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INHIBITION OF RIBONUCLEOTIDE REDUCTASE AND GROWTH OF HUMAN COLON CARCINOMA HT-29 CELLS AND MOUSE LEUKEMIA L1210 CELLS BY *N*-HYDROXY-*N'*-AMINO GUANIDINE DERIVATIVES

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Abstract—A series of *N*-hydroxy-*N'*-aminoguanidine (HAG) derivatives were studied and compared for their effects on ribonucleotide reductase activity in cell-free extracts; on nucleic acid synthesis and the growth of human colon carcinoma HT-29 cells; and on mouse leukemia L1210 cells in culture. The HAG derivatives [RCH=NNHC(=NH)NHOH-tosylate] studied could be grouped as: (1) hydroxybenzylidines; (2) methoxybenzylidines; and (3) nitrobenzylidines substituted at the R position. 2'-Hydroxybenzylidene-HAG, the lead compound, was relatively active in both HT-29 cells and L1210 cells (20 ± 5 and $13 \pm 4 \mu\text{M}$ for 50% inhibition of HT-29 and L1210 cell growth respectively). The monohydroxybenzylidene compounds were generally more active than the dihydroxy- and trihydroxybenzylidene-HAG derivatives. The methoxybenzylidene-HAGs were as active as the monohydroxybenzylidene-HAGs. 2'-Hydroxy-4'-methoxybenzylidene-HAG was much more active than 2',4'-dihydroxybenzylidene-HAG. The mononitrobenzylidene-HAGs were more active than the dinitrobenzylidene-HAG compound. In general, L1210 cells were more sensitive to the effects of the HAG compounds than were HT-29 cells. There was good agreement between the concentration of drug required to inhibit the growth of HT-29 cells and that required to inhibit the growth of L1210 cells. There was also good correlation between the ability of HAG derivatives to inhibit ribonucleotide reductase activity and to inhibit tumor cell growth. Some derivatives, such as 2',3',4'- and 3',4',5'-trihydroxybenzylidene-HAG inhibited L1210 cell growth by 50% at lower concentrations (7.8 and 11.9 μM respectively) than the concentrations needed for 50% inhibition of HT-29 cell growth (196 and 234 μM respectively) and ribonucleotide reductase activity (122 and 188 μM respectively). The studies of nucleic acid synthesis in L1210 cells using [^3H]cytidine as a precursor showed that 2',3',4'-trihydroxybenzylidene-HAG inhibited DNA synthesis at a lower concentration (29 μM for 50% inhibition) than was needed for the inhibition of RNA synthesis and formation of [^3H]deoxycytidine nucleotides in the acid-soluble fraction (320 and 820 μM for 50% inhibition respectively). These results indicate that 2',3',4'-trihydroxybenzylidene-HAG inhibits DNA synthesis in L1210 cells through other mechanisms rather than exclusively through the inhibition of ribonucleotide reductase activity.

A series of *N*-hydroxy-*N'*-aminoguanidine (HAG§) derivatives have been synthesized by Lien's group and have been shown to inhibit tumor cell growth and ribonucleotide reductase activity [1–7]. The HAG derivatives, which have a core structure resembling hydroxyurea, are believed to act through the same mechanism as hydroxyurea, a specific inhibitor of the non-heme iron subunit of ribonucleotide reductase. In a series of papers from this laboratory [4–7], it was reported that small structural changes in the R-groups attached to HAG altered the inhibitory effects of these compounds on both ribonucleotide reductase activity and L1210 cell growth. Further, it was found that there was good correlation between the ability of the HAG derivatives to inhibit L1210

cell growth and to inhibit ribonucleotide reductase activity [4]. New HAG derivatives in which a benzylidene or pyridyl ring was introduced in the R-group were synthesized [8] and studied in a systematic manner. In the present study, these derivatives were divided into several groups according to the structure of the R-groups. The inhibitory effects of each HAG derivative on (1) ribonucleotide reductase activity, (2) the growth of HT-29 cells, and (3) the growth of L1210 cells were compared. These studies indicate the importance of structural variations in the R-groups of the HAG derivatives which give rise to potential antitumor agents.

