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INHIBITORS OF RIBONUCLEOTIDE REDUCTASE

COMPARATIVE EFFECTS OF AMINO- AND HYDROXY-SUBSTITUTED PYRIDINE-2-CARBOXALDEHYDE THIOSEMICARBAZONES

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Abstract—A new series of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones (HCTs) was studied for their effects on L1210 cell growth in culture, cell cycle transit, nucleic acid biosynthesis and ribonucleotide reductase activity. 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) and 3-amino-4-methylpyridine-2-carboxaldehyde thiosemicarbazone (3-AMP) were the most active compounds tested with respect to inhibition of cell growth and ribonucleotide reductase activity. 5-Aminopyridine-2-carboxaldehyde thiosemicarbazone (5-AP) and 4-methyl-5-aminopyridine-2-carboxaldehyde thiosemicarbazone (5-AMP) were slightly less active. 3-AP, 3-AMP, 5-AP and 5-AMP inhibited the incorporation of [^{3}H]thymidine into DNA without affecting the rate of incorporation of [^{3}H]uridine into RNA. The uptake and incorporation of [^{14}C]cytidine into cellular ribonucleotides and RNA, respectively, were not decreased by 3-AP or 3-AMP; however, the incorporation of cytidine into DNA via ribonucleotide reductase was inhibited markedly. Thus, a pronounced decrease in the formation of [^{14}C]deoxyribonucleotides from radioactive cytidine occurred in the acid-soluble fraction of 3-AP- and 3-AMP-treated L1210 cells. Consistent with an inhibition of DNA replication that occurred at relatively low concentrations of 3-AP and 3-AMP, cells gradually accumulated in the S-phase of the cell cycle; at higher concentrations of 3-AP and 3-AMP, a more rapid accumulation of cells in the G_0/G_1 phase of the cell cycle occurred, with the loss of the S-phase population, implying that a second less sensitive metabolic lesion was created by the HCTs. N-Acetylation of 3-AMP resulted in a compound that was 10-fold less active as an inhibitor of ribonucleotide reductase activity and 8-fold less active as an inhibitor of L1210 cell growth. N-Acetylation of either 5-AP or 5-AMP did not alter the inhibitory properties of these compounds. The results obtained provide an experimental rationale for the further development of the HCTs, particularly 3-AP and 3-AMP, as potential drugs for clinical use in the treatment of cancer.

Key words: α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone; ribonucleotide reductase; L1210 cells; substituted pyridine-2-carboxaldehyde thiosemicarbazones; CDP reductase; inhibition of DNA synthesis

Ribonucleotide reductase, the enzyme that catalyzes the rate-limiting reaction in the *de novo* synthesis of the 2′-deoxyribonucleoside 5′-triphosphates required for DNA synthesis, is an important metabolic target for the development of cancer chemotherapeutic agents [1, 2]. Several different classes of agents that are relatively specific inhibitors of ribonucleotide reductase have been synthesized. These have included HCTs§ [3–11], *N*-hydroxy-*N*′-amino-guanidine derivatives [12–17], and polyhydroxybenzohydroxamates [18–20]. Of these, only 5-HP [21, 22] and Didox [23] have been entered into clinical trials. Although 5-HP, the only HCT to undergo clinical evaluation, exhibited significant antineoplastic activity against a spectrum of transplanted murine tumors, as well as against spontaneous lymphomas in dogs [24], in phase I studies it produced only transient decreases in peripheral blast counts in 24% of leukemic patients and no antitumor effects were noted in patients with

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§ Abbreviations: HCT, α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone; 3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AMP, 3-amino-4-methylpyridine-2-carboxaldehyde thiosemicarbazone; 3-AAMP, 3-*N*-acetylamino-4-methylpyridine-2-carboxaldehyde thiosemicarbazone; 3-HP, 3-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; 3-HMP, 3-hydroxy-4-methylpyridine-2-carboxaldehyde thiosemicarbazone; 5-AP, 5-aminopyridine-2-carboxaldehyde thiosemicarbazone; 5-AAP, 5-*N*-acetylaminopyridine-2-carboxaldehyde thiosemicarbazone; 5-AMP, 4-methyl-5-aminopyridine-2-carboxaldehyde thiosemicarbazone; 5-AAMP, 4-methyl-5-*N*-acetylaminopyridine-2-carboxaldehyde thiosemicarbazone; 5-HP, 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; 5-HMP, 4-methyl-5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; 5-HAMP, 4-methyl-5-*N*-hydroxylaminopyridine-2-carboxaldehyde thiosemicarbazone; DTT, dithiothreitol; NHI subunit, non-heme iron subunit of ribonucleotide reductase; EB subunit, effector-binding subunit of ribonucleotide reductase; MTS, 5- (3-carboxymethoxyphenyl)-2- (4,5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium, inner salt; PMS, phenazine methosulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Didox, 3,4-dihydroxybenzohydroxamic acid; MAIQ-1, 4-methyl-5-aminoisoquinoline-1-carboxaldehyde thiosemicarbazone; and MEM, minimum essential medium.

solid tumors [21, 22]. The primary toxicity of 5-HP was gastrointestinal, with myelosuppression, hemolysis, anemia, hypertension and hypotension occurring at the highest dose levels tested. No further clinical trials were conducted with this HCT. The minimal antileukemic activity of 5-HP observed in the phase I trial, which contrasted with the results obtained with transplanted tumors, appeared primarily due to rapid formation and elimination of an *O*-glucuronide conjugate.

