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EFFECT OF VARIOUS IRON CHELATING AGENTS ON DNA SYNTHESIS IN HUMAN CELLS

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Abstract—A number of hydroxamic acid derivatives, including salicyl-, octano-, decano- and dodecanohydroxamic acid and rhodotorulic acid, inhibited ^{3}H-thymidine incorporation into DNA, lowered the concentration of dATP and raised the concentration of dTTP in human PHA-stimulated lymphocytes. These effects suggest that these compounds, like desferrioxamine, inhibit ribonucleotide reductase, an iron-requiring enzyme. On the other hand, benzoic acid derivatives did not inhibit ribonucleotide reductase assessed by the dATP and dTTP pool changes, although some did inhibit DNA synthesis judged by ^{3}H-thymidine incorporation into DNA. A number of other compounds known to chelate iron also inhibited DNA synthesis without inhibiting ribonucleotide reductase. These results suggest that different iron binding compounds affect different iron pools in the cell and that some of them may have use as cytotoxic agents.

Desferrioxamine is the only effective and safe drug for treating iron overload in patients receiving regular blood transfusions for chronic refractory anaemia. Desferrioxamine, a hydroxamic acid derivative, inhibits DNA synthesis in HeLa cells [1] and it has been found to enhance the action of ribonucleoside diphosphate reductase inhibitors such as hydroxyurea in H-Ep-2 cells [2]. More recently, Hoffbrand *et al.* [3] showed that desferrioxamine in high concentrations reduces the activity of the enzyme ribonucleoside diphosphate reductase in human cells *in vitro* and causes a characteristic disturbance in concentration of the immediate DNA precursors, the deoxyribonucleoside triphosphates. Indeed, the enzyme is known to require non-haem iron as a cofactor [4,5]. A search for effective oral iron chelating agents has been initiated to obviate the need for parenteral therapy with desferrioxamine [6–11]. Most of the compounds screened are derivatives of either hydroxamic acid or benzoic acid. In the present study, 36 compounds which have previously been screened *in vitro* and *in vivo* as potential iron chelating drugs [6–11] have been tested for their effect on human ribonucleotide reductase by studying their effect on incorporation of labelled thymidine into DNA and on the concentrations of the deoxyribonucleoside triphosphates in normal human 72 hr PHA-stimulated lymphocytes. In addition, the cytotoxic action of two of the compounds which appeared to be effective inhibitors of ribonucleotide reductase on a lymphocyte cell line in culture was compared with those of desferrioxamine and hydroxyurea.

Reagent chemicals were supplied by British Drug House and Sigma Chemicals. *Micrococcus luteus* DNA polymerase and poly d(A-T) were purchased from Miles Laboratories; ^{3}H-dATP (14 Ci/mmole), ^{3}H-TTP (23.7 Ci/mmole) and methyl ^{3}H-thymidine (5 Ci/mmole) from the Radiochemical Centre, Amersham; desferrioxamine (Desferal) from Ciba Laboratories and hydroxyurea from Squibbs; Medium TC199 and phytohaemagglutinin were purchased from Burroughs Wellcome and medium RPMI and heat inactivated foetal calf serum from Flow Laboratories. Ficoll and Triosil were obtained from Pharmacia Fine Chemicals and Nyegaard & Co., Norway, respectively.

The method used for collecting human lymphocytes and conditions for lymphocyte culture have been described [12]. The centrifugation of blood on a Ficoll–Triosil gradient was used to separate lymphocytes from granulocytes [13]. Lymphocytes (3×10^6) were cultured in 3.0 ml volumes consisting of 20% autologous serum in TC199 medium and 100 μl phytohaemagglutinin.

To study the effect of potential iron chelators on ^{3}H-thymidine incorporation into DNA and on the deoxyribonucleoside triphosphate concentrations, 72 hr lymphocyte cultures from normal volunteers were incubated with the drug, previously made up in normal saline, for 4 hr at 37°. Control cultures received an equivalent volume (100 μl) of saline. Where ^{3}H-thymidine incorporation was studied, 1 μCi ^{3}H-thymidine (^{3}H-TdR) in 100 μl saline was added to cultures after 3 hr incubation with the drug and incubated for a further hour. Control cultures took up the label exponentially into DNA over the one hour period. At the end of this incubation the cells were washed twice in cold phosphate buffered saline, pH 7.4, once in 2.0 ml cold perchloric acid (0.5M) and allowed to stand in ice for 10 min. After centrifugation, the pellet was hydrolysed in 0.5 ml of 0.5M perchloric acid at 85° for 20 min. A 100 μl quantity of the centrifuged supernatant fraction was added to scintillation vials and the radioactivity counted.

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HPB-ALL, a continuous T-cell line, was cultured in medium RPMI with 10% fetal calf serum. The supernatant (old medium) was removed twice a week and the cells were recultured in fresh RPMI medium containing approximately 1.5×10^6 cells per ml. Addition of potential iron chelators was carried out at this stage, and the appropriate flask removed at time intervals for cells number studies.

For measurement of deoxyribonucleoside triphosphate (dNTP) concentrations, the cells were centrifuged at 4°, washed twice in cold phosphate buffered saline, pH 7.4, 1.0 ml of cold 60% methanol was added to the cell pellet and the suspension was stored at −20° overnight to extract the nucleotides [14]. After centrifugation, the supernatant was separated, and freeze dried. The dried extract was then diluted in a known volume of water and stored at −20° until assayed.

The assays for the measurement of dATP and dTTP concentrations have been described [15,16]. Poly d(A-T) (0.05 units) and *Micrococcus luteus* DNA polymerase (0.3 units) were used in the assay of dATP and dTTP. Assays were incubated at 37° for 35 min.

RESULTS

Table 1 shows the effect of the compounds tested on ^{3}H-TdR incorporation into DNA and on the dATP and dTTP concentrations in 72 hr normal

Table 1. Effect of various iron chelators, (10^{-3}M) for 4 hr on ^{3}H-thymidine incorporation into DNA and deoxyribonucleoside triphosphate concentrations in 72 hr PHA-stimulated normal lymphocytes

Compound	No. of Expt.	^{3}H-Thymidine incorporation (% control)	% Control dATP	% Control dTTP
Control	13	46,246 ± 13,066 (c.p.m./10^6 cells/hr)	4.2 ± 1.5 (pmoles/10^6 cells)	8.7 ± 2.2 (pmoles/10^6 cells)
(a) *Hydroxamic acid derivatives*				
Desferrioxamine	3	35	84	125
Salicylhydroxamic acid	2	2	53	158
Octanohydroxamic acid	2	1	65	167
Dodecanohydroxamic acid	1	6	69	134
Decanohydroxamic acid	1	17	85	140
Hexanohydroxamic acid	2	28	80	139
Nicotinohydroxamic acid	2	43	63	130
o-Aminobenzohydroxamic acid	2	63	68	107
Rhodotorulic acid	2	66	81	120
Histidylhydroxamic acid	2	100	98	116
Glycylhydroxamic acid	2	104	111	106
(b) *Benzoic acid derivatives*				
2,3-Dihydroxybenzoic acid	2	82	91	89
2,4-Dihydroxybenzoic acid	1	84	138	111
3,4-Dihydroxybenzoic acid	1	64	107	100
2,5-Dihydroxybenzoic acid	1	62	95	108
3,4,5-Trihydroxybenzoic acid	1	77	105	108
3,4-Diacetoxybenzoic acid	2	84	66	96
2,3-Dimethoxybenzoic acid	1	92	119	109
3,4-Dimethoxybenzoic acid	1	24	119	91
3-Acetoxy-4-methoxybenzoic acid	2	93	124	104
3-Hydroxy-4-methoxybenzoic acid	1	98	92	121
4-Hydroxy-3-methoxybenzoic acid	1	63	86	105
3,4-Methylenedioxybenzoic acid	1	102	111	101
(c) *Miscellaneous*				
Diethylenetriaminepenta-acetic acid	2	98	95	75
3,4-Dihydroxyphenyl-acetic acid	2	32	95	95
3,4-Dimethoxyphenyl-acetic acid	1	75	95	93
3,4-Dihydroxybenzamide	1	105	67	96
3,4-Dimethoxybenzamide	2	59	96	100
4-Hydroxy-3-methoxy mandelic acid	1	93	76	101
3-Methylsalicylic acid	1	70	96	95
5-Methylsalicylic acid	1	81	105	104
2,3-Dihydroxynaphthalene-6-sulphonic acid	2	86	124	90
4,8-Dihydroxyquinoline-2-carboxylic acid	2	92	113	96
Kojic acid	1	87	89	106
Maltol	1	84	103	95
D-(−)-Penicillamine	1	79	122	108
Tropolone	2	1	6	50

Table 2. Effect of various hydroxamates on cell numbers in a T-lymphocyte cell line in culture