MATERIALS AND METHODS

Materials. HAG derivatives were synthesized and characterized by Lien's group [1–3]. HT-29 cells and L1210 cells were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 culture medium, fetal calf serum and horse serum were purchased from the Grand Island Biological

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§ Abbreviations: HAG, *N*-hydroxy-*N'*-aminoguanidine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and DMSO, dimethyl sulfoxide.

Co. (Grand Island, NY). Sodium bicarbonate was obtained from Matheson, Coleman & Bell (Norwood, OH). [^{14}C]CDP (350 mCi/mmol) was purchased from the Research Products International Corp. (Mount Prospect, IL). [^3H]Cytidine (23 Ci/mmol) was purchased from Moravak Biochemicals, Inc. (Brea, CA). Other chemicals used in this study were purchased from the Sigma Chemical Co. (St Louis, MO).

Cell lines. Mouse leukemia L1210 cells were grown in suspension culture in RPMI 1640 culture medium supplemented with 10% horse serum, sodium bicarbonate (2 g/L), and gentamicin sulfate (50 mg/L). Human colon carcinoma HT-29 cells were grown as a monolayer in RPMI 1640 medium containing 10% fetal calf serum, 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4, and gentamicin sulfate (50 mg/L).

Microculture tetrazolium assay. Single cell suspensions of L1210 cells were obtained by mechanical disaggregation and of HT-29 cells by trypsinization. The cell counts were performed using a Coulter counter, model ZBI. A modification of the microculture tetrazolium assay (MTT assay) was used, as described by Carmichael *et al.* [9]. HT-29 cells were seeded into each well of 96-well plates at 1500 cells/well in 150 μL of RPMI 1640 medium. After 48 hr of incubation at 37° in a humidified atmosphere of 5% CO_2 /95% air, drugs in 50 μL of medium were added to triplicate culture wells, and plates were incubated for 6 days at 37°. Fifteen microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL in phosphate-buffered saline) was added to each well, and the plates were incubated for 4 hr. At the end of the incubation, the medium was removed from each well, and 150 μL of DMSO was added to solubilize the MTT-formazan product. Absorbance at 490 nm (test filter) and 630 nm (reference filter) was measured with a Dynatech MR700 microplate reader (Alexandria, VA). L1210 cells were seeded into wells in microculture plates in 150 μL of culture medium 1000 cells/well and were incubated at 37°. After 24 hr of incubation, drugs in 50 μL of culture medium were added to each well. After 2 days of exposure to drugs, MTT (15 μL , 5 mg/mL) was added to each well and the cells were incubated for 4 hr at 37°. The plates were centrifuged for 10 min at 500 g and the culture medium was aspirated carefully. Dimethyl sulfoxide (DMSO, 150 μL) was added to solubilize the formazan product. After thorough mixing with a multichannel pipetter, absorbance at 570 and 630 nm was measured.

Assay of ribonucleotide reductase activity. CDP reductase activity was measured by the method of Steeper and Steuart [10]. The reaction mixture contained in a final volume of 150 μL : [^{14}C]CDP (25 nmol, 0.05 μCi), dithioerythritol (900 nmol), magnesium acetate (600 nmol), ATP (300 nmol), and the partially purified enzyme from Ehrlich ascites tumor cells. Since DMSO (final concentration of 0.2%) was required to solubilize the HAG derivatives, all controls received the same amount of DMSO. All assays were carried out in triplicate. The IC_{50} values were estimated by using the method of Chou and Talalay [11].