Currently, the only known ribonucleotide reductase inhibitor in clinical use is hydroxyurea [25], which is a relatively poor inhibitor of the enzyme and, in addition, has a short serum half-life [25, 26]. Nonetheless, the utility of hydroxyurea suggests that other more potent inhibitors of ribonucleotide reductase might well be useful antineoplastic agents, particularly against rapidly proliferating cancers.

A variety of nucleoside analogs has also been generated as inhibitors of ribonucleotide reductase. These include 2′,2′-difluoro-2′-deoxycytidine [27–29], 2-fluoroadenine arabinoside [30] and 2-chloro-2′-deoxyadenosine [31], which inhibit ribonucleotide reductase following conversion to the nucleotide level. However, the 5′-triphosphate of each of these compounds appears to be a more potent inhibitor of DNA polymerase activity than of ribonucleotide reductase, making DNA polymerase and the incorporation of the analog into DNA the primary targets that are most important for the production of cytotoxicity [30–32].

In the present study, a new series of amino- and hydroxy-substituted pyridine-2-carboxaldehyde thiosemicarbazones [33, 34] has been examined. The results demonstrated that these compounds are potent inhibitors of the growth of L1210 cells in culture and that their primary action is at the level of the biosynthesis of DNA, exerted through the inhibition of ribonucleotide reductase activity.

MATERIALS AND METHODS

RPMI 1640 and MEM culture media, horse serum, calf serum and sodium bicarbonate were purchased from the Grand Island Biological Co. (Grand Island, NY). MTS [35] was a gift from Dr. Terence C. Owen, Department of Chemistry, University of South Florida (Tampa, FL). [U-^{14}C]Cytidine (400 mCi/mmol) was purchased from the Research Products International Corp. (Mount Prospect, IL). [U-^{14}C]CDP (517 mCi/mmol) was purchased from the New England Nuclear Corp. (Boston, MA), and [*methyl*-^{3}H]thymidine (5 mCi/mmol) and [5, 6-^{3}H]-uridine (42 Ci/mmol) were obtained from the Amersham Co. (Arlington Heights, IL). The unlabeled nucleosides, kanamycin, MEM non-essential amino acids, other biochemicals and snake venom (*Crotalus atrox*) were purchased from the Sigma Chemical Co. (St. Louis, MO).

Preparation of HCTs. The HCTs were prepared and characterized as previously described [33, 34].

Growth of L1210 cells in culture. L1210 cells were grown in RPMI 1640 medium supplemented with 10% horse serum, sodium bicarbonate (2 g/L) and gentamicin sulfate (50 mg/L) in a humidified incubator at 37° with 95% air/5% CO_2. The hydroxyurea-resistant subline (L1210/HU), which was generated and characterized previously [36], was maintained in the same culture medium supplemented with 750 μM hydroxyurea. These hydroxyurea-resistant cells were shown to be resistant to hydroxyurea and pyrazoloimidazole but retained sensitivity to MAIQ-1. These cells had elevated levels of ribonucleotide reductase activity with increased levels of the mRNA for the NHI subunit and amplification of the gene for the NHI subunit [37]. Before incubation in the presence of HCTs, cells were collected by centrifugation, resuspended in fresh culture medium containing no hydroxyurea, and grown overnight in the absence of drugs.

Microtiter assay for inhibition of L1210 cell growth. L1210 cell growth was assayed using the MTS/PMS method of Cory *et al.* [38]. The formazan product of MTS is water-soluble and easily measured at 492 nm in a 96-well plate reader. The 96-well plates were seeded with 2000 cells/well (150 μL) on day zero. The HCTs at various concentrations were added 24 hr later in a volume of 50 μL; 72 hr later, MTS/PMS was added and plates were incubated at 37° and read at 492 nm 2–3 hr later as previously described [38]. The 96-well plates were established with two rows of control cells (no drugs); before the addition of MTS/PMS, the contents of one row of control cells were removed from the wells and pooled, and cell numbers were determined using a Coulter particle counter, model ZF (Coulter Electronics, Hialeah, FL). This permitted the absorbance of the formazan product of control cells to be related to an actual cell count. In a typical experiment, the cells in the control wells grow to a concentration of 200,000–300,000 cells/well (approximately seven doublings). IC_{50} values were determined for each drug in at least three separate experiments. In each experiment, each drug concentration was tested in six separate wells, and at least five different concentrations of each drug were used to determine the IC_{50} value.

[^{14}C]*Cytidine metabolism in L1210 cells.* L1210 cells in log-phase growth were collected by centrifugation and resuspended in 10 mL of fresh culture medium at a concentration of 2.5×10^6 cells/mL. Cells were incubated for 2 hr in the presence and absence of 3-AP or 3-AMP in a volume of 10 mL in duplicate flasks. [^{14}C]Cytidine (400 mCi/mmol, 2 μCi/flask) was added, and the incubation was continued for 1 hr. At this time, cells were collected by centrifugation and subjected to the Schmidt–Thannhauser procedure to separate acid-soluble nucleotides, RNA and DNA [39]; aliquots of each fraction were taken for radioactivity measurements. The acid-soluble nucleotide fraction was neutralized with KOH, centrifuged to remove $KClO_4$, and the supernatant fluid lyophilized. The lyophilized material was dissolved in Tris–HCl buffer and then treated with snake venom; the resulting deoxycytidine was separated from cytidine on Dowex-1-borate columns [40].

Preparation of ribonucleotide reductase. Ehrlich ascites tumor cells adapted for growth in tissue culture were obtained through the courtesy of Dr. Richard Panniers, National Institutes of Health.

