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INHIBITION OF RIBONUCLEOTIDE REDUCTASE AND L1210 CELL GROWTH BY *N*-HYDROXY-*N'*-AMINOGUANIDINE DERIVATIVES*

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Abstract—A series of *N*-hydroxy-*N'*-aminoguanidine derivatives was studied for their effects on L1210 cell growth and ribonucleotide reductase activity. With the twelve compounds studied, there was a good correlation between the inhibition of L1210 cell growth and the inhibition of ribonucleotide reductase activity. The most potent compound required concentrations of only 1.4 and 2 μM for 50% inhibition of L1210 cell growth and ribonucleotide reductase activity respectively. These guanidine analogs specifically inhibited the conversion of [^{14}C]cytidine and deoxycytidine nucleotides in the nucleotide pool and the incorporation of [^{14}C]cytidine into DNA without altering the incorporation of [^{14}C]cytidine into RNA. Ribonucleotide reductase activity in drug-treated cells was reduced markedly. Iron-chelating agents did not either increase or decrease the inhibition caused by the *N*-hydroxy-*N'*-aminoguanidine derivatives. No evidence was obtained that these derivatives selectively inactivated one of the subunits of ribonucleotide reductase. These compounds appear to inhibit ribonucleotide reductase by a mechanism different from hydroxyurea or the thiosemicarbazone derivatives.

Ribonucleotide reductase catalyzes the key step in the synthesis of deoxyribonucleotides as precursors for DNA synthesis [1]. This enzyme site has, therefore, been a target for the design of antitumor agents. These compounds have included hydroxyurea [2], guanazole [3], 2,3-dihydro-1*H*-pyrazole[2,3-*a*] imidazole (IMPY) [4, 5], the thiosemicarbazone derivatives [6], and the polyhydroxybenzene derivatives [7]. Recently, Lien's group [8] has synthesized a series of *N*-hydroxy-*N'*-aminoguanidine derivatives that have been shown to have antitumor and antiviral activities. One series of these derivatives was shown to be effective ribonucleotide reductase inhibitors [9].

In this report, the inhibitory properties of some newer *N*-hydroxy-*N'*-aminoguanidine compounds (Fig. 1) toward ribonucleotide reductase and L1210 cell growth are compared. Further, other properties of these inhibitors of ribonucleotide reductase are defined.

METHODS AND MATERIALS

Growth of L1210 cells. L1210 cells were grown in RPMI 1640 culture medium supplemented with 10% horse serum, sodium bicarbonate (2 g/l) and gentamicin sulfate (50 mg/l). For complete growth curves, cells were incubated in flasks (30 ml) and aliquots were taken daily for cell counts. For the determination of the $[I]_{50}$ values (concentrations required for 50% inhibition), cells were seeded at 0.15×10^6 cells/ml in 24-well tissue culture plates. Drugs were added at day 0 in dimethyl sulfoxide (DMSO, 0.2% final concentration). The control cells also received DMSO. The cells were incubated at 37° in a humidified chamber flushed with 95% air/5% CO_2.

The cells were counted at day 2. At least three concentrations of drugs were used to establish the $[I]_{50}$ values. All cultures were set up in triplicate. Cell counts were made on a Coulter cell counter (model ZBI).

Assay of ribonucleotide reductase activity. CDP reductase activity was determined by the method of Steeper and Steuart [10]. The reaction mixture contained in a final volume of 0.15 ml: [^{14}C]CDP (7.5 nmoles, 0.05 μCi), dithioerythritol (900 nmoles), magnesium acetate (600 nmoles), ATP (300 nmoles), and the partially-purified enzyme from Ehrlich tumor cells [11].

For the measurement of CDP reductase activity in the crude extracts from L1210 cells, the reaction mixture contained in a final volume of 0.15 ml: [^{14}C]-CDP (3.8 nmoles, 0.05 μCi), adenylylimidodiphosphate (AMP-PNP) which was substituted for ATP (300 nmoles), dithioerythritol (900 nmoles), magnesium acetate (600 nmoles), and crude extract from L1210 cells.