Compound	No. of Expt.	Conc. (M)	Cell numbers (% control) at time interval (hr) 4	24	48	72	LD_{50} for mice (Ref. 8) except hydroxyurea
Control (× 10^6 cells/ml)	3	—	1.42	2.40	2.72	2.54	
Hydroxyurea	1	10^{-4}	100	83	76	83	200 mg/kg*
Desferrioxamine	1	10^{-3}	97	50	46	40	> 800 mg/kg
Hexanohydroxamic acid	2	10^{-3}	94	48	34	21	> 400 mg/kg
Hexanohydroxamic acid	2	10^{-4}	104	61	34	33	
Hexanohydroxamic acid	2	10^{-5}	107	78	86	120	
Hexanohydroxamic acid	1	10^{-6}	97	70	95	123	
Octanohydroxamic acid	1	10^{-3}	91	77	66	47	> 800 mg/kg
Octanohydroxamic acid	1	10^{-4}	91	73	72	60	
Octanohydroxamic acid	1	10^{-5}	93	92	91	87	

* This LD_{50} is for rabbits (McKneally *et al.* 1966. *Surgical Forum* **17**, 258).

PHA-stimulated lymphocytes.

Hydroxamic acid derivatives. The effect of derivatives of hydroxamic acid varied widely. The most dramatic effect on all three parameters measured was by salicylhydroxamic acid and octanohydroxamic acid. Both compounds suppressed ^{3}H-TdR incorporation into DNA by more than 98 per cent, increased dTTP concentration by 51–69 per cent and decreased dATP concentration by 28–52 per cent. Decanohydroxamic acid, dodecanohydroxamic acid, nicotinohydroxamic acid, hexanohydroxamic acid and rhodotorulic acid had similar but less marked effects, decreasing ^{3}H-TdR incorporation into DNA by 27–97 per cent, increasing dTTP concentration by 15–38 per cent and decreasing dATP concentration by 11–45 per cent. Although ^{3}H-TdR incorporation into DNA in PHA-transformed lymphocytes incubated with *o*-aminobenzohydroxamic acid was suppressed by a mean of 59 per cent (two experiments) and the dATP concentration was reduced by a mean of 32 per cent of control values, the dTTP concentration was not markedly increased. Histidylhydroxamic acid and glycylhydroxamic acid did not have any effect on ^{3}H-TdR incorporation into DNA nor any marked effect on the dATP and dTTP concentrations in PHA-transformed lymphocytes.

The effect of an apparently potent inhibitor of ribonucleotide reductase, octanohydroxamic acid, and a less effective inhibitor, hexanohydroxamic acid, at various concentrations and times of incubation on the cell numbers in a T-lymphocyte cell line in culture is shown in Table 2. Hexanohydroxamic acid (10^{-3} and 10^{-4}M) caused a decrease in cell numbers over a 72 hr period to 21 and 33 per cent of control cultures, respectively. Although there was a reduced number of cells in culture at 24 hr with 10^{-5} and 10^{-6} M hexanohydroxamic acid, further incubation resulted in no further fall in number of cells. Octanohydroxamic acid at 10^{-3} and 10^{-4}M caused a decrease with time in cell numbers to 47 per cent (10^{-3}M) and 60 per cent (10^{-4}M) of control cultures, but there was no marked decrease in number of cells at 10^{-5}M.

Benzoic acid derivatives. Most of the benzoic acid derived compounds studied (eight of twelve) did not show any marked effect either on the ^{3}H-TdR incorporation into DNA or on the deoxyribonucleoside triphosphate concentrations in 72 hr normal PHA-stimulated lymphocytes. The other four, i.e. 3,4-dimethoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3,4- and 2,5-dihydroxybenzoic acid all decreased the ^{3}H-TdR incorporation into DNA but did not have any consistent effect on the dATP and dTTP concentrations.

Miscellaneous. Of the compounds in this group, nine of fourteen did not have any marked or consistent effect on either ^{3}H-TdR incorporation into DNA or on the dATP and dTTP concentrations. Of the other five compounds tested, four, i.e. 3,4-dihydroxyphenylacetic acid and 3,4-dimethoxyphenylacetic acid, 3,4-dimethoxybenzamide and 3-methylsalicylic acid decreased ^{3}H-TdR incorporation by 25–68 per cent of control values, although there was no marked effect on the dATP and dTTP concentrations. Tropolone inhibited incorporation of ^{3}H-TdR into DNA and also decreased both the dATP and dTTP concentrations.

DISCUSSION

Thirty-six potential iron chelators have been screened as inhibitors of ribonucleotide reductase by studying their effect on thymidine incorporation into DNA and on the dATP and dTTP concentrations in normal stimulated lymphocytes. We have previously found this method more satisfactory than direct ribonucleotide reductase assay for inhibitor studies [3]. Except for those derived from amino acids, all of the primary monohydroxamic acids examined caused a marked reduction in ^{3}H-TdR incorporation into DNA and a concomitant change in deoxyribonucleoside triphosphates; dATP decreased while dTTP increased. These same compounds caused an alteration in iron metabolism in cultured Chang cells, although their order of effectiveness was somewhat different [10]. Particularly noteworthy is the fact that aliphatic hydroxamic acids were more effective than aromatic derivatives, salicylhydroxamic acid being an exception. While the latter compound had a moderate inhibitory effect upon ^{59}Fe incorporation into Chang cells, it almost completely blocked DNA synthesis in PHA-stimu-

lated lymphocytes. This effect was not related to cytotoxicity, although there is evidence [10] that the aliphatic hydroxamic acids may be somewhat toxic to cells. While none of the primary hydroxamic acids elicited signs of toxicity when given to hypertransfused rats for 5 days at a dose of one-fifth the LD_{50} per day, neither did they induce iron excretion [8]. On the other hand, rhodotorulic acid (RA), a quadradentate secondary hydroxamic acid, proved to be a potent iron-chelating agent in this animal model. In fact, of the 36 compounds evaluated here, rhodotorulic acid was by far the most effective *in vivo* [8]. Clinical studies have confirmed its potential usefulness as an iron-chelating drug [7]. When incubated with PHA-stimulated lymphocytes, RA caused a moderate decrease in ^{3}H-TdR incorporation and in the ratio of dATP to dTTP. Incubation with desferrioxamine, another naturally occurring secondary hydroxamate, had a similar effect upon the parameters studied, the decrease in ^{3}H-TdR incorporation being somewhat enhanced. Thus, although the secondary hydroxamates appear to be the most effective chelators *in vivo*, such is not the case *in vitro* wherein the primary derivatives seem to have a greater effect on iron metabolism and DNA synthesis. This may reflect a different mechanism of action dependent upon the type of hydroxamic acid. Nonetheless, both types had a similar effect upon the growth and division of cultured T lymphocytes, as shown by the number of cells in 72 hr cultures (Table 2).

Only four of the twelve benzoic acid derivatives (4-hydroxy-3-methoxy-, 3,4-dimethoxy-, 3,4- and 2,5-dihydroxybenzoic acid) caused a decrease in ^{3}H-TdR incorporation into DNA and among these there was no consistent effect upon the dATP and dTTP concentrations. Moreover, none of the twelve had any significant effect upon either [^{3}H]L-leucine or ^{59}Fe incorporation into cultured Chang cells [10]. On the other hand, 2,3-dihydroxybenzoic acid (2,3-DHB) was shown to induce a significant amount of urinary iron excretion in hypertransfused rats [6]. Subsequent clinical trials in patients with transfusional iron overload suggested that orally administered 2,3-DHB could retard but not prevent the accumulation of iron [17, 18]. A possible mechanism of action was suggested by White *et al.* [9]. 2,3-DHB, after preincubation with rat liver homogenate, caused a significant reduction in the amount of ^{59}Fe incorporated into the ferritin of Chang cell cultures, indicating that a metabolite may be the active compound. Unfortunately, the effect of rat liver homogenate itself on DNA synthesis in lymphocytes precluded a similar study here. Lastly, 3,4-dimethoxybenzoic acid caused a 76 per cent inhibition of ^{3}H-TdR incorporation into DNA with little or no change in the dATP and dTTP concentrations. The corresponding amide behaved similarly, the inhibition being 41 per cent. The reason for this behavior is not clear, especially since neither compound affected iron metabolism in Chang cell cultures [10] or significantly induced iron excretion *in vivo* [8].