Ehrlich cells were grown in suspension in 5-L bottles at 37° in MEM supplemented with 20 mM MOPS, 100,000 U of kanamycin, MEM non-essential amino acids and 3.75% calf serum at a final pH of 7.4 in a New Brunswick gyratory shaker. The cells were grown to a density of 0.8 to 1.2×10^6 cells/mL before collection by centrifugation. Cell pellets were washed with PBS, frozen and stored at −83°. Frozen Ehrlich cells were suspended in 2 vol. of 0.02 M Tris–HCl buffer, pH 7.0, containing 1 mM DTT. The cell suspension was subjected to homogenization using a Brinkmann polytron at full power for 2 min with cooling on ice, and the homogenate was centrifuged for 1 hr at 20,000 *g*. The supernatant fluid was treated with streptomycin sulfate (0.65% final concentration) and the resulting suspension centrifuged at 20,000 *g*. The supernatant fluid was treated with solid ammonium sulfate to reach 40% saturation, and the precipitate obtained by centrifugation was dissolved in a minimum volume of 0.02 M Tris–HCl buffer, pH 7.0, containing 1 mM DTT. This solution was dialyzed against the same buffer with a minimum of three changes of buffer over 3–12 hr; this fraction contained the ribonucleotide reductase activity.

The ammonium sulfate fraction (0–40%) was loaded onto a blue dextran-Sepharose column to separate the NHI and EB subunits by previously described methodology [41]. The NHI subunit was eluted from the blue dextran-Sepharose column with 0.02 M Tris–HCl pH 7.0, containing 1 mM DTT, concentrated by ammonium sulfate precipitation (80% saturation) and dialyzed. The blue dextran-Sepharose column was then washed with 0.02 M Tris–HCl, pH 7.0, containing 1 mM DTT and 125 mM NaCl, which resulted in the elution of a protein peak that was inactive as either the NHI or EB subunit and was discarded. Finally, the blue dextran-Sepharose column was washed with 0.02 M Tris–HCl, pH 7.0, containing 1 mM DTT and 250 mM NaCl, which eluted a fraction containing the EB subunit. This fraction was concentrated by ammonium sulfate precipitation (80% saturation) and dialyzed. The fractions containing the separated NHI and EB subunits were quick frozen in an acetone–dry ice bath and stored at −83°.

The NHI fraction was further purified by chromatography on DEAE-cellulose. The NHI fraction was loaded onto a DEAE-cellulose column and the column was washed with 0.02 M Tris–HCl, pH, 7.0, containing 1 mM DTT. The active fraction was eluted with 0.09 M Tris–HCl, pH 7.0, containing 1 mM DTT; this fraction did not contain nucleotidase or kinase activities, which would consume the substrates and/or effectors. Furthermore, neither fraction alone (i.e. the NHI or the EB subunit) had ribonucleotide reductase activity. Only when the two subunits were combined was ribonucleotide reductase activity generated.

Assay of CDP reductase activity. CDP reductase activity was assayed by the method of Steeper and Steuart [42] in a volume of 0.15 mL containing 3.75 nmol [^{14}C]CDP (0.03 μCi); 150 nmol ATP; 900 nmol DTT; and aliquots of the NHI and EB subunit fractions. The CDP reductase reaction was carried out for 30 min at 37° and terminated by heating in a boiling water bath for 4 min. After snake venom treatment to convert nucleotides to the nucleoside level [39], deoxycytidine was separated from cytidine on a Dowex-1-borate column.

Cell cycle analysis. L1210 cells in culture were washed with PBS at various times after treatment with an HCT, fixed in 70% ethanol, treated with 0.5 μM/mL of RNase A for 1 hr and stained with 50 μg/mL of propidium iodide. Each cyto-fluorometric assay was performed with 5×10^4 cells, and the percentage of cells in various phases of the cell cycle was calculated by the method of Jett [43].

[^{3}H]*Thymidine and* [^{3}H]*uridine incorporation.* The effects of HCTs on DNA and RNA synthesis were measured by the exposure of L1210 cells to 0.1 μM [^{3}H]thymidine (0.5 μCi/mL) or [^{3}H]uridine (0.5 μCi/mL) for 30 min at 37° in the presence of graded concentrations of drugs. Cells were then washed with ice-cold PBS and nucleic acids were precipitated onto Whatman GF/C filters using ice-cold 10% trichloroacetic acid. After three washes of the filters with 5 mL of ice-cold trichloroacetic acid and two washes with 95% ethanol, filters were dried and incubated with 0.25 mL of 4 N HCl at 65° for 1 hr in scintillation vials. After addition of 0.4 mL of 2.5 N NaOH and subsequent incubation for another hour, 5 mL of Ultima Gold scintillation fluid (Packard Instrument Co., Meriden, CT) was added, and the radioactivity of each sample was determined using a Beckman LS 7000 liquid scintillation spectrometer. Incorporation of [^{3}H]thymidine in untreated control cells ranged from 80,000 to 120,000 cpm/10^6 cells and incorporation of [^{3}H]uridine ranged from 45,000 to 60,000 cpm/10^6 cells in individual experiments.

Protein determination. The protein concentration of the cell-free extracts was determined by the method of Lowry *et al.* [44] using bovine plasma gamma globulin (Bio-Rad, Richmond, CA) as the standard.

RESULTS

The comparative effects of various amino- and hydroxy-derivatives of pyridine-2-carboxaldehyde thiosemicarbazone (Fig. 1) on the growth of wild-type and hydroxyurea-resistant L1210 cell lines were measured, and the results are shown in Table 1. The most active HCTs evaluated were 3-AP and 3-AMP, which had IC_{50} values of 1.3 and 1.5 μM, respectively. While the sensitivities of parental and hydroxyurea-resistant L1210 cells to 3-AP, 3-AMP, 3-HP, 5-AP and 5-AMP were similar, the IC_{50} values for 3-HMP, 5-HP, 5-HMP and 5-HAMP were 2- to 3-fold greater in the hydroxyurea-resistant cells.