For those experiments in which exogenous ribonucleotide reductase components (non-heme iron

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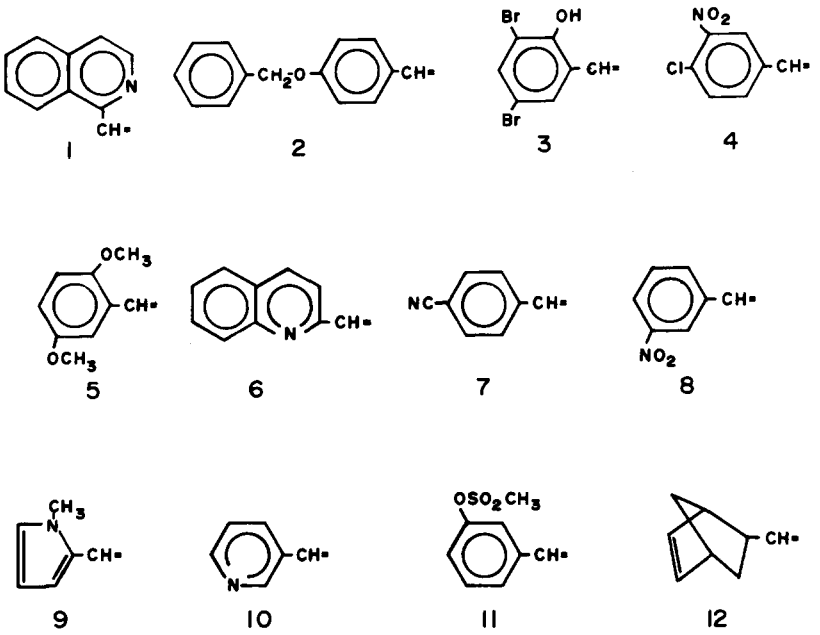

Fig. 1. Structures of the *N*-hydroxy-*N'*-aminoguanidine derivatives of the type R–NNHC(=NH)NHOH. The R groups substituted in the 1-position are as follows: compound 1, 1-isoquinolylmethylene; compound 2, 4-benzyloxybenzylidene; compound 3, 3,5-dibromo-2-hydroxybenzylidine; compound 4, 4-chloro-3-nitrobenzylidene; compound 5, 2,5-dimethoxybenzylidine; compound 6, 2-quinolylmethylene; compound 7, 4-cyanobenzylidine; compound 8, 3-nitrobenzylidene; compound 9, *N*-methyl-2-pyrrolidinylmethylene; compound 10, 3-pyridylmethylene; compound 11, 3-methylsulfonylbenzylidene; and compound 12, 5-norbonen-2-ylmethylene.

and effector-binding subunits) were added to the assay, the subunits were separated by chromatography on blue dextran-Sepharose [12].

Since the *N*-hydroxy-*N'*-aminoguanidine derivatives required the presence of DMSO (final concentration 0.2%) for solubility, all control enzyme assays received the same amount of DMSO. All assays were carried out in triplicate.

For the determination of the $[I]_{50}$, at least three concentrations of drugs were utilized. The $[I]_{50}$ values were estimated from Dixon-type plots [13].

Metabolism of [^{14}C]cytidine in L1210 cells in culture. L1210 cells, growing in log phase, were collected by centrifugation and resuspended in fresh culture medium. The cells were incubated in the presence of the appropriate drug for 90 min at 37°. [^{14}C]Cytidine (485 mCi/nmole, 0.04 μCi/ml) was added, and the incubation was continued for an additional 30 min. The cells were collected by centrifugation, and the cell pellet was subjected to a modified Schmidt–Thannhauser procedure [14]. The acid-soluble fraction was prepared by extraction of the cell pellet with 6% perchloric acid. The acid-insoluble material (RNA, DNA and protein) was treated with 1 ml of 0.5 M NaOH and incubated at 37° overnight. The solution was cooled and 60% $HClO_4$ was added to bring the solution to a final concentration of 7% $HClO_4$. The suspension was centrifuged, and the supernatant fluid (hydrolyzed RNA) and the pellet (DNA and protein) were separated. The DNA/protein pellet was solubilized in NaOH. Aliquots of the acid-soluble, RNA, or DNA fractions were taken for radioactivity measurements and for RNA [15] and DNA [16] determinations.