[^3H]Cytidine metabolism. The cells were cultured in 8 mL of fresh medium in a flask at a final concentration of 6.4×10^5 cells/mL (HT-29 cells) or 4.2×10^6 cells/mL (L1210 cells) with or without drugs. After a 90-min incubation at 37° in humidified 95% air/5% CO_2 , [^3H]cytidine (2 μCi) was added to each flask and the cells were incubated further for 30 min. At the end of the incubation period, the cell suspensions were cooled and centrifuged. The cells were collected and subjected to a modified Schmidt-Thannhauser procedure [4, 12]. The acid-soluble fraction was obtained by extraction of the cells with 1 mL of 6% perchloric acid (three times). The acid-soluble fraction was neutralized with 5 M KOH, and the KClO_4 was removed by centrifugation. The supernatant fluid was lyophilized, dissolved in 0.7 mL of 0.1 M Tris-HCl, pH 9.0, and then incubated with crude snake venom (*Crotalus atrox*, 3.2 mg) for 4 hr at 37°. The reaction was stopped by heating the tube in a boiling water bath for 4 min and the samples were applied to a 1-mL column of Dowex-1-borate to separate deoxycytidine from cytidine [13]. The acid-insoluble fraction was dissolved in 1 mL of 0.5 M NaOH and incubated overnight at 37°. The solution was cooled and 60% perchloric acid was added to reprecipitate DNA. After centrifugation, the supernatant fluid (RNA) was transferred to another tube, and the pellet was solubilized in 0.5 M NaOH.

RESULTS

Effects of HAG derivatives on the growth of HT-29 cells and L1210 cells. The microculture tetrazolium assay (MTT assay) was used to evaluate the effects of HAG derivatives on the growth of HT-29 cells and L1210 cells. The structures of the HAG derivatives [$\text{RCN}=\text{NNHC}(=\text{NH})\text{NHOH}$ tosylate] are given in Fig. 1, and the data are presented in Table 1. Group I compounds were hydroxylated-benzylidene derivatives. The monohydroxylated benzylidene-HAG (IA) was the most active in this group of compounds; 2',4'-dihydroxybenzylidene-HAG (IB) was the least active. L1210 cells were much more sensitive to compounds IC and IE than were HT-29 cells as measured by the MTT growth assay. To confirm these differences, growth studies were conducted in which cell counts were made. Cell count data (not shown) showed that these differences between the IC_{50} values for HT-29 and L1210 cells were real and were not due to an artifact of the MTT assay. Group II compounds were methoxy-substituted analogs. Compounds IIA and IIB were more active as inhibitors of tumor cell growth than was compound IIC. Group III compounds had nitro-groups substituted on the benzylidene ring. The mononitro-substituted compounds (IIIA, B and D) were more active than dinitro-substituted HAG (IIIC).

In general, L1210 cells were more sensitive to the growth inhibitory effects of these HAG compounds than were the HT-29 cells.

Effects of HAG derivatives on ribonucleotide reductase activity. The effects of HAG derivatives on ribonucleotide reductase activity were studied using CDP as the substrate for ribonucleotide reductase. As seen in Table 1, in general there was