The effects of the two most potent HCT inhibitors of the growth of L1210 cells, 3-AP and 3-AMP, on the cell cycle distribution of L1210 cells were measured, and the results are shown in Fig. 2. At a relatively low concentration of 3-AP or 3-AMP (1 μM), which approximated the IC_{50} value for cell growth, an accumulation of cells in the S-phase of the cell cycle occurred. This was particularly evident with 3-AP, with 90% of the cell population accumulating in the S-phase after 24 hr of exposure to this agent. At a higher concentration of 3-AP or

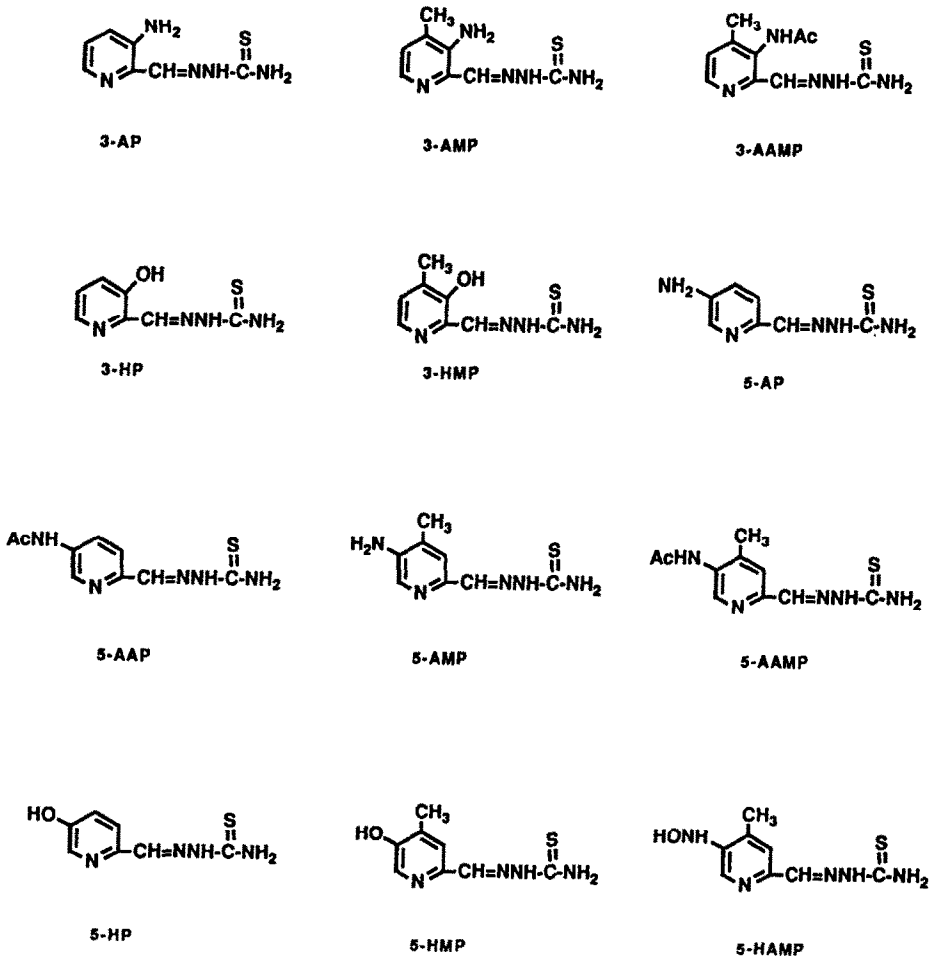

Fig. 1. Structures of amino- and hydroxy-derivatives of pyridine-2-carboxaldehyde thiosemicarbazones.

3-AMP, cells accumulated in the G_0/G_1 phase of the cell cycle, with the loss of cells in the S-phase of the population, suggesting that a second less sensitive metabolic lesion was created by the HCTs. These changes were observed as early as 6 hr after exposure of cells to 3-AP or 3-AMP.

Table 1. Comparison of the effects of HCTs on the growth of wild-type and hydroxyurea-resistant L1210 cells

	$IC_{50}(\mu M)$	
Compound	L1210	L1210/HU
3-AP	1.3 ± 0.5 (4)*	1.6 ± 0.1 (2)
3-AMP	1.5 ± 0.3 (4)	2.3 ± 0.8 (2)
3-HP	2.2 ± 0.6 (2)	3.8 ± 0.7 (2)
3-HMP	3.6 ± 0.6 (2)	7.8 ± 1.7 (2)
5-AP	3.0 ± 0.6 (5)	3.7 ± 0.9 (2)
5-AMP	2.8 ± 0.9 (5)	3.1 ± 0.2 (2)
5-HP	4.2 ± 0.8 (2)	12.0 ± 2.0 (2)
5-HMP	4.9 ± 1.9 (2)	15.1 ± 0.9 (2)
5-HAMP	8.2 ± 1.6 (2)	27.3 ± 1.0 (2)

* Numbers in parentheses are the number of individual determinations made. With 4 or 5 experiments, values are shown as means ± SD; with 2 experiments, the average ± range is given.

The primary metabolic lesion created by HCTs is interference with the biosynthesis of DNA, and this action is primarily due to potent inhibition of ribonucleotide reductase activity [9]. For this reason, the capacity of the amino- and hydroxy-substituted pyridine-2-carboxaldehyde thiosemicarbazones to inhibit CDP reductase activity was measured, and the results are shown in Table 2. In agreement with the effects of these agents on the growth of L1210 cells, 3-AP and 3-AMP were the most active of the HCTs as inhibitors of CDP reductase activity, causing 50% inhibition of enzymatic activity at a concentration of 0.3 μM. As such, they were 4–6 times more potent than the corresponding 3-hydroxy-substituted derivatives. In contrast, 5-AP and 5-AMP were only slightly more effective than the corresponding derivatives, 5-HP and 5-HMP. While 3-AP and 3-AMP were more active than 5-AP and 5-AMP, there did not appear to be pronounced differences between 3-HP and 3-HMP and 5-HP and 5-HMP.

Since acetylation of the amino function of amino-substituted pyridine-2-carboxaldehyde thiosemicarbazones eliminated the antileukemic effects of these HCTs in L1210-bearing mice [34], the effects of acetylation of amino-substituents on the growth

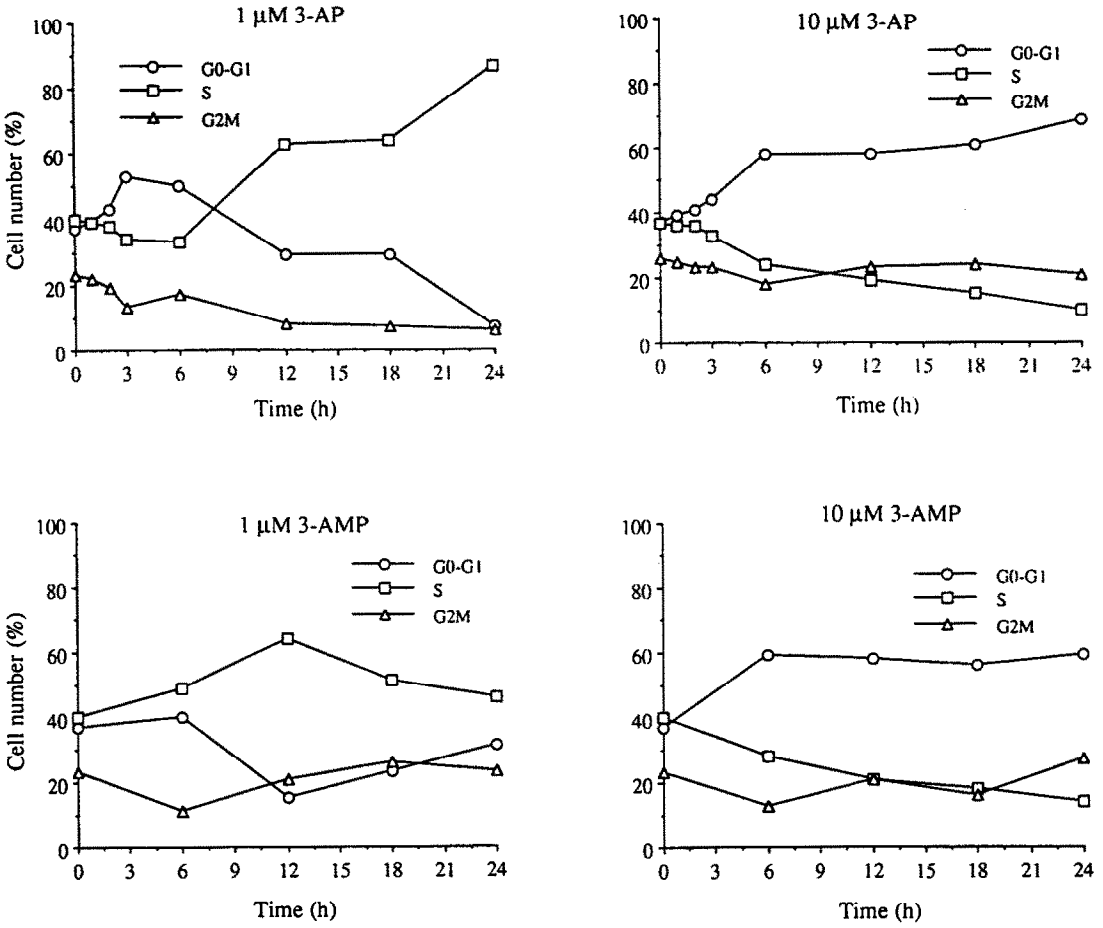

Fig. 2. Effects of various concentrations of 3-AP and 3-AMP on the cell cycle distribution of L1210 cells. Cells were exposed to the indicated concentrations of HCTs for different times, washed with PBS, fixed with 70% ethanol, treated with RNase, stained with propidium iodide and analyzed by flow cytometry. Data are the average of two independent experiments in which each point was determined in triplicate.

of L1210 cells and on ribonucleotide reductase activity were measured. As seen from the data in Table 3, acetylation of the amino group on the pyridine ring had different effects on the inhibitory activity of these agents, depending on whether the amino group was on the 3- or 5-position. Thus, acetylation of the amino group of 3-AMP, which results in the production of 3-AAMP, generated an agent that was 10-fold less active as an inhibitor of CDP reductase activity and 8-fold less active as an inhibitor of the growth of L1210 cells than the corresponding non-acetylated agent, 3-AMP. In

Table 2. Comparison of the effects of HCTs on CDP reductase activity

Compound	CDP reductase $IC_{50}(\mu M)$
3-AP	0.3*
3-AMP	0.3
3-HP	1.2
3-HMP	2.0
5-AP	1.3
5-AMP	0.9
5-HP	1.8
5-HMP	2.3
5-HAMP	0.9

* Average of duplicate experiments; the variation between experiments was less than 10%.

Table 3. Effects of acetylation of HCTs on CDP reductase activity and L1210 cell growth in culture

	IC_{50} (μM)	
Compound	CDP reductase activity	L1210 cell growth
3-AMP	0.5*	1.4 ± 0.3 (4)†
3-AAMP	5.0	11.8 ± 0.2 (4)
5-AP	2.0	3.0 ± 0.6 (5)
5-AAP	2.0	8.2 ± 0.8 (3)
5-AMP	1.5	2.8 ± 0.9 (5)
5-AAMP	2.0	1.9 ± 0.4 (3)

* Average of duplicate experiments; the variation between experiments was less than 10%.

† Number of individual determinations with standard deviations.

contrast, acetylation of the amino group of 5-AP did not alter the effectiveness of the resulting agent, 5-AAP, as an inhibitor of CDP reductase, but did increase by 2.7-fold the IC_{50} value for inhibition of the growth of the L1210 cells, whereas acetylation of the amino group on 5-AMP did not alter the IC_{50} values for CDP reductase activity or L1210 cell growth.

To ensure that the effects of the HCTs on ribonucleotide reductase activity were expressed at the level of DNA in intact cells, the effects of 3-AP, 3-AMP, 5-AP and 5-AMP on the incorporation of [^{3}H]thymidine and [^{3}H]uridine into acid-insoluble material were measured. The results obtained are depicted in Fig. 3. 3-AP, 3-AMP, 5-AP and 5-AMP caused rapid inhibition of the incorporation of [^{3}H]-thymidine into DNA, in a manner consistent with interference with the activity of ribonucleotide reductase, without a corresponding decrease in the incorporation of [^{3}H]uridine into RNA.

To provide evidence that these agents are capable of inhibiting ribonucleotide reductase activity *in situ*, the effects of 3-AP and 3-AMP on [^{14}C]cytidine metabolism were measured in intact L1210 cells. The findings shown in Fig. 4 demonstrate the effects of 3-AP and 3-AMP on the incorporation of [^{14}C]-cytidine into cytidine nucleotides and RNA, and the movement of this label through ribonucleotide reductase to form deoxycytidine nucleotides, which are incorporated into DNA. Consistent with the findings obtained with radioactive uridine and thymidine, both 3-AP and 3-AMP inhibited the incorporation of cytidine into DNA without affecting the incorporation of this pyrimidine ribonucleoside into RNA. Neither 3-AP nor 3-AMP decreased cytidine conversion to the ribonucleotide level, but the movement of [^{14}C]cytidine into deoxycytidine nucleotides was decreased by these HCTs. The IC_{50} values for the inhibition of the formation of deoxycytidine nucleotides and for the incorporation

