Comparative Studies of the Cytostatic Action and Metabolism of 5-Azacytidine and 5,6-Dihydro-5-azacytidine

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SUMMARY

5,6-Dihydro-5-azacytidine hydrochloride, a chemically stable, soluble analog of 5-azacytidine, has cytostatic activity against mouse leukemic L1210 cells grown in culture, but concentrations on the order of 10 µM, 10-fold higher, than the parent drug, are necessary to inhibit cell growth. The addition of either cytidine or uridine protected against growth inhibition by 5-azacytidine and 5,6-dihydro-5-azacytidine, whereas thymidine potentiated the cytostatic action of both drugs. Deoxycytidine also enhanced the action of 5-azacytidine but had no effect with the reduced analog. Cell suspensions of L1210 cells were able to phosphorylate 5-azacytidine and, to a lesser extent, 5,6-dihydro-5-azacytidine. In cell-free extracts in the presence of ATP and Mg²⁺, but both drugs were converted to nucleotides but at less than 5% the rate of cytidine. As a substrate for mouse kidney cytidine deaminase, the apparent Kₘ value for 5,6-dihydro-5-azacytidine (33 µM) is of the same order of magnitude as that for cytidine (37 µM) but less than that for 5-azacytidine (2.1 x 10⁻³ µM). The Vₘ for deamination of the reduced analog is one-tenth that for 5-azacytidine. 3,4,5,6-Tetrahydouridine, a potent inhibitor of cytidine deaminase, is more effective in blocking deamination of 5-azacytidine than 5,6-dihydro-5-azacytidine.

INTRODUCTION

aza-C¹ was first described in 1964 (23), and has been found to be clinically useful in the treatment of acute myelocytic leukemia (13, 20). However, when the drug is given as a bolus, severe nausea and vomiting occur. These troublesome gastrointestinal toxicities can be overcome by continuous infusion of the drug without loss of clinical effectiveness (11, 17, 18, 25). Because of the instability of aza-C in aqueous solutions, fresh preparations are made and administered every 4 hr (18).

Hydrolytic decomposition of aza-C at neutral pH values leads primarily to opening of the triazine ring (22), producing compounds of unknown biological effects. In order to overcome this problem of instability, Beisler et al. (2, 3) have reduced the hydrolytically susceptible 5,6-imino double bond of aza-C with sodium borohydride, forming DHaza-C. The reduced analog is not susceptible to hydrolytic attack, has good solubility, and is stable in aqueous solutions at 25° for weeks. Although the optimal dose of DHaza-C is greater than that of aza-C, the antitumor activity in terms of extension of life-span is comparable to that of aza-C (2). At an optimal dose of 3 mg/kg daily for 9 days, aza-C gave a 120% increase in life-span, compared with a 100% increase at an optimal dose of 100 mg/kg for the dihydro derivative (26) on the same schedule.

The purpose of this communication is to compare the cytostatic actions of aza-C and DHaza-C in tissue culture with the use of murine leukemic L1210 cells, and to investigate the phosphorylation of these drugs by whole-cell suspensions and supernatant extracts. In addition, the deamination of aza-C and DHaza-C by cytidine deaminase, the enzyme which may, in part, be responsible for the biological inactivation of aza-C (5), is discussed. The ability of THU, a potent inhibitor of cytidine deaminase, to block deamination of DHaza-C is also presented.

MATERIALS AND METHODS

Materials. Cytidine, thymidine, uridine, and deoxycytidine were purchased from Sigma Chemical Co., St. Louis, Mo. aza-C was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, and the chloride salt of DHaza-C was synthesized by reduction of aza-C with NaBH₄. The detailed synthesis, spectral characterization, and purity of DHaza-C have been described (2, 3). Glutamate dehydrogenase (1200 units/ml) was purchased from Boehringer Mannheim, Mannheim, Germany. THU (NSC 112907) was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. [2-¹⁴C]Cytidine (40 µCi/µmole) was obtained from Schwarz/Mann, Orangeburg, N. Y., and [4-¹⁴C]aza-C (45 µCi/µmole) was from Monsanto Research Corp., Dayton, Ohio. [4-¹⁴C]DHaza-C was synthesized from labeled aza-C by the borohydride reduction procedure and had a specific activity of 0.41 µCi/µmole. DEAE-cellulose was obtained from Whatman, Clifton, N. J.