The acid-soluble fraction from above was neutralized with KOH. After removal of the $KClO_4$, the supernatant fluid was lyophilized. The lyophilized samples were dissolved in 0.7 ml of 0.1 M Tris–HCl, pH 9.0, and crude snake venom (*Crotalus atrox*, 20 mg/ml) was added. After incubation for 4 hr at 37°, the reactions were stopped by heating in a boiling water bath (4 min), cooled on ice, and centrifuged. The supernatant fluids were put over Dowex-1-borate columns to separate deoxycytidine from cytidine [17].

Preparation of crude extracts from L1210 cells. L1210 cells in log phase were collected by centrifugation. The cells were incubated in fresh culture medium containing the drug under study. After a 2-hr incubation period in the presence of drug, the cells were collected by centrifugation. The cell pellet was homogenized in 1 ml of 0.05 M Tris–HCl, pH 7.0, containing 1 M dithioerythritol. The homogenate was centrifuged at 28,000 *g* for 1 hr, and the supernatant fluid was used as the source of enzyme extract from L1210 cells.

Synthesis of N-hydroxy-N'-aminoguanidine compounds. The *N*-hydroxy-*N'*-aminoguanidine derivatives were synthesized and characterized as previously described [18].

Chemicals. The nucleotides and biochemicals used in these studies were purchased from the Sigma Chemical Co., St. Louis, MO. The horse serum, RPMI 1640 culture medium, and the sodium bicar-

bonate were purchased from the Grand Island Biological Co., Grand Island, NY. The [^{14}C]cytidine (485 mCi/mmole) and [^{14}C]CDP (350 mCi/mmole) were purchased from the Research Products International Corp., Mount Prospect, IL. Desferal was a gift from the Ciba–Geigy Corp., Summit, NJ.

RESULTS

Comparison of effects of N-*hydroxy* N′-*aminoguanidine derivatives on L*1210 *cell growth and ribonucleotide reductase activity.* The guanidine derivatives were studied for their effects on L1210 cell growth in culture and on ribonucleotide reductase activity utilizing CDP as the substrate. As seen in the data presented in Table 1, there was a fairly good correlation between the ability of these compounds to inhibit L1210 cell growth and to inhibit ribonucleotide reductase activity as measured by the respective $[I]_{50}$ values. Compound 1 was the most potent derivative in terms of inhibiting L1210 cell growth and ribonucleotide reductase activity. Only compound 5, which was a good inhibitor of L1210 cell growth, but a very poor inhibitor of ribonucleotide reductase activity, did not give a good correlation. Compounds 10, 11 and 12, which were the poorest inhibitors of L1210 cell growth, were also poor inhibitors of ribonucleotide reductase activity. The parent compound, hydroxyguanidine, inhibited ribonucleotide reductase activity only 11% at a concentration of 1 mM.

The measurement of growth curves for the L1210 cells in the presence of the drugs for a 5-day period showed that, with time, the tumor cells grew out. As seen in Fig. 2, compound 1, at a concentration of 2.5 μM, inhibited L1210 cell growth by 70% at day 2, but by only 15% on day 5. Higher concentrations of compound 1 (10 μM) were required to sustain the inhibition of cell growth over a 5-day period. Also shown in Fig. 2 is the growth curve for L1210 cells in the presence of compound 11, one of the least active *N*-hydroxy-*N*′-aminoguanidine derivatives. Even at a concentration of 100 μM, compound 11 did not completely inhibit L1210 cell growth over the 5-day period.

*Ribonucleotide reductase activity in drug-treated L*1210 *cells.* L1210 cells were incubated in culture in the presence of the *N*-hydroxy-*N*′-aminoguanidine derivatives (compounds 1, 2, 7 and 12). Cell-free extracts were prepared and ribonucleotide reductase activity was determined. The data in Table 2 show that, in those cells incubated in the presence of the guanidine derivatives, the level of ribonucleotide reductase in the cell-free extract was reduced in a concentration-dependent manner, and with approximately the same order of sensitivity as shown in Table 1. In an attempt to define the subunit of ribonucleotide reductase which was sensitive to these compounds, the cell-free extracts from the control and drug-treated cells were supplemented with either excess exogenous non-heme iron subunit (NHI) or effector-binding subunit (EB) [12, 19]. These data are also included in Table 2. Neither exogenous NHI-subunit nor EB-subunit restored ribonucleotide reducase activity in the extracts from the drug-treated cells. Compound 7 was the exception in that the NHI subunit restored the activity.

Effect of N-*Hydroxy*-N′-*aminoguanidine derivatives on [*^{14}*C] cytidine metabolism in L*1210 *cells.* The effects of several of the *N*-hydroxy-*N*′-aminoguanidine derivatives on [^{14}C]cytidine metabolism in L1210 cells were studied. In these experiments, the incorporation of [^{14}C]cytidine into the acid-soluble, RNA, and DNA fractions, as well as the formation of deoxycytidine in the acid-soluble fraction, was determined. These drugs had no effect on the incorporation of [^{14}C]cytidine into the acid-soluble or RNA fractions. As seen in the data in Table 3, compound 1 was most effective in inhibiting the incorporation of [^{14}C]cytidine into DNA and in the

Table 1. Effects of *N*-hydroxy-*N*′-aminoguanidine derivatives on L1210 cell growth and ribonucleotide reductase activity

R=NNHC(=NH)NHOH · tosylate Compound	$[I]_{50}$ (μM) L1210 cell growth*	$[I]_{50}$ (μM) Ribonucleotide reductase†
1	1.4	2
2	5.6	30
3	5.8	22
4	16.5	20
5	17.0	>780
6	20.0	25
7	22.5	25
8	30.0	42
9	65.0	90
10	120.0	120
11	123.0	69
12	>150.0	500

* $[I]_{50}$, concentration of drug required to inhibit L1210 cell growth in culture by 50%. The $[I]_{50}$ values were estimated from Dixon-type plots [13]. At least three concentrations were used for each drug.

† $[I]_{50}$, concentration of drug required to inhibit CDP reductase by 50%. The $[I]_{50}$ values were estimated from Dixon-type plots [13]. At least three concentrations were used for each drug.

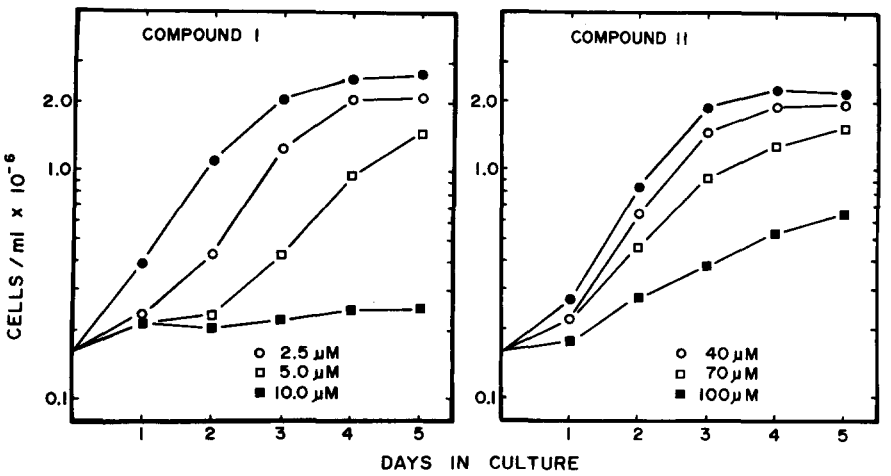

Fig. 2. Effects of compounds 1 and 11 on L1210 cell growth in culture. Compound 1 was added at final concentrations of 2.5 μM (○), 5.0 μM (□) and 10.0 μM (■); compound 11 was added at final concentrations of 40 μM (○), 70 μM (□) and 100 μM (■). The control cultures (●) received no drug, but were grown in 0.2% DMSO. The cultures were set up in triplicate, and duplicate samples were taken daily for cell counts.

formation of [^{14}C]deoxycytidine nucleotides (as a measure of ribonucleotide reductase activity *in situ*). Compounds 7 and 12 were least active based on the concentrations required to achieve inhibition of DNA synthesis. The data reported in Table 3 represent the concentrations of compounds 1, 2, 7 and 12 which gave approximately the same degree of inhibition of [^{14}C]cytidine incorporation into DNA.

