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Effects of *N*-Hydroxy-*N'*-aminoguanidine Derivatives on Ribonucleotide Reductase Activity, Nucleic Acid Synthesis, Clonogenicity, and Cell Cycle of L1210 Cells[1]

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ABSTRACT

Derivatives of *N*-hydroxy-*N'*-aminoguanidine were recently shown to be efficient inhibitors of mammalian ribonucleotide reductase and cancer cell growth. We investigated the effects of the 1-isoquinolylmethylene and the 2-quinolylmethylene derivatives of *N*-hydroxy-*N'*-aminoguanidine on intracellular targets, cell viability, and cell cycle of L1210 mouse leukemia cells. A 2-h exposure of L1210 cells to either drug in the low micromolar concentration range led to inhibition of intracellular ribonucleotide reductase activity and DNA synthesis. After a 24-h incubation in the presence of these drugs, RNA synthesis was also markedly diminished. The clonogenicity of L1210 cells was inhibited after treatment with the drugs for 24 and 48 h, the I_{50} values being comparable to the drug concentrations required for 50% inhibition of DNA synthesis and cell proliferation. The isoquinoline compound was always more inhibitory to reductase activity, nucleic acid synthesis, and clonogenicity than the quinoline compound. As shown by flow cytometry, the *N*-hydroxy-*N'*-aminoguanidine isoquinoline derivative at 0.5–10 μM led to an elevation of G_0/G_1 cells and a decrease of G_2/M and S cells. At 10 μM of the drug this shift remained unchanged over 48 h. L1210 cells treated with 0.5, 1, and 2 μM of the drug overcame the block after 4 to 12 h of exposure and progressed through S- and G_2/M-phase in a synchronized manner.

INTRODUCTION

N-Hydroxy-*N'*-aminoguanidine derivatives have been synthesized recently (1–3) and proven active against cancer cells and viral infections (2–4). These compounds are inhibitors of ribonucleotide reductase as it was demonstrated for the isolated enzyme from Novikoff rat tumors (1) as well as for the enzyme in L1210 cells (4). Ribonucleotide reductase controls cell proliferation by catalyzing the 2'-reduction of ribonucleoside 5'-diphosphates, the rate-limiting step en route to DNA synthesis (5, 6). Accordingly, a series of 12 differently substituted HAG[3] derivatives inhibited growth of L1210 cells, the I_{50} values being closely correlated with the respective drug concentration required for 50% inhibition of ribonucleotide reductase activity (4).

In this paper, we further characterize the isoquinoline derivative of HAG since it is the most potent inhibitor of the 12 drugs tested (3, 4) and compare it with the structurally related quinoline derivative (Fig. 1). The study focuses on drug effects on intracellular targets, cell viability, and cell cycle of L1210 mouse leukemia cells.

MATERIALS AND METHODS

Materials. HAG derivatives were synthesized and characterized by Lien's group (1–3). Trypan blue was provided by Matheson Coleman & Bell (Norwood, OH). Seakem ME Agarose came from FMC Co. (Rockland, ME). Propidium iodide and RNase (90 Kunitz U/mg) were purchased from Calbiochem (La Jolla, CA). [U-^{14}C]Cyd (450 Ci/mol) was supplied by Research Products International Co. (Mount Prospect, IL).

Labeling Experiments. The L1210 mouse leukemia cells were purchased from American Culture Collection (Rockville, MD). In 6-week intervals, the cells were cultured from thawed stocks kept in liquid nitrogen. Using the BRL Myco Tect test (Gaithersburg, MD) the cells were routinely checked for mycoplasma. The cells were suspended in RPMI 1640 medium supplemented with 10% horse serum (v/v) and sodium bicarbonate (2 g/liter), all of which came from Grand Island Biological Co. (Grand Island, NY). Additionally, the medium contained gentamycin (50 mg/liter) supplied by Sigma Chemical Co. (St. Louis, MO).

For the 2-h experiments L1210 cells in log phase were harvested and resuspended in fresh medium at a density of 3–5 × 10^6 cells/ml. After a 30-min preincubation at 37°C the cells were added to plastic flasks containing the drug. Physiological sodium chloride solution containing 2% dimethyl sulfoxide was used as the solvent for the HAG derivatives and was also added to control cultures so that the final dimethyl sulfoxide concentration was 0.03% throughout. The incubation was at 37°C, and in the final 30 min the cells were exposed to [^{14}C]Cyd (0.13 μCi/ml). Using a modified Schmidt-Thannhauser procedure (4, 7) cells were extracted with perchloric acid (6%) and the pellet further treated with NaOH. This allowed determination of radioactivity incorporated into DNA and RNA (acid-insoluble fraction) as well as into the dCyd pool (acid-soluble fraction). Conversion of [^{14}C]Cyd to [^{14}C]dCyd is a measure of intracellular ribonucleotide reductase activity (8).