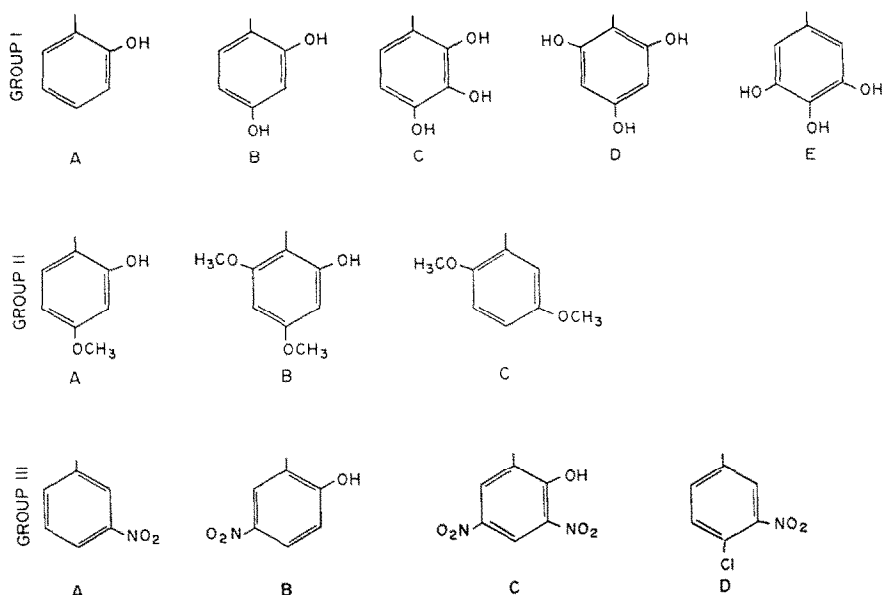


Fig. 1. Structure of substituted *N*-hydroxy-*N'*-aminoguanidine compounds. The R—CH= groups substituted on RCH=NNHC (=NH)NHOH-tosylate are as follows: Group I: (A) 2'-hydroxybenzylidene-, (B) 2',4'-dihydroxybenzylidene-, (C) 2',3',4'-trihydroxybenzylidene-, (D) 2',4',6'-trihydroxybenzylidene-, and (E) 3',4',5'-trihydroxybenzylidene-; Group II: (A) 2'-hydroxy-4'-methoxybenzylidene-, (B) 2'-hydroxy-4',6'-dimethoxybenzylidene-, and (C) 3',6'-dimethoxybenzylidene-; Group III: (A) 3'-nitrobenzylidene-, (B) 2'-hydroxy-5'-nitrobenzylidene-, (C) 2'-hydroxy-3',5'-dinitrobenzylidene-, and (D) 3'-nitro-4'-chlorobenzylidene-.

Table 1. Effects of HAG-derivatives on HT-29 and L1210 cell growth in culture and ribonucleotide reductase activity

Compound	HT-29	IC ₅₀ (μM) L1210	Ribonucleotide reductase
I: A	20 ± 5	13 ± 4	76.4
B	796 ± 70	279 ± 89	519
C	196 ± 65	7.8 ± 1.0	122
D	197 ± 28	50.3 ± 3.0	80.2
E	234 ± 29	11.9 ± 0.2	188
II: A	31 ± 10	17.7 ± 1.3	49
B	25 ± 3	13.0 ± 3.0	73
C	50 ± 12	48.5 ± 2.0	>780*
III: A	31 ± 3	51 ± 1	42*
B	21 ± 2	4.9 ± 3.4	138
C	>100	>100	>100
D	43 ± 18	32 ± 2	20*

Values for HT-29 and L1210 cells are means ± SD, N = 3; values for ribonucleotide reductase are the means of triplicate determinations.

* Data taken from Cory *et al.* [4].

good correlation between the effects of these compounds on ribonucleotide reductase activity and on tumor cell growth. For some derivatives, such as 3',6'-dimethoxybenzylidene-HAG (IIC) and 2'-hydroxy-5'-nitrobenzylidene-HAG (IIIB), relatively higher concentrations of these compounds were required to inhibit ribonucleotide reductase activity than to inhibit tumor cell growth. For other compounds such as 3',4',5'- (IE) and 2',3',4'-trihydroxybenzylidene-HAG (IC), there was not a good correlation between the inhibition of ribonucleotide reductase and the inhibition of L1210 cell growth. These compounds inhibited L1210 cell growth at

much lower concentrations than those required for the inhibition of ribonucleotide reductase activity.

Effect of 2',3',4'-trihydroxybenzylidene-HAG on [³H]cytidine metabolism in HT-29 cells and L1210 cells. 2',3',4'-Trihydroxybenzylidene-HAG (IC), which showed a large difference in inhibition between HT-29 cells (IC₅₀ = 196 ± 65 μM) and L1210 cells (IC₅₀ = 7.8 ± 1.0 μM), was chosen for the study of its effects on [³H]cytidine metabolism in these two cell lines. In these experiments, incorporation of [³H]cytidine into acid-soluble nucleotides, RNA and DNA fractions, and the formation of [³H]deoxycytidine nucleotides in the acid-soluble fraction