Reversibility of inhibition of ribonucleotide reductase by N-*hydroxy*-N′-*aminoguanidine derivatives.* On a concentration basis, compound 1 was the most potent inhibitor of ribonucleotide reductase activity ($[I]_{50}$ of 2 μM). Experiments were carried out to determine if the inhibition by this guanidine derivative was reversible. The enzyme was incubated in the presence and absence of compound 1 (100 μM) for 1 hr on ice. Aliquots were taken for assay while the rest of the samples were put over Sephadex G-25 columns (25 × 1 cm). The protein peak eluting in the void volume was concentrated in an Amicon B-15 cell and assayed. Under these conditions, the inhibition by compound 1 (15 μM final concentration

Table 2. Ribonucleotide reductase activity in crude extracts from *N*-hydroxy-*N*′-aminoguanidine derivative-treated L1210 cells

Compound*	(μM)	Ribonucleotide reductase Activity (nmoles/30 min/mg protein)	+NHI† (% control)	+EB†
None		0.53 (100)‡	100	100
1	1.0	0.17 (32)	48	46
	2.5	0.02 (3)	16	13
	5.0	0.00 (0)	0	0
2	5.0	0.47 (88)	92	79
	30.0	0.08 (15)	23	12
	60.0	0.01 (1)	4	0
7	50.0	0.16 (30)	101	34
	100.0	0.03 (6)	41	5
12	50.0	0.36 (67)	68	69
	500.0	0.28 (52)	64	56
	1000.0	0.07 (14)	20	15

* The L1210 cells were incubated in the presence of drug for 1 hr. The cell concentration was 1 × 10^6 cells/ml. Cells from appropriate flasks were pooled, and cell-free extracts were prepared. The cell-free extract (1.0 ml) was prepared from 6 × 10^6 cells. Aliquots (100 μl) were used for the enzyme assays.

† NHI, exogenous non-heme iron subunit, was added in excess to the assays prior to the addition of substrate; or the EB, exogenous effector-binding subunit, was added in excess to the assays prior to the addition of substrate.

‡ Numbers in parentheses are the percent of control values.

Table 3. Effects of *N*-hydroxy-*N'*-aminoguanidine derivatives on [^{14}C]cytidine metabolism in L1210 cells*

Compound	RNA	DNA (% control)	dCyd
1, 5 μM	117	14	35
2, 38 μM	92	15	38
7, 100 μM	100	5	44
12, 500 μM	96	19	63

* The experiments were set up as described in the Methods and Materials. The control values for compounds 1 and 2 in terms of cpm per 10^6 cells were: 16,800, 4200 and 450 for RNA, DNA and deoxycytidine respectively. The control values for compounds 7 and 12 in terms of cpm for 10^6 cells were: 20,000, 5000 and 520 for RNA, DNA and deoxycytidine respectively. For each drug, three different concentrations were used. For compound 1, the concentrations were 0.5, 1.0 and 5 μM; for compound 2, the concentrations were 3.7, 7.5 and 37.5 μM; for compounds 7 and 12, the concentrations were 100, 500 and 1000 μM.

in the reaction mixture), which gave 70% inhibition of CDP reduction prior to gel chromatography, was reversed completely after removal by gel chromatography (Table 4).

Effect of iron-chelating agent on inhibition of ribonucleotide reductase by N-*hydroxy*-N'-*aminoguanidine derivatives.* Previous studies showed that the iron-chelating agents Desferal, EDTA, and 8-hydroxyquinoline potentiated the inhibition of ribonucleotide reductase by hydroxyurea, IMPY and guanazole, while completely abolishing the inhibition caused by 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone and 1-formylisoquinoline thiosemicarbazone (MAIQ and IQ) [20]. The effects of Desferal or EDTA on the inhibition of reductase activity by some of the *N*-hydroxy-*N'*-aminoguanidine derivatives were studied. In contrast to the results seen with hydroxyurea or IMPY and MAIQ or IQ, the iron-chelating agents neither increased nor decreased the inhibition caused by compounds 1, 2, 6, 7 and 11 (the only derivatives studied in these experiments, but which span the range of inhibiting activities).