Cell Culture. L1210 cells were grown in Roswell Park Memorial Institute Medium 1630 supplemented with 10% fetal calf serum. Growth inhibition studies were performed at 37° by incubating 5-ml volumes of cells (1 x 10⁸ cells/ml) in tissue culture flasks (30 ml, 25 sq cm) with various concentrations of test compounds. Drugs and nucleosides, dissolved in phosphate buffer at pH 7.4, containing 0.85% NaCl solution, were sterilized by passage through Millipore Swinnex-13 filter units and were immediately added to the exponentially growing cultures in a volume of 0.1 ml.

¹ The abbreviations used are: aza-C, 5-azacytidine; DHaza-C, 5,6-dihydro-5-azacytidine-HCl; THU, 3,4,5,6-tetrahydouridine.

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designated time intervals, cell counts were determined on a Model B Coulter counter. The data shown represent results of typical experiments repeated 3 or more times. In the trypan blue exclusion test for viability, cells were mixed with 0.4% trypan blue and then were counted microscopically within 5 min; the number of stained cells was expressed as a percentage of the total.

**Enzyme Assays.** Cytidine kinase assays were carried out at 37° in 50 mM Tris-Cl buffer at pH 7.7 containing 5 mM ATP, 5 mM MgCl₂, and different concentrations of [2-14C]cytidine (1.20 µCi/µmole), [4-14C]aza-C (4.0 µCi/µmole), and [4-14C]DHaza-C (0.41 µCi/µmole). The source of cytidine kinase was a supernatant extract (100,000 × g, 1 hr at 4°) from homogenized L1210 cells (30% suspension in 20 mM Tris-Cl buffer at pH 7.7). The conversion of pyrimidines to their monophosphates was determined by the method of Furlong (10). At different incubation periods, 25 µl were removed and spotted on Whatman DE81 filter paper discs (2.5 cm), and labeled nucleoside was removed by 3 water washes. Deamination of cytidine was measured according to the spectrophotometric assay described by Tomchick et al. (24), and is based on the loss of absorbance in acid when cytidine is converted to uridine at 290 nm. The assays were conducted in 20 mM potassium phosphate buffer at pH 6.8 at 37°. DHaza-C and aza-C deamination was measured by a coupled assay to glutamate dehydrogenase (14). The determinations were made at 37° in 20 mM phosphate buffer at pH 7.2 using 0.3 mM NADH, 20 mM α-ketoglutarate, 18 units glutamic dehydrogenase per ml, various concentrations of nucleosides, and 0.05 ml of cytidine deaminase preparation. The decrease in absorption of NADH converted to NAD was followed in a Gilford spectrophotometer at 340 nm. Activity is expressed as µmoles of substrate deaminated per hr per ml reaction mixture.

**Purification of Cytidine Deaminase.** Kidneys were excised from 100 male C57BL × DBA F₁ mice (7 to 8 weeks old) and were added to 60 ml of chilled 0.15 M KCl. The kidneys were homogenized in a Ten Broeck tissue grinder and the homogenate was centrifuged at 90,000 × g for 60 min. The pellet was discarded and the supernatant fluid was heated at 60° for 6 min and then immediately placed on ice. The supernatant after heat treatment was centrifuged at 90,000 × g for 30 min and the pellet was discarded. Solid (NH₄)₂SO₄ was added to the supernatant over a period of 30 min to attain 30% saturation. The preparation was then stirred for an additional 30 min and centrifuged at 15,000 × g for 20 min. (NH₄)₂SO₄ was added to the supernatant over a 20-min period to increase the saturation to 50%. The solution was stirred slowly for 1 hr, followed by centrifugation at 15,000 × g for 30 min. The supernatant was discarded and the pellet was suspended in 10 ml of 0.01 M potassium phosphate at pH 6.8 and dialyzed twice for 5 hr against 1000 ml of buffer at 4°. This preparation was used for kinetic assays and could be stored at −20° for several weeks without loss of cytidine deaminase activity. No cytidine deaminase activity was detected in L1210 extracts. The specific activity of cytidine deaminase from the final isolation step was 15 µmoles deaminated per hr per mg of protein, as determined by Lowry et al. (19), and represented a 6-fold purification over the 100,000 × g crude supernatant.