The 24-h experiments were started with a seeding concentration of 2 × 10^5 cells/ml so that the control cells approximately doubled twice and were still in log phase when [^{14}C]Cyd was added. The incubation was carried out under sterile conditions at 37°C in a humidified atmosphere of 90% air/10% CO_2. After 23 h the cell concentrations were adjusted to the value that was determined in the culture with the most pronounced growth inhibition: aliquots of the cell suspensions were counted in a Coulter Counter (model ZBI). If required, a calculated volume of the cell suspension was removed leaving equal amounts of cells in all flasks. The suspensions removed were centrifuged at 1500 rpm for 7 min and the cell-free supernatants were added back to the respective flasks. At 23.5 h the cells were pulse-labeled with [^{14}C]Cyd (1.5 μCi/ml). Further processing of the cells for the determination of [^{14}C]Cyd incorporation into DNA, RNA, and dCyd was as described for the 2-h experiment. Initial experiments without adjustment of the cell concentrations resulted in an increase of labeling per 10^6 cells with increasing drug concentrations.

When HPLC analyses were performed the perchloric acid extracts of the cells were split. One half was neutralized for HPLC while the other half was lyophilized, taken up in 0.1 M Tris, pH 8.0, and treated with snake venom prior to passage over Dowex-1-borate for analysis of the radioactivity in the dCyd pool (4).

Colony-forming Assays. In plastic trays with 2-ml wells, L1210 cells were exposed to the drugs for 24 and 48 h at 37°C in a humidified atmosphere of 90% air/10% CO_2. Cell growth was determined with a Coulter Counter and 1 ml of each suspension was processed for plating (9). The cells were washed with drug-free medium and resuspended (750 cells/ml medium). In the meantime, plastic petri dishes (60 mm in diameter) were coated with an agar layer. This and the other layers consisted of RPMI 1640 medium, 20% horse serum, and 0.24% Seakem ME agar. After solidification of the bottom layer, the middle layer (3 ml) containing 75 cells was poured, allowed to solidify, and then covered

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[3] The abbreviations used are: HAG, *N*-hydroxy-*N'*-aminoguanidine; Cyd, cytidine; dCyd, deoxycytidine; HPLC, high-performance liquid chromatography.

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with the top layer (3 ml). The dishes were incubated for 3 weeks at 37°C in a humidified atmosphere of 90% air/10% CO_2. Finally, colonies were determined by use of a New Brunswick Bactronic Colony Counter. In control cultures, 70 and 20% of the cells plated after incubations of 24 and 48 h, respectively, formed colonies.

Viability by Trypan Blue. The staining solution consisted of trypan blue [4 g/liter of phosphate buffered saline (Dulbecco)]. Cell suspensions (0.5 ml) were mixed with 0.2 ml of staining solution and analyzed within 30 min using a hemocytometer. The viability was defined as the ratio of unstained cells over total cells counted.

HPLC Analyses. Neutralized perchloric acid extracts (80 µl, corresponding to 6×10^5 cells) were analyzed on a Partisil 10 SAX column (Whatman, Clifton, NJ). Ammonium phosphate buffer was used as mobile phase with a flow rate of 2 ml/min (10). The starting buffer was 10 mM, pH 2.8, and the final buffer was 500 mM, pH 4.8; the 35-min gradient was linear. The nucleotides were detected at 254 nm and the quantitation was done on the basis of response factors using a computing integrator. These were obtained with standard nucleotides analyzed in the same system.

Cell Cycle Experiments. L1210 cells were exposed to the isoquinoline derivative as described above. At the times indicated 10^6 cells were pelleted at 1000 rpm and 6 min. After pouring off the supernatant the cells were resuspended in the remaining fluid (approximately 100 µl) and mixed with 0.5 ml of staining solution (3.4 mM sodium citrate plus 0.05 mg/ml propidium iodide) (11). This suspension was kept 10 min at 0°C in the dark. RNase was added to give a final activity of 4 U/ml and the incubation was continued at room temperature for 30 min. Before flow cytometry, the stained cells were kept overnight in the refrigerator. The analyses were carried out with an EPICS C flow cytometer (Coulter Electronics, EPICS Division, Hialeah, FL) with a 2-W argon laser. The excitation wavelength was set at 488 nm. The ungated fluorescence signals of 20,000 stained L1210 cells were accumulated and expressed as a linear histogram of red fluorescence.

RESULTS

Intracellular Targets of *N*-Hydroxy-*N'*-aminoguanidine Derivatives. L1210 cells were exposed to the isoquinoline or the quinoline derivative of HAG for 2 and 24 h (Figs. 2 and 3). After pulse-labeling these cells with [^{14}C]Cyd, the incorporation of radioactivity into DNA, RNA, and the dCyd pool was quantitated. Both drugs inhibited ribonucleotide reductase as indicated by the decreased labeling of the dCyd pool. The inhibition was concentration-dependent and was observed after the short and the extended incubation period. Under each condition of Figs. 2 and 3, DNA synthesis was reduced by both drugs. As compared to ribonucleotide reductase activity, DNA synthesis appeared always to be slightly more sensitive to the treatment. RNA synthesis differed from DNA synthesis in that the incorporation of [^{14}C]Cyd into RNA was almost unaffected by the 2-h exposure to either drug. However, the extended incubation period led to an inhibition of the RNA labeling comparable to that observed with DNA. The comparison of Figs. 2 and 3 shows that the isoquinoline derivative of HAG was more inhibitory than the quinoline derivative to the parameters tested.

Cytotoxicity. In order to test the capacity of the HAG derivatives to kill cells, L1210 cells were incubated for 48 h in the presence of the quinoline or the isoquinoline compound before plating in soft agar. Under these conditions, the drug markedly diminished the formation of colonies. The cytotoxic and the cytostatic effects were observed in a similar concentration range. For 50% growth inhibition, 3 and 14 µM of the isoquinoline and the quinoline derivative, respectively, were required as compared to 6 and 17 µM necessary for a 50% loss in viability. Clonogenicity of cells treated with the isoquinoline compound was also tested after a 24-h exposure. The I_{50} values for cell growth and colony formation were 2 and 10 µM,

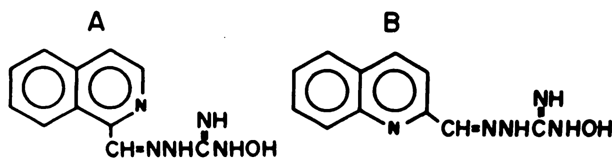

Fig. 1. Structures of the 1-isoquinolylmethylene (*A*) and 2-quinolylmethylene (*B*) derivatives of HAG.

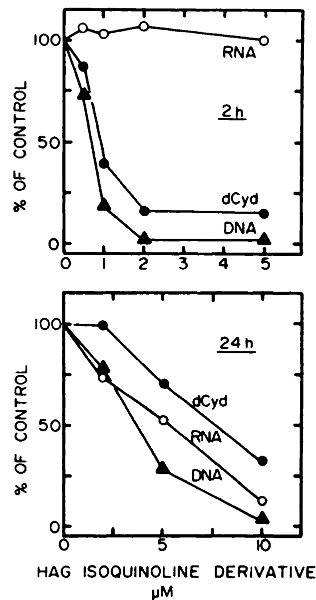

Fig. 2. Effects of the HAG isoquinoline compound on ribonucleotide reductase activity and nucleic acid synthesis in L1210 cells. Cells were exposed to the drug for 2 or 24 h and pulse labeled with [^{14}C]Cyd during the final 30 min of the incubation. An acid-soluble and an acid-insoluble fraction were prepared from the cells and further processed according to a modified Schmidt-Thannhauser procedure (4, 7). This allowed separate determination of the amount of radioactivity incorporated into DNA, RNA, and into the dCyd pool. The ratio of [^{14}C]dCyd over [^{14}C]Cyd represents cellular ribonucleotide reductase activity. Shown are the mean values of triplicate experiments. Control values (cpm/10^6 cells) were 11010 (RNA), 920 (DNA), and 130 (dCyd) for the 2-h incubation as compared to 21010 (RNA), 2030 (DNA), and 330 (dCyd) in the 24-h incubation.

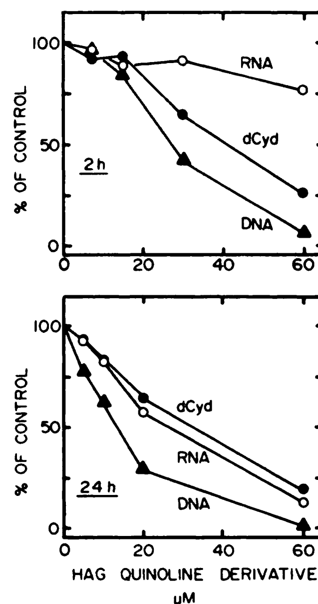

Fig. 3. Ribonucleotide reductase activity and nucleic acid synthesis in L1210 cells treated with the HAG quinoline derivative. Incubation and analysis was carried out exactly as described in Fig. 2. Control values (cpm/10^6 cells) were 25960 (RNA), 2050 (DNA), and 280 (dCyd) for the 2-h incubation as compared to 19260 (RNA), 3040 (DNA), and 480 (dCyd) after the 24-h incubation.

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respectively (data not shown). For comparison, the determination of cell viability by the uptake of trypan blue resulted in an I_{50} value of 3 μM for a 36-h exposure to the isoquinoline derivative.

These results were in line with HPLC measurements of the nucleoside 5'-triphosphate content of drug-treated cells. At 10 μM of the isoquinoline derivative, the cellular contents of ATP, GTP, CTP, and UTP dropped to 4, 11, 4, and 2% of those of untreated control cells, respectively.

Effect of the Isoquinoline Derivative on Cell Cycle. G_1/early S cells express the highest level of ribonucleotide reductase activity, M and early G_1 the lowest (12, 13). We therefore characterized the time and concentration dependency of effects exerted by the HAG isoquinoline compound on the cell cycle (Fig. 4). Each drug concentration (0.5, 1, 2, and 10 μM) used caused an increase of G_0/G_1 cells at the expense of G_2/M and S cells within the first 4 h of exposure. At the lowest drug concentration cells were released from the block between G_1 and early S after 4 h of incubation, and traversed S phase in a synchronized manner to arrive in G_2/M-phase; after one cycle period these cultures exhibited histograms comparable to those of controls (Fig. 4). In the presence of 1 and 2 μM of the drug the arrest of cells in G_0/G_1-phase was extended to 8 and 12 h, respectively. The subsequent depletion of G_0/G_1 cells was more pronounced and lasted longer than under 0.5 μM. Again, the cells progressed highly synchronized through S- and G_2/M-phases. By 48 h, S and G_2/M cells were still above and G_0/G_1 cells were below control values. The highly toxic concentration of 10 μM (Fig. 2) blocked the cells for at least 48 h. Thus, an increased G_0/G_1 fraction as well as decreased G_2/M and S fractions were characteristic of each histogram obtained after several periods of exposure to 10 μM of the drug (Fig. 4).

DISCUSSION

In order to define the mechanism underlying the antiproliferative effect of HAG derivatives (4), labeling experiments after short as well as extended exposure periods were carried out. The results presented in Figs. 2 and 3 indicate that the inhibition of ribonucleotide reductase activity and DNA synthesis is a long-lasting event with an early onset. This finding could account for the observed cytostatic and cytotoxic properties since the drug concentrations for inhibition of cell growth and clonogenicity are similar to those required for inhibition of DNA synthesis and ribonucleotide reductase activity (Figs. 2 and 3). After short and extended periods of exposure to either the HAG isoquinoline or the quinoline derivative, the concentration dependency curve for the inhibition of the reductase always followed the respective curve for the inhibition of DNA synthesis (Figs. 2 and 3). This enhanced susceptibility of DNA synthesis towards inhibition agrees with previous investigations showing a sigmoidal relationship between the concentrations of deoxynucleoside triphosphates and the rate of DNA synthesis (14).

It is a common feature of inhibitors of ribonucleotide reductase that they inhibit DNA synthesis because of deoxynucleotide depletion but leave RNA synthesis unaffected (14). This inhibition pattern was also confirmed for the HAG derivatives after a 2-h exposure period (Figs. 2 and 3). However, extension of the incubation time to 24 h strongly reduced the labeling of RNA by [^{14}C]Cyd. The decrease in RNA synthesis is probably secondary to the long-lasting block of DNA formation. In addition, ATP, GTP, UTP, and CTP were markedly decreased after the extended incubation period.

While earlier studies established the antiproliferative action of HAG derivatives (2–4) this report extends these data by demonstrating that the drug treatment is also cytotoxic. The differences between the concentrations required for the cytostatic and the cytotoxic effects are small and confirm the potential of these agents for cancer chemotherapy.