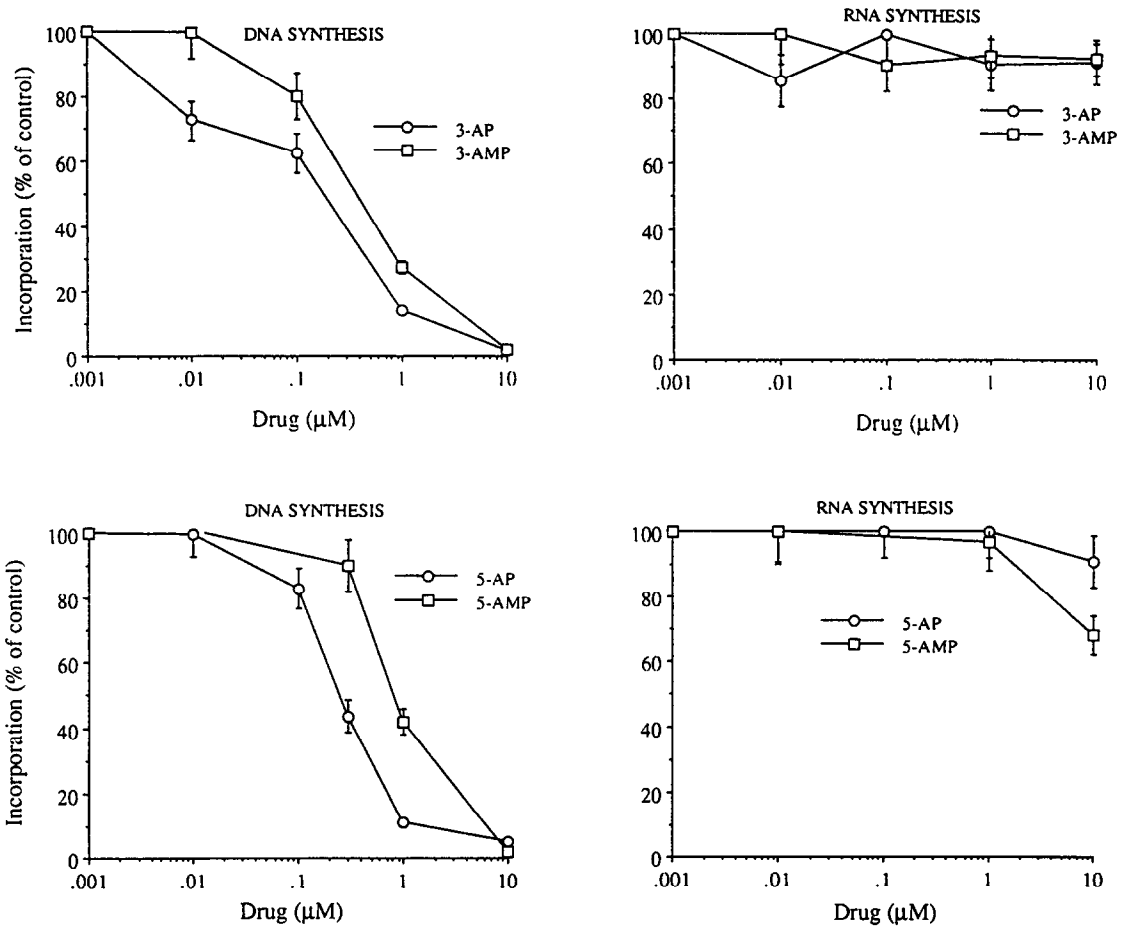

Fig. 3. Effects of 3-AP, 3-AMP, 5-AP and 5-AMP on the incorporation of [^{3}H]thymidine and [^{3}H]-uridine into DNA and RNA of L1210 cells. Cells were exposed simultaneously to the indicated concentrations of HCTs and [^{3}H]thymidine or [^{3}H]uridine for 30 min. Incorporation of radioactive label into trichloroacetic acid-precipitable material was determined as described in Materials and Methods. Points represent the means ± SD of 3 to 4 independent experiments. [^{3}H]Thymidine incorporation into DNA ranged from 80,000 to 120,000 cpm/10^6 cells and [^{3}H]uridine incorporation into RNA ranged from 45,000 to 60,000 cpm/10^6 cells in the various experiments.

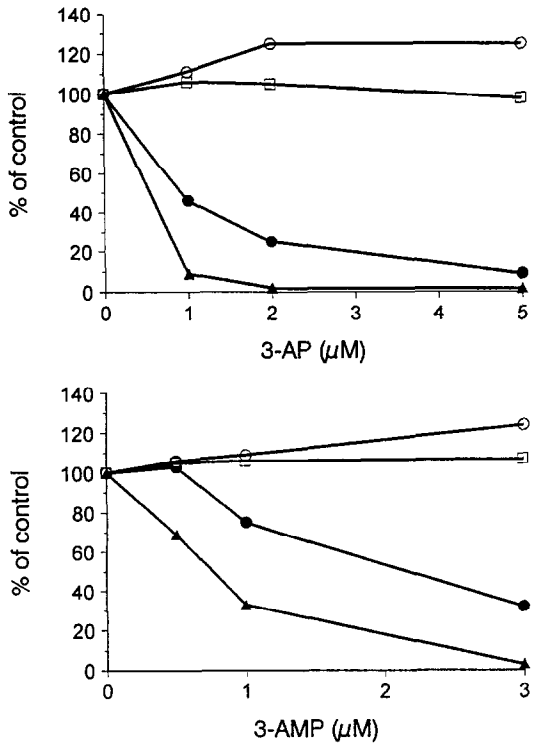

Fig. 4. Effects of 3-AP and 3-AMP on the metabolism of [^{14}C]cytidine by L1210 cells in culture. L1210 cells were incubated in the presence or absence of 3-AP or 3-AMP at the concentrations indicated. After 2 hr in the presence of drug, [^{14}C]cytidine (0.2 μCi/mL) was added and the incubation continued for an additional hour. The cells were collected and treated as described in Material and Methods to separate the acid-soluble (○), RNA (□), DNA (▲) and deoxycytidine (●) fractions. The non-drug-treated controls had 23,200, 37,000, 7500 and 400 cpm/10^6 cells for the total acid-soluble, RNA, DNA and deoxycytidine fractions, respectively. Each value represents the average of duplicate determinations.

of radioactive cytidine into DNA for 3-AP were 1.0 and 0.53 μM, respectively, and for 3-AMP they were 2.0 and 0.75 μM, respectively.

The effects of Desferal, an iron-chelating agent, on the inhibition of CDP reductase activity by 3-AP and 5-AP were compared, and the results are shown in Table 4. Desferal (100 μM) essentially completely prevented the inhibition of CDP reductase by both 3-AP and 5-AP. This action corresponded to a similar effect of Desferal on the inhibition of CDP reductase activity by MAIQ-1, which was included as a control.

Table 4. Effects of Desferal on the inhibition of CDP reductase activity by 3-AP and 5-AP

Compound	Desferal (μM)	% of Control*
3-AP, 1.0 μM		22
3-AP, 1.0 μM	100	92
5-AP, 5.0 μM		16
5-AP, 5.0 μM	100	94
MAIQ-1, 0.2 μM		27
MAIQ-1, 0.2 μM	100	80

* Control CDP reductase activity was 12.42 nmol/30 min/mg of protein.

DISCUSSION

Ribonucleotide reductase appears to be the rate-limiting enzyme in the biosynthetic pathway for DNA; consequently, it is a critical enzyme for cellular replication. The HCTs are among the most potent known inhibitors of ribonucleotide reductase and, therefore, appear to have considerable potential as anticancer agents [3–11, 33, 34]. Of this class of compounds, only 5-HP has undergone clinical evaluation, albeit a limited one through a phase I trial [21, 22]. Other thiosemicarbazones such as MAIQ-1 did not receive a clinical evaluation because of perceived problems in adequately formulating this agent for use in humans. In an effort to circumvent the problem of rapid conjugation of the heteroaromatic ring hydroxyl group that appeared to limit the efficacy of 5-HP in human trials and to produce agents capable of being adequately formulated for use in humans, several hydroxy- and amino-substituted pyridine-2-carboxaldehyde thiosemicarbazones were synthesized, and a methyl substituent was introduced adjacent to the substituted hydroxy or amino moiety to provide bulk in an effort to decrease metabolic inactivation via glucuronidation or acetylation. Of the newly synthesized agents, 3-AP, 3-AMP, 5-AP and 5-AMP were the most active as antineoplastic agents against the L1210 leukemia in CD_2F_1 female mice [33, 34].

Evaluation of these agents as inhibitors of the growth of L1210 leukemia cells in culture demonstrated that the 3-amino-substituted derivatives were slightly more potent than the corresponding 5-amino-substituted HCTs; this finding corresponded to greater potency as inhibitors of ribonucleotide reductase activity (Tables 1 and 2). The 3-amino-containing pyridine-2-carboxaldehyde thiosemicarbazones were also slightly more active than the corresponding 3-hydroxy-substituted derivatives. The growth of L1210 cells in culture is inhibited by hydroxurea, with an IC_{50} value of 85 μM. For comparison, the IC_{50} value for 3-AP, one of the most active agents in this series, was only 1.3 μM, 65-fold less. An L1210 cell line that was selected for resistance to hydroxurea, with an IC_{50} value of 2000 μM for hydroxyurea [36], did not show a corresponding increase in resistance to these pyridine-2-carboxaldehyde thiosemicarbazones. The 3-hydroxy- and 5-hydroxy-derivatives, however, showed slight increases in IC_{50} values (1.7-fold to 3.3-fold) in these hydroxyurea-resistant L1210 cells. This finding corresponded to the previous report that hydroxyurea-resistant cell lines were not cross-resistant to MAIQ-1 [36]. Studies with the isolated enzyme, CDP reductase, indicated that the mechanisms by which hydroxyurea and MAIQ-1 inhibited ribonucleotide reductase activity were not identical [45]. Thus, iron-chelating agents such as Desferal, EDTA and 8-hydroxyquinoline potentiated the inhibition of ribonucleotide reductase by

hydroxyurea, while attenuating the effects of MAIQ-1 and the related agent, 1-formylisoquinoline thiosemicarbazone. As seen from the data in Table 4, the iron-chelating agent Desferal markedly reduced the inhibition of ribonucleotide reductase activity by 3-AP and 5-AP. These data are in agreement with the conclusions of Preidecker, *et al.* [11] and of Thelander and Graslund [46], who showed that the inhibitory species was the iron–thiosemicarbazone complex rather than the free thiosemicarbazone.

Since metabolic inactivation of amino-substituted derivatives can occur through acetylation [6, 34], the effects of the N-acetylated derivatives of 3-AMP, 5-AP and 5-AMP on ribonucleotide reductase activity and on the replication of L1210 cells were evaluated. Although French and Blanz [4] have reported that 5-AAP has antitumor activity against L1210 leukemia, we did not confirm their findings [34]; this difference in response may be due to differences between sublines of L1210 leukemia. Acetylation of 3-AMP, which also yielded a derivative with no activity *in vivo* against the L1210 leukemia, resulted in an agent that was 8- to 10-fold less active than its non-acetylated counterpart as both an inhibitor of the replication of L1210 cells and of CDP reductase activity (Table 3). This may result from the proximity of the amino group in the 3-position of the pyridine ring to the thiosemicarbazone side chain which allows an additional coordination site for iron and/or the possibility that acetylation of this amino group interferes with the formation of the iron complex of the thiosemicarbazone, which appears to be the active species. In contrast, acetylation of 5-AP or 5-AMP did not alter the inhibitory properties of these derivatives against L1210 cells *in vitro* or against ribonucleotide reductase to any significant degree. The differences observed between the results with the purified reductase enzyme, L1210 cell growth in culture and antitumor effects with L1210 tumor-bearing mice point to pharmacokinetic effects of N-acetylation rather than to the simple loss of activity at the enzymatic target site upon acetylation of the amino function.

The effects of 3-AP, 3-AMP, 5-AP and 5-AMP were clearly the result of the specific inhibition of DNA synthesis. This was shown by the fact that the inhibitions of incorporation of [^{3}H]thymidine and of [^{14}C]cytidine into DNA were comparable, with the IC_{50} values for these effects being similar to the IC_{50} values for cell growth. In contrast, neither [^{3}H]-uridine nor [^{14}C]cytidine incorporation into RNA was inhibited by these agents, even at concentrations that produced essentially complete inhibition of DNA synthesis. The conversion of [^{14}C]cytidine to deoxycytidine nucleotides, a measure of the throughput of this radioactive precursor via ribonucleotide reductase, was inhibited markedly by both 3-AP and 3-AMP, providing strong evidence for the *in situ* inhibition of ribonucleotide reductase by these HCTs. The inhibitory effects of the HCTs on the incorporation of [^{3}H]thymidine into DNA or the conversion of [^{14}C]cytidine to deoxycytidine nucleotides and incorporation into DNA were seen after very short times of incubation of the cells with the drugs. Clearly, these effects are consistent with the direct inhibition of ribonucleotide reductase activity and are not due to cell cycle transit effects. Consistent with an inhibition of DNA replication at relatively low concentrations ($<IC_{50}$) of 3-AP or 3-AMP, cells gradually accumulated in the S-phase of the cell cycle; at higher concentrations of 3-AP and 3-AMP (7-fold greater than the IC_{50}), a more rapid accumulation of cells in the G_0/G_1 phase of the cell cycle occurred, with the loss of the S-phase population, implying that a second less sensitive metabolic lesion was created by the HCTs.

The studies presented in this report demonstrated that 3- and 5-amino-substituted pyridine-2-carboxaldehyde thiosemicarbazones do not require metabolic activation to be potent inhibitors of ribonucleotide reductase activity. Further, these agents are effective inhibitors of tumor cell growth in culture. Since these latter findings correspond to the results obtained with these agents as inhibitors of the L1210 leukemia *in vivo* [33, 34], it appears that selectivity occurs with respect to the effects of these agents on tumor cells relative to host tissues. 3-AP and 3-AMP, the most potent of the HCTs examined in this series, particularly appear to hold promise for further development for clinical use.

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