4. Effect of deferoxamine on hypoxanthine metabolism. Cells (2×10^6) were incubated for 24 hr with a range of deferoxamine concentrations. Cells were washed and incubated with 1 μCi of radioactive hypoxanthine for 1 hr. Incorporation of radioactivity into nucleic acids (A) and nucleotides (B) was analysed.

acid and free nucleotides (Fig. 5). High concentrations of deferoxamine (1500 μM) caused only 25% of inhibition in HL-60 cells (results not shown). No incorporation of radioactive glycine into nucleosides and bases was detected.

Effect of deferoxamine on the metabolism of purine and pyrimidine nucleosides and bases. The effect of

Table 1. Effect of deferoxamine on ribonucleotide reduction

Substrate	Deferoxamine (μM)	dATP (cpm/10^6 cells)	dATP (% of control)	ATP (cpm/10^6 cells)
Adenine	0	2250	100	47,040
	300	1080	48	59,220
	1500	870	39	42,480

Cells (2×10^6/ml) were incubated with deferoxamine for 24 hr. Cells were washed and incubated with 2 μCi of radioactive adenine for 3 hr. Incorporation of radioactivity into ribonucleotides and deoxyribonucleotides was analyzed.

Table 2. Effect of deferoxamine on intracellular ATP and GTP levels in HL-60 cells

Deferoxamine (μM)	ATP (pmol/10^6 cells)	GTP
0	948	242
50	1109	251
300	1147	275

Cells (10×10^6/5 ml) were incubated with deferoxamine for 24 hr. Nucleotides were extracted and quantitated on HPLC.

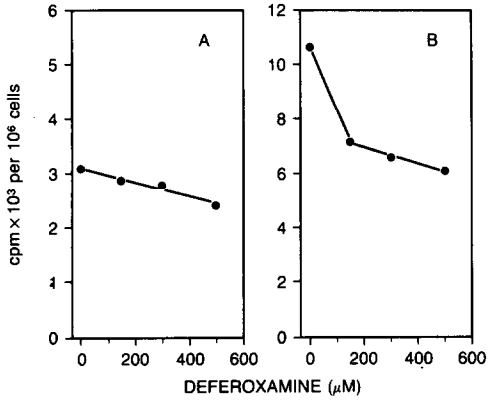

Fig. 5. Effect of deferoxamine on purine biosynthesis *de novo*. Cells (2×10^6) were incubated for 24 hr with a range of deferoxamine concentrations. Cells were washed and incubated with 1 μCi of radioactive glycine for 3 hr. Incorporation of radioactivity into nucleic acids (A) and nucleotides (B) was analysed.

deferoxamine on purine and pyrimidine nucleoside and base incorporation into nucleotides and nucleic acids was also studied. Incorporation of thymidine and deoxyguanosine into nucleic acids was inhibited by 50% or more, and incorporation of adenine, guanosine, and deoxyadenosine was inhibited between 10 and 50% (Table 3). On the contrary,

Table 3. Effect on deferoxamine on the metabolism of purine and pyrimidine nucleoside and bases

Substrate	Nucleic acids (% of control)	Nucleotides
Adenine	71.1	124.7
Guanosine	62.6	75.5
Thymidine	40.8	122.2
Cytidine	165.0	129.0
Deoxyadenosine	76.4	108.8
Deoxyguanosine	50.8	99.9

Cells (2×10^6/ml) were incubated for 24 hr with 300 μM deferoxamine. Cells were then washed and incubated with radioactive substrates for 3 hr. Radioactivity incorporation into nucleic acids and nucleotides was measured. Radioactivity incorporated into nucleic acids and nucleotides in cells incubated as a control in the absence of deferoxamine was, respectively, in cpm/10^6 cells: for adenine 17,470 and 34,210; for guanosine 3,420 and 2,850; for thymidine 24,980 and 2,640; for cytidine 2,790 and 2,200; for deoxyadenosine 22,080 and 24,510; for deoxyguanosine 54,330 and 85,250.

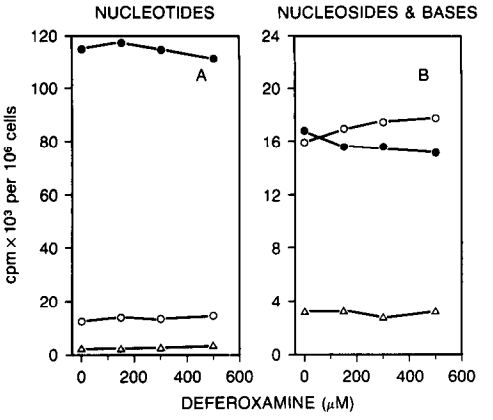

Fig. 6. Effect of deferoxamine on ATP catabolism. Cells (2×10^6) were incubated for 1 hr with 2 μCi of radioactive adenine. Unincorporated adenine was washed out, and cells were incubated for 30 min in a range of deferoxamine concentrations. Radioactivity in nucleotides [ATP (●), ADP (○) and AMP (△)] and in nucleosides and bases [inosine (●), adenosine (△) and hypoxanthine (○)] was measured.

cytidine incorporation into nucleic acids was increased. On the other hand, deferoxamine slightly increased incorporation of adenine, cytidine, thymidine and deoxyadenosine into the free nucleotide pool.

