Effects of Deoxynucleosides on Cultured Human Leukemia Cell Growth and Deoxynucleotide Pools

Douglas D. Ross, Steven A. Akman, Anthony W. Schrecker, and Nicholas R. Bachur

Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, Baltimore, Maryland 21201

ABSTRACT

We investigated the mechanism of cell growth inhibition caused by the deoxyribonucleosides thymidine (dThd), deoxyguanosine (dGuo), deoxyadenosine (dAdo), and deoxycytidine (dCyd). Growth of the cultured human leukemic cells HL-60 and K-562 was measured by cloning in soft agar. Of the deoxyribonucleosides, dGuo was the most potent cell growth inhibitor; however, the potency of added dAdo was probably attenuated by the presence of adenosine deaminase in the tissue culture growth medium. The concentrations of nucleoside causing 50% inhibition of HL-60 cloning were: dCyd, >10,000 μM; dAdo, 500 μM; dThd, 5,000 μM; and dGuo, 80 μM. For K-562 cloning, the concentrations causing 50% inhibition of cloning were dCyd, >10,000 μM; dAdo, 1,600 μM; dThd, 880 μM; and dGuo, 100 μM. Measurement of deoxycytidine 5'-triphosphate (dCTP) pool size in HL-60 cells following dThd, 880 μM; and dGuo, 100 μM. Measurement of deoxycytidine 5'-triphosphate (dCTP) pool size in HL-60 cells following incubation with 750 μM deoxyribonucleosides revealed that dGuo caused the greatest reduction of dCTP pools, both in early (passage 10)- and late (passage 71)-passage-derived HL-60 cell cultures (35 and 18% of control, respectively), compared to dThd (61 and 26% of control, respectively) and dAdo (39% of control of HL-60 passage 10). In K-562 cells, reductions in dCTP pool size caused by dAdo, dThd, and dGuo were 67, 46, and 35% of control, respectively.

Incorporation of [3H]dCyd into DNA of HL-60 and K-562 cells was enhanced by dThd and dGuo, but the degree of enhancement was greater for dThd than for dGuo. Despite its effect in reducing HL-60 dCTP pool size, dAdo failed to enhance [3H]dCyd incorporation in either HL-60 or K-562 cells. Addition of dCyd to the cultures could only partially rescue the inhibition of HL-60 cloning caused by dThd or dGuo, suggesting that inhibition of cytidine 5'-diphosphate reduction by ribonucleotide reductase is not the only mechanism whereby these nucleosides inhibit leukemic cell cloning. These data suggest that, in addition to inhibiting de novo dCTP production via ribonucleotide reductase, these nucleosides may affect other processes in the salvage pathway such as cellular uptake and phosphorylation or the DNA polymerase reaction itself.

INTRODUCTION

Recently, the naturally occurring nucleoside, dThd³ has been under intense investigation as a cancer chemotherapeutic agent (2, 7, 14, 15, 18, 19). Previous work in our laboratory (1) has shown that high (μM) concentrations of dThd are required for growth inhibition of HL-60 and K-562 cultured human leukemic cells. The role of deoxynucleoside triphosphates in regulating DNA synthesis is well known (25, 26). The deoxynucleosides dGuo and dAdo, like dThd, inhibit cell growth in culture (25, 26), with one mechanism of growth inhibition postulated as conversion to the corresponding deoxynucleoside triphosphate and subsequent inhibition of CDP reduction to dCDP by ribonucleotide reductase, thus depleting the cell of dCTP essential for DNA synthesis. In fact, Moore et al. (24) have shown that dATP and dGTP are potent inhibitors of Novikoff hepatoma cell ribonucleotide reductase compared to dTTP.

The studies presented in this paper compare the effects of the naturally occurring nucleosides dThd, dGuo, dAdo, and dCyd on cell growth and the intracellular pools of dCTP and dGTP in cultured human leukemic cell lines HL-60 and K-562. Our data indicate that dGuo is a more potent inhibitor of HL-60 and K-562 cell cloning and that it decreases dCTP pools more extensively than do the other naturally occurring deoxynucleosides.

MATERIALS AND METHODS

Cell Culture. HL-60, a cell line derived from peripheral blood leukocytes of a female patient with acute progranulocytic leukemia, was supplied by Dr. R. Gallagher and Dr. S. Collins, National Cancer Institute, Bethesda, Md. (8). These cells were maintained in continuous liquid suspension culture at an initial cell concentration of 2.5 x 10⁵ cells/ml in Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-inactivated fetal calf serum; L-glutamine, 0.3 mg/ml (2 mw); penicillin, 50 IU/ml; and streptomycin, 50 μg/ml. Unless stated otherwise, HL-60 cell cultures derived from cells cryopreserved after the 71st passage (p-71) were used. The population doubling time of these cells is approximately 48 hr.

K-562 cells are derived from the pleural fluid of a patient with chronic myelogenous leukemia in blast crisis (21). K-562 cells are maintained in continuous liquid suspension culture at an initial cell concentration of 25,000 cells/ml, in Roswell Park Memorial Institute Medium 1640, supplemented with 10% heat-inactivated fetal calf serum; L-glutamine, 0.3 mg/ml (2 mw); penicillin, 50 IU/ml; and streptomycin, 50 μg/ml. The population doubling time is approximately 24 hr.

Soft Agar Experiments. HL-60 cells clone in soft agar spontaneously without exogenous stimulus at an efficiency of approximately 10 to 20%. K-562 cells also clone spontaneously at 20 to 50% efficiency. Nucleosides were tested against HL-60 cloning by incubating 5000 HL-60 cells/ml in the medium described above, plus 0.3% agar and the appropriate concentrations of nucleoside for 2 weeks in 35-mm Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37°, 100% humidity, and 7.5% CO₂. After 2 weeks, colonies greater than 50 cells were counted through a X20 dissecting microscope. K-562 cloning assays were performed in exactly the same manner, except that an initial cell concentration of 500 cells/ml was used.

Chemicals and Radioisotopes. Nucleosides (dThd, dCyd, dAdo, and dGuo) were obtained from Sigma Chemical Co., St. Louis, Mo. [8-
\[^{3}H\]dGuo (8.7 Ci/mmol), \[^{8-3}H\]dAdo (12.9 Ci/mmol), and \[^{5-3}H\]dCyd (27 Ci/mmol) were obtained from ICN, Irvine, Calif. \[^{5}-\text{methyl-}^{3}H\]dTdh (2 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. dCTP, dGTP, poly(dG-dC), and poly(dT-dC) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. \[^{5-3}H\]dCTP (24 Ci/mmol) and \[^{8-3}H\]dGTP (8 to 12 Ci/mmol) were obtained from New England Nuclear. Their purity was determined periodically by thin-layer chromatography on plastic sheets coated with poly(ethyleneimine) cellulose (Macherey-Nagel, obtained from Brinkmann Instruments, Inc., Westbury, N.Y.). The chromatograms were developed with 2 M LiCl:2 M HCOOH, 1:2 (33).

Escherichia coli DNA polymerase I was obtained from Bethesda Research Laboratories, Inc., Rockville, Md. Micrococcus luteus (Micrococcus lysodeikticus) DNA polymerase was purchased from Miles Laboratories, Elkhart, Ind. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co., Chicago, Ill.

Radioisotope Incorporation Studies. HL-60 or K-562 cells were grown in batch suspension culture in logarithmic growth phase (confirmed by daily cell counts via a Coulter Counter). Cells were transferred to sterile culture tubes (16 x 125 mm) in the presence of varying amounts of nonradioactive nucleoside at an initial concentration of 500,000 cells/ml. Triplicate tubes were made for each nucleoside concentration. Tubes were incubated at 37° in 7.5% CO\(_2\) and 100% humidity for 24 hr, after which a 60-min pulse (1 µCi/ml) of a different radiolabeled nucleoside was given, following which the tubes were placed on ice. Radioactivity incorporated into the acid-insoluble fraction was determined by pouring the cell suspensions onto Whatman (Whatman Inc., Clifton, N.J.) GF/C glass fiber filters, washing the filters twice with ice-cold NaCl (154 mM), once with ice-cold 5% trichloroacetic acid, and once with ice-cold absolute methanol. Filters were then placed in glass scintillation vials with 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.), and the vials were allowed to stand overnight at room temperature. Glacial acetic acid (0.1 ml) was added, followed by 10 ml of Aquasol liquid scintillation cocktail (New England Nuclear). Radioactivity was determined in a Searle Mark III liquid scintillation counter.

Nucleoside Triphosphate Pool Studies. HL-60 or K-562 cells in logarithmic growth phase (72 hr after dilution with fresh medium) were adjusted to a cell concentration of 800,000 cells/ml in liquid suspension culture. To aliquots containing 20 to 50 million cells, dGuo, dThd, or dAdo in Roswell Park Memorial Institute Medium 1640 were added to a final concentration of 750 µM. Controls received medium alone. Cultures were then incubated for 16 hr at 37° (7.5% CO\(_2\) and 100% humidity). Pool sizes of dCTP and dGTP were measured by modifications of previous methods (16, 27, 36, 39). Cells were collected by centrifugation (500 x g for 10 min at 4°), washed once with cold phosphate-buffered saline (8.7 mM potassium phosphate in 154 mM NaCl, pH 7.4 (9)), then extracted twice with 1 ml of 60% methanol (27, 35) for 10 min at 30°, and centrifuged (1000 x g for 10 min at 4°).

The combined 60% methanol extracts were evaporated to dryness on a rotary evaporator and then dissolved in 0.8 to 1.2 ml of 10 mM Tris-HCl buffer, pH 8.2. The solutions were clarified by centrifugation at 40,000 x g for 10 min. Pools of dCTP were determined in reaction mixtures (120 µl total volume) containing 40 µl of either cell extract in duplicate (appropriately diluted, if necessary) or of known dCTP standards (5 to 7 concentrations in amounts of 0 to 8.4 pmol/sample in duplicate), 50 mM Tris-HCl (pH 8.2), 2.2 mM magnesium diacetate, 2 mM dithiothreitol, 12 mM NaCl, bovine serum albumin (100 µg/ml), \[^{8-3}H\]dGTP (2.5 µCi), and 2 to 8 µM unlabeled dGTP, 2.7 µM poly(dG-dC) (0.1 µg) (17), and E. coli DNA polymerase I (0.24 unit). The mixtures were incubated in siliconized Pyrex culture tubes (10 x 75 mm) at 20° (suggestion of Dr. R. E. Handschumacher) for 30 min, and trichloroacetic acid-insoluble radioactivity was determined as described previously (34), except that Whatman GF/F filters were used instead of Millipore filters. The amounts of dCTP in the extracts were computed from those present in the standards by linear regression analysis. The results were corrected for isotope dilution (27) caused by the dGTP present in the cell extracts. When high levels of dGTP were present in the cells treated with dGuo, equivalent amounts of unlabeled dGTP were also added in the assays of the dCTP standards and other extracts.

Pools of dGTP were determined similarly with the following modifications: Tris-HCl buffer was pH 8.3; known dGTP standards ranged from 0 to 12 pmol/sample; \[^{8-3}H\]dCTP was 1.0 µCi (dCTP + \[^{3}H\]dCTP, 1 µM); NaCl was 5.5 mM; the template was 1.7 µM poly(dG-dC) (0.06 µg); and the enzyme was M. luteus DNA polymerase (0.75 unit). Both the dCTP and dGTP standards gave linear results, and the amounts of unknown nucleotide determined were proportional to amounts of extract. In early experiments in which incubation times were varied, retardation of \[^{3}H\]dGTP incorporation by an inhibitor in the cell extracts (30, 40) was noticed but was overcome by using a sufficient amount of enzyme.

Adenosine Deaminase Assay. Activity of adenosine deaminase (EC 3.5.4.4) in the tissue culture medium used in these experiments was determined by the method of Gustin and Kemp (13).

RESULTS

Deoxynucleoside Effects on Leukemic Cell Cloning. Of the deoxyribonucleosides, dGuo is the most potent inhibitor of K-562 and H-60 cell cloning (Chart 1), having an IC\(_{50}\) of 80 µM for HL-60 and 100 µM for K-562. dAdo is 5 to 10 times more effective than dThd in inhibiting HL-60 colony growth (IC\(_{50}\) is 500 to 1,000 µM for dAdo, 5,000 µM for dThd). For K-562 colony growth, the IC\(_{50}\) for dAdo and dThd are 1,600 and 880 µM, respectively. dCyd is relatively nontoxic to HL-60 and K-562 cells, with IC\(_{50}\) > 10,000 µM for each cell line.

Deoxynucleoside Effects on Incorporation of Labeled Deoxynucleotide Into Acid-insoluble Material. