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New Ribonucleotide Reductase Inhibitors with Antineoplastic Activity[1]

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ABSTRACT

For the purpose of developing an effective anticancer agent with a mode of action directed against ribonucleotide reductase, a number of acyl and aryl hydroxamic acids and their congeners were synthesized and tested for their ability to inhibit ribonucleotide reductase *in vitro* and to prolong the life span of L1210 leukemia-bearing mice. Benzohydroxamic acid and other six-member aromatic ring hydroxamic acids were found to be as inhibitory as was hydroxyurea *in vitro*, and they increased the life span of L1210 leukemia-bearing mice. Addition of hydroxy groups to the benzene ring of benzohydroxamic acid increased both inhibition of ribonucleotide reductase and life span of L1210 leukemic mice. Di- and trihydroxybenzohydroxamic acids, particularly when the hydroxyl groups were adjacent, were even more potent both *in vitro* and *in vivo*. For example, in comparison to hydroxyurea, 2,3,4-trihydroxybenzohydroxamic acid was 160 times more potent as an inhibitor of ribonucleotide reductase and increased the life span of L1210-leukemic mice at a lower dosage. The hydroxamic acid moiety was not essential for activity, since 2,3,4-trihydroxybenzamide was 100 times more potent than was hydroxyurea *in vitro*. Of the compounds tested, 3,4-dihydroxybenzohydroxamic acid was most effective in prolonging the life span of L1210-leukemic mice, increasing survival time over 100% and at one-third the dosage of hydroxyurea.

INTRODUCTION

The reaction that converts ribonucleotides to deoxyribonucleotides is a logical target site for the development of an anticancer agent because of the crucial role that this reaction plays in the regulation of DNA synthesis and cellular replication (6, 15, 22). Hydroxyurea is the only drug in general clinical use for which the primary mode of action is inhibition of this reaction (5, 16, 20). The disadvantage of this drug is that it requires frequent large doses in order to maintain an effective concentration required for activity. Another group of compounds which are strong inhibitors of ribonucleotide reductase *in vitro* and which increase the life span of animals bearing neoplasia, the α-(*N*)-heterocyclic carboxyaldehyde thiosemicarbazone derivatives of isoquinoline and pyridine (1), are presently being tested as possible cancer chemotherapeutic agents. However, preliminary data indicate that these compounds are toxic and are not effective in humans (4, 14).

In order to develop an effective chemotherapeutic agent for which the mode of action is inhibition of ribonucleotide reductase activity, a number of aliphatic and aromatic hydroxamic acids and their corresponding amides and esters were synthesized and evaluated for inhibition of ribonucleotide reductase and for their ability to prolong the life span of L1210-leukemic mice.

Several polyhydroxybenzohydroxamic acids and some of their corresponding amides and esters were found to be more effective inhibitors of ribonucleotide reductase than was hydroxyurea. Additionally, several of these compounds increased the life span of L1210-leukemic mice to a greater extent and at a lower dosage than did hydroxyurea on a similar regimen.

MATERIALS AND METHODS

Chemicals. The synthesis and properties of the substituted benzohydroxamic acid compounds and their congeners which are discussed in this report are the subject of another report.[3] Methyl 2,4-dihydroxybenzoate, methyl 3,5-dihydroxybenzoate, 3,4,5-trihydroxybenzoic acid (gallic acid), and pyrogallol were all purchased from Aldrich, Milwaukee, Wis. Hydroxyurea was obtained from Squibb, New Brunswick, N. J.; [^{3}H]CDP was acquired from Amersham/Searle Corporation, Arlington Heights, Ill.

Antineoplastic Activity. The assessment of antineoplastic activity was conducted on 20-g L1210 leukemia-bearing female C57BL/6 × DBA/2 F_1 (hereafter called B6D2F_1) mice. L1210 leukemia was maintained by weekly passage of 10^5 L1210 cells i.p. into DBA/2 mice. Diluted ascitic fluid, 0.1 ml (10^5 cells), was administered i.p. to female B6D2F_1 mice. The comparisons between drug-treated and control mice were conducted on groups of 8 mice. Drugs were administered i.p. beginning 24 hr after tumor transplantation and were continued once daily for 8 days. Compounds were generally tested at 3 dose levels in the range of 100 to 1000 mg/kg, each dose in 8 mice. Other dose levels were given dependent upon toxicity and biological activity. Compounds showing activity were retested at a more narrow dose range centered around the optimum dose obtained from the initial test.

Ribonucleotide Reductase Inhibition. Ribonucleotide reductase was partially purified from Novikoff hepatoma by a procedure similar to that previously outlined (8). The 0 to 40% ammonium sulfate fraction was used in these experi-

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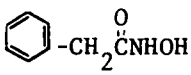
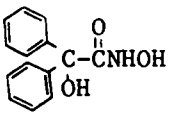
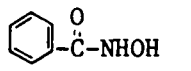
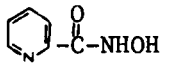
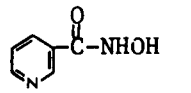
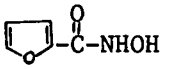
[3] B. van't Riet, G. L. Wampler, and H. L. Elford. Synthesis of hydroxy and amino substituted benzohydroxamic acids: inhibition of ribonucleotide reductase and antitumor activity. *Journal of Medicinal Chemistry*, 1979, in press.

Table 1

Ribonucleotide reductase inhibition and antitumor activity of some hydroxamic acids

Assays for ribonucleotide reductase inhibition and assessment of antineoplastic activity are described in "Materials and Methods."

Compound	Structure	*In vitro* ID_{50} (μM)	*In vivo* T/C %[a]	*In vivo* Dose[b] mg/kg	*In vivo* Dose[b] mmol/kg
Hydroxyurea	$H_2NCNHOH$ (O)	500	162 ± 10[c]	2500	33
Acetohydroxamic acid	CH_3CNHOH (O)	1000	155 ± 9	500	6.7
Malonohydroxamic acid	$CH_2(CNHOH)_2$ (O)	Inactive[d]	Inactive[e]	250[f]	1.9[f]
Phenylacetohydroxamic acid		1000	128 ± 3	500	3.2
Benzilohydroxamic acid		500	Toxic	5	0.02
Benzohydroxamic acid		400	144 ± 16	500	3.6
Picolinohydroxamic acid		500	Inactive[e]	15[f]	0.11[f]
Nicotinohydroxamic acid		800	132 ± 9	500	3.6
Furanohydroxamic acid		1000	Toxic	25	0.20

[a] Percentage of life span of 8 drug-treated, L1210-bearing mice compared to 8 control mice.
[b] Drug dose given i.p. for 8 days which gave the maximum life span of L1210-bearing mice.
[c] Average ± S.E.
[d] Less than 20% inhibition at 1000 μM.
[e] Less than 25% increase in life span compared to controls.
[f] The maximum dose tested which did not depress the life span compared to controls.

ments. Activity of the enzyme was assayed by monitoring the reductive conversion of CDP to dCDP by the use of a slightly modified assay procedure (8) originally developed by Reichard *et al.* (17). The assay mixture (0.34 ml) contained 3 μCi [^{3}H]CDP (specific activity, 14 or 19 Ci/mmol), 3.3 mM ATP, 5.9 mM $MgCl_2$, 8.8 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.5), 15 mM dithiothreitol, and enzyme protein between 0.4 and 1.3 mg. Incubation was at 30° for 40 min.

The inhibitors were dissolved in water or in water-ethanol or water-dimethyl sulfoxide. The maximum concentrations of ethanol and dimethyl sulfoxide in the assay mixture were 1 and 2%, respectively. At these concentrations, neither compound was inhibitory. Each inhibitor was tested at a minimum of 3 concentrations. The active compounds were reassayed at least one additional time. The ID_{50}[4] (μM) was estimated from graphs summarizing the effects of each compound.

RESULTS

The effects of several aliphatic and aromatic hydroxamic acids on ribonucleotide reductase *in vitro* and on the survival of L1210 mice are presented in Table 1. The substitution of an acyl group for an amino group of hydroxyurea, an active anticancer compound, resulted in a range of responses. The replacement of the amino group with a

[4] The abbreviation used is: ID_{50}, μM concentration of drug required to inhibit by 50% the observed activity of the partially purified ribonucleotide reductase of Novikoff rat liver tumor.

methyl group decreased *in vitro* activity. On the other hand, the substitution of an aromatic 6-member ring on the basic hydroxamic acid structure resulted in ID_{50}'s similar to those of hydroxyurea and were as effective in prolonging the life span of L1210 mice at similar or lower dosages as was hydroxyurea. The 5-member ring compound, furanohydroxamic acid, was not very inhibitory *in vitro* and was toxic *in vivo*. The separation of the phenyl ring from the hydroxamic acid moiety by a methylene group caused a significant decrease in both *in vitro* and *in vivo* activity. The inclusion of more than one phenyl group on the methyl substitution enhanced *in vitro* activity to a level comparable to that of hydroxyurea; however, the compound was toxic *in vivo*.

In order to increase activity, various monosubstituted benzohydroxamic acids were synthesized and tested for activity. The results are reported in Table 2. The introduction of a hydroxyl group increased inhibition of ribonucleotide reductase, and the hydroxy-substituted compounds were fairly active against L1210 leukemia. The *o*- and *p*-substituted hydroxybenzohydroxamic acids were more active *in vitro* than the *m* form, but 3-hydroxybenzohydroxamic acid was more potent in antitumor activity. Again, dosage to achieve maximal increase in life span was lower than with hydroxyurea. The introduction of a nitro functional group on the benzene ring instead of a hydroxyl group resulted in a decrease in inhibition of enzyme and a loss of antitumor activity because of increased toxicity of the compound. The substitution of a methyl group for the hydrogen atom of the hydroxyl group resulted in a significant loss of both enzyme inhibition and antineoplastic activity.

The addition of a second hydroxyl group increased inhibition of ribonucleotide reductase over corresponding monosubstituted compounds (Table 2). When the hydroxyl groups were not adjacent, the inhibition was approximately double that of the monohydroxylated compound. However, a much greater increase of about 1 log unit was achieved when the hydroxy groups were adjacent. The antileukemia activity of the adjacent hydroxy compounds was also increased as indicated by the smaller dosages of the adjacent hydroxy compounds that were required to achieve maximum prolongation of life span of L1210-leukemic mice. *In vitro* activity was enhanced even further by the introduction of a third adjacent hydroxy group.

As seen with the monohydroxy-substituted compounds, replacement of hydroxy groups in the trihydroxy-substituted compound with methoxy groups resulted in a decrease of *in vitro* activity and a loss of *in vivo* activity.

Further studies were conducted with hydroxybenzene compounds lacking the hydroxamic group in order to determine the contribution of the hydroxylated benzene ring to overall inhibition by the polyhydroxylated benzohydroxamic acids. The hydroxamic acid moiety was replaced by either a hydrogen, carboxylic acid, ester, or amide group. One significant result of this study was the discovery that certain hydroxylated benzene derivatives without a hydroxamic acid moiety nevertheless strongly inhibited ribonucleotide reductase (Table 3). Furthermore, in order for strong inhibition to occur *in vitro*, 2 or more adjacent hydroxyl groups were required on the aromatic ring. Within a series of identically hydroxylated benzene rings, the amide derivative was the most active inhibitor followed by the ester derivative, the lowest amount of inhibition being effected by the carboxylic acid or unsubstituted polyhydroxybenzene compounds. In comparison with their hydroxamic acid analogs, the free and substituted amides of 3,4-dihydroxy- and 2,3,4- and 3,4,5-trihydroxybenzene compounds were similar in potency of inhibition of ribonucleotide reductase *in vitro*.

The trihydroxy amides, as well as the substituted amide, *N*-methyl-3,4,5-trihydroxybenzamide, contain antitumor therapeutic activity comparable to their trihydroxybenzohydroxamic acid counterparts. However, 3,4-dihydroxybenzamide, although containing appreciable *in vitro* activity, does not prolong the life span of the L1210-leukemic mice as well as does 3,4-dihydroxybenzohydroxamic acid. Also, the methyl esters of the adjacent hydroxybenzoates were effective reductase inhibitors with ID_{50}'s of 100 μM or less but were inactive antineoplastic agents, except for methyl 3,4,5-trihydroxybenzoate. Possibly, the dihydroxy esters are hydrolyzed to free acids by esterases, and the acids are less active *in vivo*.

The relative *in vitro* activities of selected compounds from Tables 1, 2, and 3 compared to hydroxyurea, shown in Table 4, indicate clearly that there is generally an increase in *in vitro* activity correlated with an increase in the number of hydroxyl groups on the benzene ring. There was an increase in inhibitory activity of about 2- to 4-fold after the introduction of a single hydroxyl group; further hydroxylation provided a dramatic increase in activity of about 10-fold when the new hydroxyl group was introduced in an adjacent position, but only a very modest increase of 2-fold or less when the position was nonadjacent. A third adjacent hydroxyl group increased inhibitory activity still further as in the case of 2,3,4-trihydroxybenzohydroxamic acid, which was more than 160-fold more potent than hydroxyurea. The hydroxamic acid structure is not essential for activity since the 2,3,4-trihydroxybenzamide was 100-fold more active than hydroxyurea.

Previous work has shown that increasing the concentration of dithiosulfhydryl compounds in the assay mixture or the addition of Fe^{2+} ions partially decreases the inhibition of ribonucleotide reductase by hydroxyurea (16). As seen in Table 5, a decrease in inhibition by benzohydroxamic acid and 2,3,4-trihydroxybenzohydroxamic acid also occurred when the dithiothreitol concentration was increased or when Fe^{2+} in the form of ferrous ammonium sulfate was added to the incubation mixture. Increasing the concentration of Fe^{2+} or dithiothreitol above the stated values did not further increase activity.

DISCUSSION

This study was undertaken for the purpose of developing a class of cancer chemotherapeutic agents for which the mode of action is inhibition of ribonucleotide reductase. Because the pool size of deoxynucleotides is not large enough to support DNA synthesis for more than a brief period (19, 24), deoxynucleotides must be formed immediately prior to their incorporation into DNA. In addition, ribonucleotide reductase activity has been shown to correlate positively with the rate of cellular proliferation (8, 22).

Table 2

Ribonucleotide reductase inhibition and antitumor activity of some substituted benzohydroxamic acids

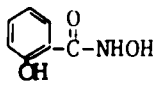
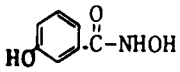
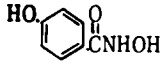
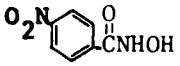
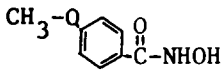
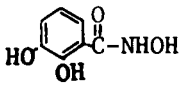
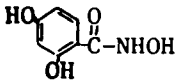
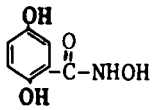
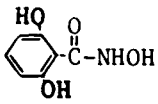
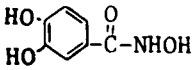
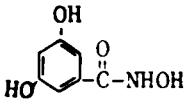
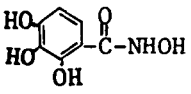
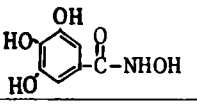
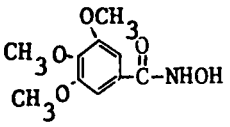
Compound	Structure	*In vitro* ID_{50} (μM)	*In vivo* T/C %[a]	*In vivo* Dose[b] mg/kg	*In vivo* Dose[b] mmol/kg
Salicylhydroxamic acid		150	147 ± 7[c]	500	3.3
3-Hydroxybenzohydroxamic acid		350	177 ± 12	500	3.3
4-Hydroxybenzohydroxamic acid		250	124 ± 10	400	2.6
4-Nitrobenzohydroxamic acid		500	Inactive[d]	10[e]	0.055[e]
4-Methoxybenzohydroxamic acid		500	Inactive	250	1.5
2,3-Dihydroxybenzohydroxamic acid		8	136 ± 11	200	1.2
2,4-Dihydroxybenzohydroxamic acid		250	142 ± 7	500	3.0
2,5-Dihydroxybenzohydroxamic acid		200	130 ± 14	300	1.8
2,6-Dihydroxybenzohydroxamic acid		100			
3,4-Dihydroxybenzohydroxamic acid		30	203 ± 15	600	3.6
3,5-Dihydroxybenzohydroxamic acid		400	152 ± 22	1000	5.9
2,3,4-Trihydroxybenzohydroxamic acid		3.5	130 ± 7	125	0.62
3,4,5-Trihydroxybenzohydroxamic acid		10	153 ± 19	400	2.2

Table 2 continued

Compound	Structure	*In vitro* ID_{50} (μM)	*In vivo* T/C %[a]	*In vivo* Dose[b] mg/kg	*In vivo* Dose[b] mmol/kg
3,4,5-Trimethoxybenzohydroxamic acid		80	Inactive[d]	500[e]	2.2[e]

[a] Percentage of life span of 8 drug-treated, L1210-bearing mice compared to 8 control mice.
[b] Drug dose given i.p. for 8 days which gave the maximum life span of L1210-bearing mice.
[c] Average ± S.E.
[d] Inactive, less than 25% increase in life span compared to controls.
[e] The maximum dose tested which did not depress the life span compared to controls.

At the present time, hydroxyurea is the only drug in general clinical use for which the primary biochemical focus of action is inhibition of ribonucleotide reductase (5, 21). However, it requires frequent large doses in order to be effective; therefore, it was hoped that by modifying the structure a more effective chemotherapeutic agent could be developed.

Structural activity relationship studies by Young *et al.* (25) on the effects of hydroxyurea and its congeners on DNA synthesis in HeLa cells showed that, to obtain inhibition of DNA synthesis without affecting RNA or protein synthesis, the —NOH group is required, the carbonyl is not, the proton on the hydroxyl group must be free, and inhibitory activity is increased by substituents which make the hydroxy pK_a resemble that of an alcohol.

Hydroxyurea is a relatively weak inhibitor of ribonucleotide reductase *in vitro* (5, 16), requiring 500 μM to inhibit 50%. Data presented in this paper indicate that, in comparison to hydroxyurea, several newly synthesized compounds, 3,4-dihydroxy-, 2,3,4-trihydroxy-, and 3,4,5-trihydroxybenzohydroxamic acids, *N*-methyl-3,4,5-trihydroxybenzamide, and phenyl-3,4,5-trihydroxybenzoate are more potent inhibitors of ribonucleotide reductase and increased the life span of L1210 leukemia-bearing mice at lower dosages. Of the compounds that we tested, 2,3,4-trihydroxybenzohydroxamic acid was the strongest ribonucleotide reductase inhibitor, 160 times more potent than hydroxyurea; and 3,4-dihydroxybenzohydroxamic acid was the most active antitumor compound, increasing survival time of L1210 mice more than 100% at one-third the dosage of hydroxyurea.

There was a moderate degree of correspondence between enzyme inhibition and antineoplastic activity. All of the hydroxybenzohydroxamic compounds were more effective ribonucleotide reductase inhibitors than was hydroxyurea, and a number of them exhibited better chemotherapeutic activity, either by increasing the life span of L1210-bearing mice more than did hydroxyurea or by requiring a smaller amount of drug to achieve a life span comparable to that obtained with hydroxyurea. The best enzyme inhibitor, 2,3,4-trihydroxybenzohydroxamic acid, required only $^1/_{20}$ of the dose of hydroxyurea to achieve antitumor activity.

However, the relationship between ribonucleotide reductase inhibition and life span increase of L1210 mice is more of an indication than an exact correlation. For example, 3-hydroxybenzohydroxamic acid is a better anticancer agent than is the 4-hydroxy compound even though the latter was a slightly better enzyme inhibitor. We did not find any compound that was active *in vivo* that was inactive *in vitro*.

The toxicity of several of the aryl hydroxamic acids limited the dosage so that the expected optimal level could not be given for comparative purposes. Such was the case for benzilohydroxamic acid, picolinohydroxamic acid, furanohydroxamic acid (Table 1), and 4-nitrobenzohydroxamic acid (Table 2).

Another factor which may distort the correlation between enzyme inhibition and antitumor activity is the pharmacological properties of the drugs. Some structures are probably more susceptible to enzymatic breakdown, while others may possess better distribution characteristics. Therefore, more information about some of the pharmacological properties of these drugs is required in order to understand more fully the *in vitro* and *in vivo* relationship.

It must also be taken into consideration that the conditions used in this study for the purposes of comparison of antineoplastic activity were not optimum for this class of inhibitors. Hydroxyurea and other S-phase-specific antitumor agents have been shown to be most effective on an intermittent schedule (10, 18, 23). Therefore, the antineoplastic activity of the compounds tested in this study can be expected to be improved under optimized conditions.

Some structural features necessary for activity of this group of compounds have been revealed by this study. When the amino group of hydroxyurea was replaced by an acyl group, activity was reduced but still present, indicating that the amino group of hydroxyurea is not essential for activity. This is in agreement with the structural studies of Young *et al.* (25) discussed previously. When the amino group was replaced with a benzyl group, the activity both *in vitro* and *in vivo* of the resulting benzohydroxamic acid was comparable to that of hydroxyurea. This presented the opportunity of an attachment site for additional substituents. Activity was increased by addition of hydroxyl groups on the phenyl ring, especially when the hydroxyl groups were adjacent.

Table 3

Ribonucleotide reductase inhibition and antitumor activity of some benzoic acid derivatives

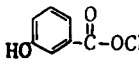
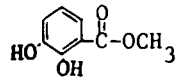
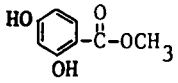
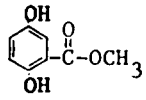
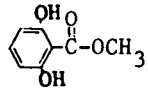
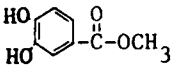
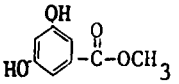
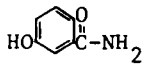
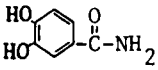
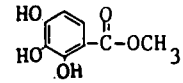
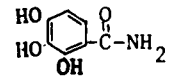
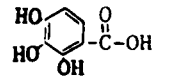
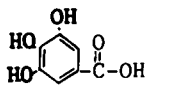
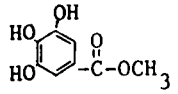
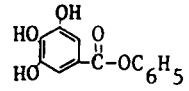
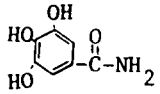
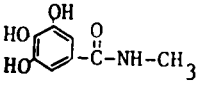
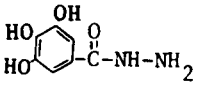
Compound	Structure	*In vitro* ID_{50} (μM)	*In vivo* T/C (%)[a]	*In vivo* Dose[b] mg/kg	*In vivo* Dose[b] mmol/kg
Methyl 3-hydroxybenzoate		1000	Inactive[c]	500[d]	3.3
Methyl 2,3-dihydroxybenzoate		100	Inactive[c]	500[d]	3.0
Methyl 2,4-dihydroxybenzoate		>1000	Inactive[c]	1000	6.0
Methyl 2,5-dihydroxybenzoate		800			
Methyl 2,6-dihydroxybenzoate		200			
Methyl 3,4-dihydroxybenzoate		70	Inactive[c]	500[d]	3.0
Methyl 3,5-dihydroxybenzoate		>1000	Inactive[c]	1000	6.0
3-Hydroxybenzamide		Inactive[e]	Inactive[c]	1000	7.3
3,4-Dihydroxybenzamide		50	135 ± 11	1200	7.8
Methyl 2,3,4-trihydroxybenzoate		8	Inactive[c]	800	4.4
2,3,4-Trihydroxybenzamide		5	148 ± 7[f]	200	1.2
2,3,4-Trihydroxybenzoic acid		100	Inactive[c]	500[d]	2.9
3,4,5-Trihydroxybenzoic acid		100	139 ± 9	1500	7.8
Methyl 3,4,5-trihydroxybenzoate		30	145 ± 4	250	1.4

Table 3 continued

Compound	Structure	*In vitro* ID_{50} (μM)	*In vivo* T/C (%)[a]	*In vivo* Dose[b] mg/kg	*In vivo* Dose[b] mmol/kg
Phenyl 3,4,5-trihydroxybenzoate		15			
3,4,5-Trihydroxybenzamide		10	142 ± 5	250	1.5
N-Methyl 3,4,5-trihydroxybenzamide		25			
3,4,5-Trihydroxybenzhydrazide		15	Toxic	500	2.3
Pyrogallol		60	Toxic	100	0.8

[a] Percentage of life span of 8 drug-treated, L1210-bearing mice compared to 8 control mice.
[b] Drug dose given i.p. for 8 days which gave the maximum life span of L1210-bearing mice.
[c] Less than 25% increase in life span compared to controls.
[d] Toxic at 1000 mg/kg.
[e] Less than 20 inhibition at 1000 μM.
[f] Average ± S.E.

Table 4

Relative inhibition of ribonucleotide reductase by hydroxyurea and its analogs

	Inhibitory potency of ribonucleotide reductase[a] (*in vitro*)
Hydroxyurea	1.0
Acetohydroxamic acid	0.5
Benzohydroxamic acid	1.2
Salicylhydroxamic acid	3.3
p-Nitrobenzohydroxamic acid	0.6
3,5-Dihydroxybenzohydroxamic acid	1.2
2,4-Dihydroxybenzohydroxamic acid	2
2,5-Dihydroxybenzohydroxamic acid	2.5
2,6-Dihydroxybenzohydroxamic acid	5
2,3-Dihydroxybenzohydroxamic acid	63
3,4-Dihydroxybenzohydroxamic acid	20
3-Hydroxybenzamide	0
3,4-Dihydroxybenzamide	25
3,4,5-Trihydroxybenzoic acid	5
3,4,5-Trihydroxybenzohydroxamic acid	50
3,4,5-Trihydroxybenzamide	50
2,3,4-Trihydroxybenzoic acid	25
2,3,4-Trihydroxybenzohydroxamic acid	167
2,3,4-Trihydroxybenzamide	100

[a] Inhibition of ribonucleotide reductase relative to hydroxyurea. The ID_{50} of hydroxyurea is 500 μM.

Table 5

Effect of iron and dithiothreitol on hydroxamic acid inhibition of ribonucleotide reductase

The assay mix is described in "Materials and Methods." The concentrations of additions were: benzohydroxamic acid, 143 μM; 2,3,4-trihydroxybenzohydroxamic acid, 25 μM; and $Fe(NH_4)_2(SO_4)_2$, 5 μM. Dithiothreitol (7.5 mM) was added to the assay mix which already contained 15 mM dithiothreitol.

Inhibitor	Addition	% of inhibition
None	None	
Benzohydroxamic acid	None	69
Benzohydroxamic acid	Dithiothreitol	35
Benzohydroxamic acid	$Fe(NH_4)_2(SO_4)_2$	33
2,3,4-Trihydroxybenzohydroxamic acid	None	87
2,3,4-Trihydroxybenzohydroxamic acid	Dithiothreitol	66
2,3,4-Trihydroxybenzohydroxamic acid	$Fe(NH_4)_2(SO_4)_2$	67

A major piece of information about the structural requirements for activity that resulted from this study is that the hydroxamic acid moiety is not necessary for activity. The di- and trihydroxybenzamides in which the hydroxyl groups were adjacent were active ribonucleotide reductase inhibitors and antineoplastic agents. This information, coupled with the fact that pyrogallol (1,2,3-trihydroxybenzene) is inhibitory to reductase at 60 μM, suggests that there is an interaction between ribonucleotide reductase and the adjacent hydroxyl groups similar to its interaction with hydroxamic acid.

The polyhydroxyphenyl compounds do not appear to cause an irreversible molecular lesion because, when HeLa cells were incubated with 3,4,5-trihydroxybenzohydroxamic acid and cell replication was reduced 80% or more, removal

of the drug and replacement with fresh media resulted in doubling of cells in the normal generation time of 16 hr.

Although none of the compounds in this report previously has been tested for inhibition of ribonucleotide reductase, a number of aryl hydroxamic acids previously have been synthesized and tested for inhibition of thymidine uptake by Gale *et al.* (12, 13). Several of these compounds caused selective inhibition of DNA synthesis in Ehrlich ascites tumor cells *in vitro*. They reported that salicylhydroxamic acid and 2,3-dihydroxybenzohydroxamic acid were most active in selectively inhibiting DNA synthesis. As can be seen in Table 2, when we tested these compounds as part of our series we found that they inhibited ribonucleotide reductase, 2,3-dihydroxybenzohydroxamic acid being the most potent inhibitor of those compounds examined by Gale and Hynes (12).

The mode of action of hydroxyurea and its analogs has been attributed to their ability to chelate transition metals (16). Iron is thought to be present in mammalian ribonucleotide reductase. This is based on the fact that the *Escherichia coli* enzyme B_2 subunit contains iron (3) and that the mammalian enzyme resembles it in many respects, as well as the fact that ribonucleotide reductase is stimulated by ferrous ions under certain assay conditions and is inhibited by a numer of metal-chelating drugs such as the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones (1), guanazole (2), and deferoxamine (11). However, metal chelation cannot adequately explain the mechanism of action of hydroxyurea and its analogs. In the first place, although ferrous salts have been shown to partially protect ribonucleotide reductase against hydroxyurea inhibition, they could not completely reverse the inhibition even at very high levels (16). The complexity of the inhibition is indicated by the partial protection afforded by increasing the concentration of the chemical reducing agent, dithiothreitol (16). Similar results were seen with the polyhydroxybenzohydroxamic acid compounds in this study (Table 5), indicating a similarity in the mode of action of these compounds to that of hydroxyurea.

Furthermore, the best metal-chelating agents are not always the best inhibitors. For instance, acetohydroxamic acid has been reported to be a stronger chelating agent than is hydroxyurea, but it is a weaker inhibitor *in vitro* (25). In addition, Robertson and van't Riet[5] found little difference in the ferric-complexing abilities of various hydroxy-substituted benzohydroxamic acids, although they exhibited a wide range of enzyme inhibition. Therefore, the mechanism of action of the compounds discussed here is apparently not due solely to metal chelation.

An alternative explanation to metal chelation as the mode of action of this class of compounds is that the hydroxamic acids and their congeners may interfere with a free radical that is present during the enzymatic reaction. An organic free radical has been observed in the B_2 subunit of *E. coli* ribonucleotide reductase (3).

The data that we have reported in this paper provide the basis for a new class of compounds which inhibit ribonucleotide reductase and which may prove to be useful anticancer chemotherapeutic agents.

[5] R. H. Robertson and B. van't Riet, unpublished observations.

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