**Phosphorylation of Cytidine, aza-C, and DHaza-C by Intact L1210 Cells.** Cells in log growth were harvested and washed with incubation medium [by the method described by Agarwal et al. (1)] containing 75 mM NaCl, 2 mM MgSO₄, 5 mM potassium phosphate buffer at pH 7.4, 10 mM glucose, penicillin (100 units/ml), and streptomycin (100 µg/ml). A 10% suspension of cells in the incubation medium was exposed to 1 mM of [2-14C]cytidine (0.22 µCi/µmole), [4-14C]aza-C (3.21 µCi/µmole), or [4-14C]DHaza-C (0.41 µCi/µmole) at 37° for 1 hr. Reactions were stopped by the addition of perchloric acid (final concentration, 4%) and allowed to stand on ice for 15 min. The acid extracts were spun for 5 min at 2000 × g. The supernatants were adjusted to neutrality with KOH, and potassium perchlorate was removed by centrifugation. The clear extracts (2 ml) were added to DEAE-cellulose columns at room temperature (Whatman DE52, 0.9 x 8 cm), and nucleosides were washed from the resin with approximately 20 ml of H₂O until radioactivity in the effluent was at background. Nucleotides were eluted with 30 ml of 0.2 M potassium phosphate buffer at pH 7.4. Radioactivity of the elutions was measured in acidified aquasol at a counting efficiency of 92%.

**RESULTS**

**Growth Inhibition Studies.** The growth curves presented in Chart 1 show that both aza-C and its reduced analog, DHaza-C, inhibit the logarithmic growth of L1210 leukemia cells. However, the effective concentration of DHaza-C (5.0 to 20.0 µM) is 10-fold higher than that of aza-C (0.5 to 2.0 µM). At the highest concentration of each drug, a cessation of growth was observed between 12 and 24 hr after treat-
When the cells were exposed to trypan blue at 36 hr, only 3.7 and 4.8% of the cells took up the stain with 1 μM aza-C and 10 μM DHaza-C, respectively. Only 0.5% of the control cells appeared to be stained.

Effect of Cytidine and Uridine on Growth Inhibition by aza-C and DHaza-C. Chart 2 shows that, when either cytidine or uridine is added to the cultures simultaneously with aza-C or its reduced derivative, both protect against the cytostatic action of aza-C and DHaza-C. As the concentration of either cytidine or uridine is raised from 1 μM to 100 μM, there is an increase in protection against aza-C and DHaza-C.

The highest concentration (100 μM) of cytidine (Chart 2A), but not of uridine (Chart 2C), slightly inhibits the growth rate, compared with that of untreated cells. Although a 10-fold higher concentration of DHaza-C is needed to achieve an equivalent inhibitory response shown by aza-C, higher concentrations of cytidine and uridine were not required to overcome its cytostatic action. When cytidine or uridine at 100 μM was added 12 hr after treatment with aza-C or DHaza-C, no protective effect was observed.