In the miscellaneous group of iron chelators only tropolone had a marked effect on all three parameters of DNA synthesis which were studied. The effect, though, was quite different from that of the other iron chelators, in that tropolone (10^{-3} M) almost completely inhibited ^{3}H-TdR incorporation into DNA, while the concentration of dATP was reduced by 94 per cent and that of dTTP by 50 per cent. Similar results were obtained at a concentration of 10^{-4} M. This together with the pattern of inhibition suggests that the compound is cytotoxic. A similar conclusion was reached by White *et al.* [11] who found that although tropolone was able to remove iron from transferrin *in vitro* and could interfere with iron metabolism in cultured Chang cells, it significantly inhibited protein synthesis at concentrations in excess of 10^{-3} M. None of the other miscellaneous chelators had a marked or consistent effect upon the dATP and dTTP concentrations, although a few did cause a decrease in ^{3}H-TdR incorporation into DNA. In particular, 3,4-dihydroxyphenylacetic acid caused a decrease of nearly 70 per cent. Diethylenetriaminepentaacetic acid, once widely used clinically in iron-chelation therapy, had no effect upon the three parameters. This was somewhat surprising, since it was markedly cytotoxic to cultured Chang cells at a concentration of 6×10^{-3} M [9]. Likewise, maltol and kojic acid had no effect on DNA synthesis. In cultured Chang cell systems, however, iron incorporation into both whole cells and ferritin was reduced significantly, particularly by maltol. Neither compound was an effective chelator *in vivo*, however.

The results presented here are consistent with the concept that different types of iron chelators may act at different sites within the cell. It appears that only hydroxamic acids have an effect upon DNA synthesis, presumably via inhibition of ribonucleotide reductase. In this regard, they, like their congener hydroxyurea, may be of therapeutic use as cytotoxic agents. On the other hand, such a physiological effect might limit their usefulness as potential iron-chelating drugs. Although no evidence of such an effect has been noted in the case of desferrioxamine, a periodic evaluation of rapidly proliferating tissues such as bone marrow seems warranted, particularly in patients whose iron stores have been brought to near normal.

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REFERENCES

1. E. Robbins and T. Peterson, *Proc. natn. Acad. Sci. U.S.A.* **66**, 1244 (1970).
2. R. W. Brockman, *Cancer Chemother. Rep.* Part II, **4**, 115 (1974).
3. A. V. Hoffbrand, K. Ganeshaguru, J. W. L. Hooton and M. H. N. Tattersall, *Br. J. Haemat.* **33**, 517 (1976).
4. N. C. Brown, R. Eliasson, P. Reichard and L. Thelander, *Eur. J. Biochem.* **9**, 512 (1969).
5. E. C. Moore, M. S. Zodeck, K. C. Agrawal and A. C. Sartorelli, *Biochemistry* **9**, 4492 (1970).
6. J. H. Graziano, R. W. Grady and A. Cerami, *J. Pharmac. exp. Ther.* **190**, 570 (1974).
7. R. W. Grady, C. M. Peterson, R. L. Jones, J. H. Graziano, K. K. Bhargava, V. A. Berdoukas, G. Kokkini, D. Loukopoulos and A. Cerami, *J. Pharmac. exp. Ther.* **209**, 342 (1979).

8. R. W. Grady, J. H. Graziano, H. A. Akers and A. Cerami, *J. Pharmac. exp. Ther.* **196**, 478 (1976).
9. G. P. White, R. Bailey-Wood and A. Jacobs, *Clin. Sci. Molec. Med.* **50**, 145 (1976).
10. G. P. White, A. Jacobs, R. W. Grady and A. Cerami, *Br. J. Haemat.* **33**, 487 (1976).
11. G. P. White, A. Jacobs, R. W. Grady and A. Cerami, *Blood* **48**, 923 (1976).
12. K. C. Das and A. V. Hoffbrand, *Br. J. Haemat.* **19**, 203 (1970).
13. A. Boyum, *Scand. J. clin. Lab. Invest.* **21**, (Suppl), 97 (1968).
14. L. Skoog and B. Nordenskjold, *Eur. J. Biochem.* **19**, 81 (1971).
15. U. Lindberg and L. Skoog, *Analyt. Biochem.* **34**, 152 (1970).
16. M. H. N. Tattersall, A. Lavoie, K. Ganeshaguru, E. Tripp and A. V. Hoffbrand, *Eur. J. clin. Invest.* **5**, 191 (1975).
17. C. M. Peterson, J. H. Graziano, R. W. Grady, R. L. Jones, H. V. Vlassara, V. C. Canale, D. R. Miller and A. Cerami, *Br. J. Haemat.* **33**, 477 (1976).
18. C. M. Peterson, J. H. Graziano, R. W. Grady, R. L. Jones, A. Markenson, V. Levi, V. C. Canale, G. E. Gray, A. Cerami and D. R. Miller, *Exp. Hemat.* **7**, 74 (1979).