Effect of deferoxamine on ATP catabolism. Deferoxamine at different concentrations had only a small effect on ATP catabolism. The small decrease in ATP level observed was associated with a minor increase of hypoxanthine excretion (Fig. 6), and no significant changes in inosine or adenosine excretion were found.

Effect of deferoxamine on protein synthesis. Incorporation of a radioactive valine, threonine and leucine mixture by HL-60 cells into protein was inhibited only slightly by increased concentrations of deferoxamine (Fig. 7).

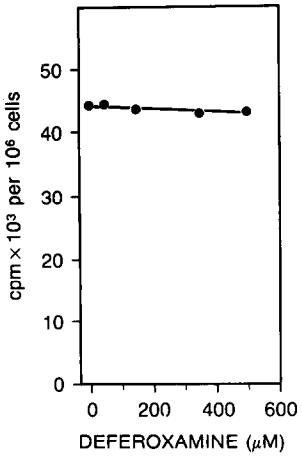

Fig. 7. Effect of deferoxamine on protein synthesis. Cells (2×10^6) were incubated for 24 hr in a range of deferoxamine concentrations. Cells were washed and incubated with 2 μCi of a radioactive mixture of valine, treonine and leucine for 3 hr. Incorporation of radioactivity into the acid-insoluble pellet was measured.

DISCUSSION

The findings that deferoxamine in low-micromolar concentrations can markedly inhibit cell proliferation, and that the drug has minimal toxicity, make deferoxamine a very attractive subject for study as a possible immune regulator and potential antileukemic agent. Although deferoxamine has been known for years as the drug used to treat iron-overload patients [1, 2], its antiproliferative action is not well defined yet. It has been reported that deferoxamine inhibits synthesis of nucleic acids [4], and RNA synthesis inhibition was probably secondary to inhibition of DNA formation. Indeed, the intracellular pool of deoxyribonucleotides decreased markedly after deferoxamine treatment, suggesting that deferoxamine likely affected the ribonucleotide reductase enzyme which contains iron [3]. To explain the mechanism of the antiproliferative action of deferoxamine, we studied in detail the effect of deferoxamine on particular pathways of nucleotide metabolism and nucleic acid synthesis.

We have found that the iron-containing enzyme ribonucleotide reductase is likely the most important target for the antiproliferative action of deferoxamine. But our results also showed that the alteration of cellular metabolism by deferoxamine is more complex than can be explained by inhibition of ribonucleotide reductase activity alone (Table 1). Deferoxamine markedly inhibited the incorporation of deoxyribonucleosides and ribonucleosides into nucleic acids (Table 3, Figs. 1–4). On the other hand, deferoxamine has shown only little or no inhibitory effect on such pathways as purine biosynthesis *de novo* (Fig. 5), salvage of nucleosides and bases to nucleotides (Table 3; Figs. 1–4) ATP catabolism (Fig. 6) or intracellular concentrations of ribonucleotides (Table 2).

Results presented in this paper indicate that the mechanism of deferoxamine action includes the inhibition of ribonucleotide reductase activity and, as a consequence, depletion of deoxyribonucleotides for DNA synthesis. Although this effect of deferoxamine on DNA synthesis is understandable because ribonucleotide reductase contains ferrous atoms, the inhibition of RNA synthesis, as well as inhibition of ribonucleoside and deoxyribonucleoside incorporation, into nucleic acids and its effect on salvage pathways remain unexplained.

REFERENCES

1. B. Modell, *Prog. Hemat.* **11**, 267 (1979).
2. B. Modell, E. A. Letsky, D. M. Flynn, R. Peto and D. J. Weatherall, *Br. med. J.* **284**, 1081 (1982).
3. E. Robbins, J. Fant and W. Norton, *Proc. natn. Acad. Sci. U.S.A.* **69**, 3708 (1972).
4. E. Robbins and T. Pederson, *Proc. natn. Acad. Sci. U.S.A.* **66**, 1244 (1970).
5. A. V. Hoffbrand, K. Ganeshaguru, J. W. L. Hooton and M. H. N. Tatersell, *Br. J. Haemat.* **33**, 517 (1976).
6. K. Ganeshaguru, A. V. Hoffbrand, R. W. Grady and A. Cerami, *Biochem. Pharmac.* **29**, 1275 (1980).
7. J. L. Lipsky and P. S. Leitman, *J. Immun.* **2**, 179 (1980).
8. H. M. Lederman, A. Cohen, J. W. W. Lee, M. H. Freedmen and E. W. Gelfand, *Blood* **64**, 748 (1984).
9. H. L. Elford, M. Freese, E. Passamani and H. P. Morris, *J. biol. Chem.* **245**, 5228 (1970).
10. J. X. Khym, *Clin. Chem.* **21**, 1245 (1975).
11. J. F. Henderson, J. H. Fraser and E. E. McCoy, *Clin. Biochem.* **7**, 339 (1974).
12. D. Hunting, J. Horden and J. F. Henderson, *Can J. Biochem.* **59**, 838 (1981).
13. J. Barankiewicz and A. Cohen, *J. biol. Chem.* **259**, 15178 (1984).