Effect of Thymidine and Deoxycytidine on Growth Inhibition by aza-C and DHaza-C. Chart 3 shows the effects of thymidine on the growth of L1210 cells in the presence or absence of either 1 μM aza-C or 10 μM DHaza-C. Thymidine, alone, at 100 μM (Chart 3, A and B) caused a lag in the growth of the cells, but after 1 generation time (12 hr), the cells recovered and the curve appeared to be parallel to controls. The combination of aza-C or DHaza-C with 100 μM thymidine (Chart 3, A and B) arrested growth of the cells completely. Ten μM thymidine (Chart 3, C and D) did not affect the growth curves, but in combination with aza-C (1 μM), growth was prevented completely, or in combination with DHaza-C (10 μM), the growth rate was drastically reduced. When the experiments were repeated with lesser concentrations of aza-C (0.5 μM) and DHaza-C (5 μM) (Chart 4, A and B), 100 μM thymidine was still capable of completely preventing cellular growth. The addition of 10 μM thymidine also potentiates the action of aza-C and DHaza-C at low concentrations (Chart 4, C and D) but is not as effective as the higher pyrimidine concentration. Chart 5A shows that deoxycytidine, like thymidine, is capable of enhancing the cytostatic action of aza-C, and in the absence of drug at 100 μM, unlike thymidine, no effect on cellular growth is observed. However, deoxycytidine (1 to 100 μM) neither protected nor potentiated the action of DHaza-C (Chart 5B).

Phosphorylation of Cytidine, aza-C, and DHaza-C. Whole-cell suspensions of L1210 cells are able to phosphorylate cytidine, aza-C, and DHaza-C. However, after 1 hr of incubation, 24 nmoles of cytidine are phosphorylated per 10^9 cells, compared with the formation of 15 nmoles of aza-C nucleotides and 2 nmoles of DHaza-C nucleotides. The curves shown in Chart 6 indicate that both aza-C and DHaza-C are phosphorylated by L1210 cell-free extracts but at only 5% the rate of the natural substrate, cytidine, at a concentration of 2 mM. When whole homogenates were used as the enzyme source, phosphorylation of cytidine and the 2 analogs was not improved. The concentrations (2 mM)
aza-C and DHaza-C

**Chart 4.** Enhancement of thymidine on the cytotoxic action of aza-C (0.5 μM) and DHaza-C (5 μM) on the growth of L1210 cells. The nomenclature used here is the same as that given in the legend for Chart 3.

**Chart 5.** Effects of deoxycytidine on the cytotoxic action of aza-C (1 μM) and DHaza-C (10 μM) on the growth of L1210 cells. A: □, control; ●, 100 μM deoxycytidine; ◦, aza-C; ○, aza-C plus 1 μM deoxycytidine; ○, aza-C plus 10 μM deoxycytidine; ○, DHaza-C; ○, DHaza-C plus 1 μM deoxycytidine; ○, DHaza-C plus 10 μM deoxycytidine. B: □, control; ◦, DHaza-C; □, DHaza-C plus 1 μM deoxycytidine; ○, DHaza-C plus 10 μM deoxycytidine.

were 200 times the highest concentrations used in the cell inhibition studies and 28 times the apparent Km (70 μM) of cytidine, with the use of the cell-free extract as the enzyme source (data not shown).

aza-C and DHaza-C as Substrates for Cytidine Deaminase. Double-reciprocal plots for cytidine deaminase using cytidine, aza-C, and DHaza-C as substrates are shown in Chart 7. From these plots, apparent Km and Vm values were calculated and are given in Table 1. The kinetic constants indicate that the binding of DHaza-C to cytidine deaminase is comparable to the natural substrate, whereas aza-C appears to bind to the enzyme to a lesser extent. However, the

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (μM)</th>
<th>Vm (μmole/hr/ml)</th>
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<tbody>
<tr>
<td>Cytidine</td>
<td>42 ± 3 (80)</td>
<td>0.107 ± 0.002</td>
</tr>
<tr>
<td>aza-C</td>
<td>(1.81 ± 0.29) x 10³</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>DHaza-C</td>
<td>29 ± 3 (48)</td>
<td>0.023 ± 0.005</td>
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</tbody>
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* Mean ± S.E.
* This value in parentheses is the Km calculated from the direct spectral determination of cytidine at 290 nm.
* This value in parentheses represents the Vm value calculated from competitive inhibition plots using the direct spectral determination with cytidine as substrate.
DISCUSSION

The results presented in this report show that, similar to aza-C, the reduced analog DHaza-C inhibits growth of L1210 cells in culture, but 10-fold higher concentrations of DHaza-C are necessary to achieve growth inhibition comparable to the parent drug. Continuous exposure of L1210 cells to either aza-C or DHaza-C for 36 hr killed only a small percentage of cells as determined by the trypan blue test for viability.

In agreement with the studies of Li et al. (16), our findings indicate (Chart 2) that uridine and cytidine decrease the inhibitory action of aza-C, and as well, our work revealed a similar decrease with DHaza-C. However, thymidine and deoxycytidine were found to enhance the cytostatic action of aza-C, whereas thymidine but not deoxycytidine potentiated the effect of the reduced analog. The latter finding represents the only major difference between aza-C and DHaza-C observed thus far.

Thymidine enhances the action of both aza-C and DHaza-C, even at concentrations (aza-C, 0.5 μM; DHaza-C, 5 μM) that, alone, only slightly inhibit cell growth (Chart 4). Reasons for the potentiation effect of thymidine in combination with aza-C or DHaza-C and of deoxycytidine for aza-C, but not DHaza-C, are at present unknown. However, studies are underway to investigate the effects of deoxynucleosides in combination with aza-C and DHaza-C on cellular nucleotide pool sizes and incorporation of these drugs into nucleic acids.

The mechanism of the antineoplastic effect exerted by aza-C has not been completely defined, although the drug is known to exert effects on DNA, RNA, and protein metabolism (7, 12, 27). It appears that both aza-C and DHaza-C must be phosphorylated for activity via uridine-cytidine kinase, since uridine and cytidine are capable of reversing its action (9, 12, 16). Lee et al. (15) have shown that aza-C is phosphorylated by calf thymus uridine-cytidine kinase at 42% the rate of cytidine, and a K_m value was obtained that was 4-fold higher than that for the natural nucleosides. Data presented in Chart 6 also show that both aza-C and DHaza-C are poorer substrates for uridine-cytidine kinase extracted from L1210 cells, compared with cytidine, yet whole cell suspensions are able to phosphorylate aza-C and DHaza-C to a greater extent than one would anticipate from the cell-free cytidine kinase experiments (Chart 6). Therefore, because aza-C and DHaza-C are poorer substrates for uridine-cytidine kinase and higher concentrations of the natural nucleosides than drugs are necessary for protection (Chart 2), possibly other metabolic routes may, in part, be responsible for the drug-inhibitory effect, and/or only small quantities of phosphorylated aza-C and DHaza-C are needed to be effective.

Caminer (4) has shown that THU is a potent inhibitor of cytidine deaminase isolated from human liver. Their kinetic analysis showed THU to be a “partial type” inhibitor in which the inhibited enzyme was still able to form product, but at a reduced rate. These results are in agreement with our findings with mouse kidney cytidine deaminase where, at saturating concentrations of inhibitor, residual activity still remains with all 3 substrates (Chart 9). However, it is possible that another deaminase that is not inhibited by THU is present and is capable of deaminating these substrates at slower rates.

Caminer (4) reported that, for maximum inhibition of the liver enzyme, a 2- to 6-min incubation period at 37° with THU was necessary. Chabner et al. (6) reported that inhibition of cytidine deaminase isolated from human granulocytes was also slightly enhanced by preincubation of enzyme and inhibitor, whereas THU inhibition of the bacterial enzyme isolated from Escherichia coli was instantaneous (8). Similar to the bacterial enzyme, no enhancement of the inhibitory action of THU with mouse kidney enzyme was observed by preincubation.

These comparative studies between aza-C and its reduced analog indicate that DHaza-C is less potent than aza-C.
C as a cytostatic agent against L1210 cells. DHaza-C probably would undergo deamination faster at the lower therapeutic concentrations typical of physiological conditions. THU blocks deamination of aza-C and DHaza-C in vitro and, therefore, might be effective in prolonging the life of both drugs in vivo. Neil et al. (21) have shown that THU coadministered with aza-C increases the p.o. activity of the drug against L1210 leukemia in mice. The advantages of good solubility and increased stability of DHaza-C over aza-C may outweigh the higher doses required. The usefulness of combining aza-C or DHaza-C with deoxypyrimidines (e.g., thymidine) to enhance the cytostatic action of these drugs merits further investigation, in that lower dose levels could be used with less toxic effects.

REFERENCES
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