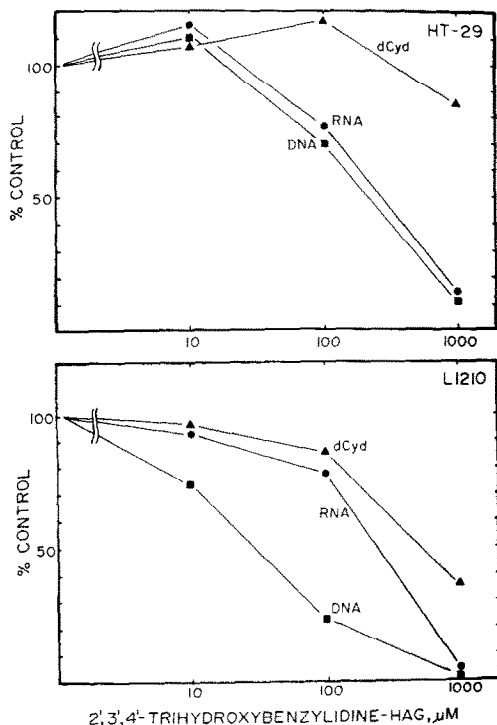


Fig. 2. Effects of 2',3',4'-trihydroxybenzylidene-HAG on [³H]cytidine metabolism in HT-29 and L1210 cells. HT-29 or L1210 cells were incubated in the presence and absence of 2',3',4'-trihydroxybenzylidene-HAG for 90 min at the concentrations of drug indicated. [³H]Cytidine (2 μCi/8 mL) was added and the incubation was continued for 30 min. The cells were subjected to the Schmidt-Thannhauser procedure [12] to separate the nucleotide pool, RNA and DNA fractions. Deoxycytidine nucleotides were separated from cytidine nucleotides on Dowex-1-borate [13]. The control values for the HT-29 cells were: deoxycytidine nucleotides, 1470 cpm/10⁷ cells; RNA, 179,300 cpm/10⁷ cells; and DNA, 2770 cpm/10⁷ cells. The control values for the L1210 cells were: deoxycytidine nucleotides, 2440 cpm/10⁷ cells; RNA, 153,100 cpm/10⁷ cells; and DNA, 7970 cpm/10⁷ cells.

(*in situ* ribonucleotide reductase activity) were measured. As shown in Fig. 2, the incorporation of [³H]cytidine into RNA and DNA fractions was inhibited by 2',3',4'-trihydroxybenzylidene-HAG (IC) in HT-29 cells in a similar fashion; the formation of [³H]deoxycytidine nucleotide required higher concentrations of 2',3',4'-trihydroxybenzylidene-HAG (IC). In L1210 cells, 2',3',4'-trihydroxybenzylidene-HAG (IC) inhibited DNA synthesis to a greater extent than either RNA synthesis or deoxycytidine nucleotide formation.

DISCUSSION

The reaction in which ribonucleoside 5'-diphosphates are reduced to the corresponding 2'-deoxyribonucleoside 5'-diphosphates is catalyzed by ribonucleotide reductase. This step is the rate-limiting process in the *de novo* synthesis of the deoxyribonucleoside triphosphates required for DNA synthesis [14-16]. Ribonucleotide reductase activity is cell cycle dependent and increases dramatically at

late G₁-early S phase [17, 18]. Further, it has been shown that the activity of this enzyme is elevated in relation to the growth rate of a tumor [19, 20]. Thus, ribonucleotide reductase is an appropriate site for enzyme-targeted chemotherapy. The results of our studies indicate that the inhibition of tumor cell growth by the HAG derivatives is generally related to their inhibitory effects on ribonucleotide reductase activity. Some HAG derivatives, such as 2',3',4'-trihydroxybenzylidene-HAG (IC), may have additional mechanisms of action. Elford *et al.* [21] have studied a series of polyhydroxybenzene derivatives of hydroxamic acid. Using an assay with the stable free-radical, diphenylpicrylhydrazyl, it was shown that the trihydroxy-derivatives of benzohydroxamic acids consumed the free-radical at a rate at least 80 times that of the monohydroxybenzohydroxamic acid. It is possible that 2',3',4'-trihydroxybenzylidene-HAG (IC) also has other intracellular sites of action involving an interaction with free radicals.

2',3',4'-Trihydroxybenzylidene-HAG (IC) also inhibited RNA synthesis. This result differs from that of hydroxyurea, in that hydroxyurea (and other HAG derivatives) does not inhibit RNA synthesis on short-term incubation [5]. It is not clear whether the inhibition of RNA synthesis by this compound is essential for the inhibition of tumor cell growth.

Small structural changes in the R-group of HAG derivatives greatly altered the potency of these derivatives. The potency of the HAG derivatives was compared on the basis of their IC₅₀ values. The IC₅₀ values for tumor cell growth were determined by MTT assay. These values corresponded very closely to the IC₅₀ values that were determined for some of the HAG compounds by direct cell count using the Coulter counter. With respect to the hydroxybenzylidene-HAGs, the monohydroxybenzylidene-HAG (IA) was the most potent in inhibiting the growth of HT-29 cells and L1210 cells. The addition of a second hydroxy substituent at the 4'-position of 2'-hydroxybenzylidene-HAG greatly reduced the effects of 2'-hydroxybenzylidene-HAG (IA) on ribonucleotide reductase activity in cell-free extracts and on HT-29 and L1210 cell growth.

In contrast, the substitution of a methoxy group on the 4-position (IIA) did not alter the inhibitory nature of the HAG derivatives. The presence of a hydroxy substituent at the 3'-position of the R-group seemed to play an important role in inhibiting L1210 cell growth. In the methoxybenzylidene-HAG group of compounds, placement of a methoxy substituent(s) on the benzylidene ring at either the 4'- or 6'-position did not alter the effect of 2'-hydroxybenzylidene-HAG (IA) on ribonucleotide reductase activity and tumor cell growth. However, 3,6-dimethoxybenzylidene-HAG (IIC) was less active in both HT-29 cells and L1210 cells than was 2'-hydroxy-4'-methoxybenzylidene-HAG (IIA) or 2'-hydroxy-4',6'-dimethoxybenzylidene-HAG (IIB), and it had almost no activity as an inhibitor of ribonucleotide reductase activity.

The addition of a chloro substituent on 3'-nitrobenzylidene-HAG did not alter its inhibitory properties. When the activity of 2'-hydroxy-5'-nitrobenzylidene-HAG (IIIB) was compared with

that of 2'-hydroxybenzylidene-HAG (IA), it was seen that a nitro substituent at the 5'-position of the R-group increased the activity of 2'-hydroxybenzylidene-HAG in L1210 cells 5.5-fold and decreased its ribonucleotide reductase inhibitory activity by 55%. When the activity of 2'-hydroxy-3',5'-dinitrobenzylidene-HAG (IIIC) was compared with that of 2'-hydroxy-5'-nitrobenzylidene-HAG (IIIB), it was seen that the addition of the nitro substituent at the 3'-position of the phenyl ring decreased the activity of this compound as an inhibitor of the growth of HT-29 and L1210 cells. The presence of a nitro substituent either increased or decreased the inhibition of tumor cell growth and ribonucleotide reductase activity relative to 2'-hydroxybenzylidene-HAG. It appears that the effect of a nitro substituent depends on the ring position or the number of nitro substituents on the phenyl ring of 2'-hydroxybenzylidene-HAGs.

In conclusion, our results show that most of the HAG derivatives examined in this series were more active in both HT-29 cells and L1210 cells than hydroxyurea (IC_{50} values for HT-29 and L1210 cells were 206 and 38 μ M respectively). These compounds appeared to inhibit tumor cell growth by inhibiting cellular ribonucleotide reductase activity, while some of the compounds appeared to inhibit L1210 cell growth at lower concentrations by targeting other metabolic sites as well as ribonucleotide reductase. The potency of the HAG derivatives as inhibitors of ribonucleotide reductase and tumor cell growth depended not only on the nature of the substituent but also on the location of the substituent on the phenyl ring.

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