DISCUSSION

The results of these studies indicate that the *N*-hydroxy-*N'*-aminoguanidine derivatives are inhibitors of CDP reductase activity with essentially a corresponding inhibition of L1210 cell growth. The inhibition of ribonucleotude reductase by these analogs was observed in partially-purified enzyme preparations, and in extracts from drug-treated L1210 cells. These derivatives also inhibited the incorporation of [^{14}C]cytidine into DNA, a process which required the ribonucleotide reductase step *in situ.* There was good correlation among the variables measured (L1210 cell growth, ribonucleotide reductase activity, and [^{14}C]cytidine metabolism) for the various derivatives. Compound 1, for example, was most active, while compound 12 was least active in terms of inhibiting L1210 cell growth, inhibiting ribonucleotide reductase activity, or inhibiting [^{14}C]-cytidine metabolism. Replacement of the H- in *N*-hydroxy-*N'*-aminoguanidine with a bulky R-group markedly potentiated the inhibitory properties of these drugs. However, structural variations in the R-group also altered greatly the potency of these compounds (e.g. compound 1 vs compound 12). Further, slight structural variations also altered the sensitivity of these drugs at least 10-fold. The isoquinoline derivative was at least 10-fold more active than the quinoline derivative of *N*-hydroxy-*N'*-aminoguanidine. In the thiosemicarbazone series of compounds, the isoquinoline derivative was also much more potent in inhibiting ribonucleotide reductase activity and thymidine incorporation into DNA than the quinoline derivative [19]. Another interesting comparison is that the pyridine analog was less active than the isoquinoline analog in both the *N*-hydroxy-*N'*-aminoguanidine and thiosemicarbazone series [19].

While the data in this report show that the *N*-hydroxy-*N'*-aminoguanidine analogs inhibited ribonucleotide reductase activity and there was good correlation with the inhibition of L1210 cell growth, it is possible that there are other metabolic targets for these compounds or that some of the compounds are converted to a more active form in intact cells. This may be particularly relevant to compound 5 which was much more inhibitory to L1210 cell growth than to ribonucleotide reductase activity.

The inhibition of ribonucleotide reductase by compound 1 was reversible either by dilution (data not shown) or by separation by chromatography on Sephadex G-25. It was not possible to demonstrate which subunit of ribonucleotide reductase was inhibited by these guanidine derivatives. Reductase activity in the extracts from drug-treated L1210 cells was not restored by the addition of either exogenous non-heme iron or effector-binding subunits. In this

Table 4. Reversibility of inhibition of ribonucleotide reductase by *N*-hydroxy-*N'*-aminoguanidine derivative

Sample	Reductase activity (nmoles/30 min/mg protein)
(A) Control	1.61
(B) Compound 1, 15 μM*	0.50
(C) Control (after Sephadex)	1.29
(D) Compound 1 (after Sephadex)	1.34

* The concentration of compound 1 incubated with the enzyme was 100 μM. The final concentration of compound 1 in the reaction mixture during the reductase assay was 15 μM.

respect, these compounds differ from the previously studied ribonucleotide reductase inhibitors [20]. Further, the inhibition of reductase by the guanidine analogs was neither stimulated nor reversed by the iron-chelating agents Desferal or EDTA. This, again, was in contrast to the effects of the iron-chelating agents on the inhibition of reductase by hydroxyurea, guanazole and IMPY, and MAIQ and IQ [21].

The *N*-hydroxy-*N'*-aminoguanidine derivatives appear to inhibit ribonucleotide reductase by mechanisms dissimilar from hydroxyurea, guanazole and IMPY as one group, or MAIQ and IQ as the other type of reductase inhibitors.

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