The isoquinoline and the quinoline compound share the same intracellular targets and act with a similar time dependency. However, independent of the parameter tested, cell growth,

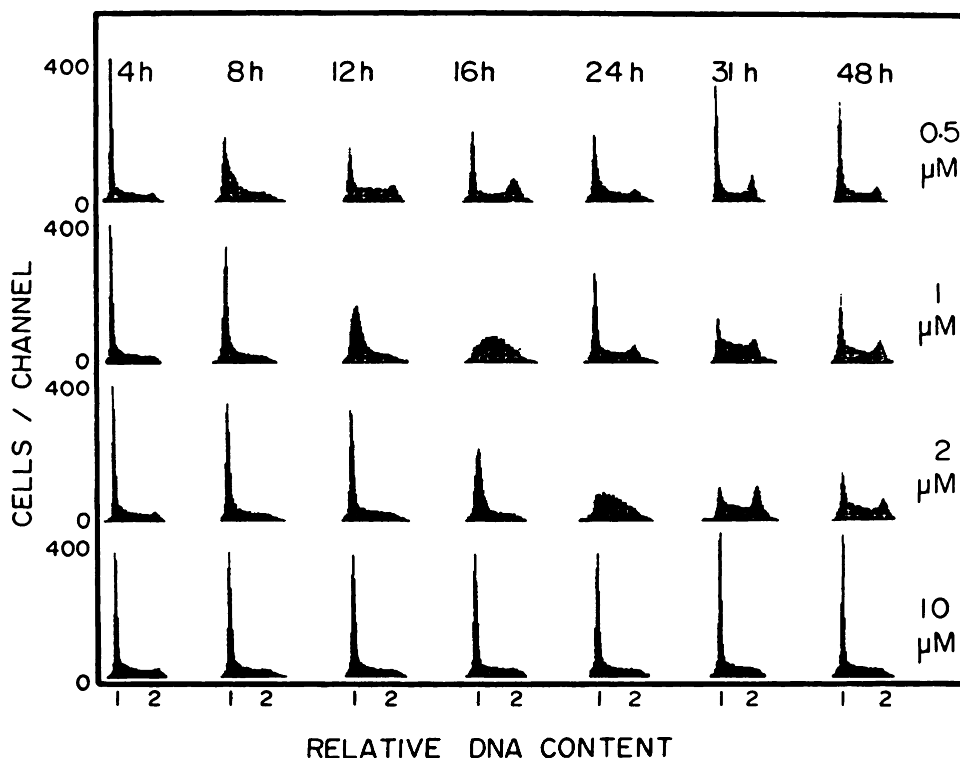

Fig. 4. Effects of the HAG isoquinoline derivative on the cycle of L1210 cells. The cells were exposed to the drug for the various times indicated, harvested, stained with propidium iodide and analyzed by flow cytometry. Control histograms obtained from untreated cultures at each of the times indicated showed the same pattern as the histogram at 0.5 μM/48 h.

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colony formation, ribonucleotide reductase activity, or nucleic acid synthesis, the isoquinoline compound was always more inhibitory than the quinoline derivative. This relationship was basically also demonstrated for the structurally similar thiosemicarbazone derivatives (15). 1-Formylisoquinoline thiosemicarbazone is a much stronger inhibitor of ribonucleotide reductase activity and DNA synthesis than the 2-formylguinoline derivative.

In the cell cycle studies, exposure to the HAG isoquinoline compound induced an expansion of the G_0/G_1 fraction and a concomitant loss of S and G_2/M cells. This initial shift in the fraction sizes was largely reversible between 0.5 and 2 μM, but it remained unchanged up to 48 h in the presence of 10 μM of the drug. Even at the highest concentration used (10 μM), cells are apparently able to complete mitosis and enter G_1-phase (Fig. 4). The increase of G_0/G_1 cells and the failure to replenish M cells indicate that the cell cycle block induced by 10 μM of the HAG isoquinoline derivative affects the whole S-phase. The time period in which the cells are reversibly arrested is a function of the drug concentration. This gives rise to the array of histograms in Fig. 4 where histograms showing a similar DNA distribution are found on a diagonal as best seen with 0.5 μM/8 h, 1 μM/12 h, and 2 μM/16 h. In the concentration range 0.5–2 μM, the drug strongly inhibited ribonucleotide reductase activity and DNA synthesis in L1210 cells after a 2-h exposure (Fig. 2), but it had negligible effects at 24 h (Fig. 2) or 36 h (data not shown). This might suggest a sequence of events leading from inhibition of ribonucleotide reductase via diminished DNA synthesis to a block of cells in S-phase. The reversibility of the action of the HAG isoquinoline compound suggests that L1210 cells metabolize the drug. The treatment with the HAG isoquinoline compound gave results comparable to the DNA histograms of CCRF-CEM cells treated with the ribonucleotide reductase inhibitor hydroxyurea (16, 17).

In conclusion, the HAG compounds potently inhibit ribonucleotide reductase activity and nucleic acid synthesis which account for their cytostatic and cytotoxic effects. The capacity of the HAG isoquinoline derivative to synchronize tumor cells might be utilized in combination with chemotherapeutic agents that predominantly kill cells in S- or G_2/M-phase.

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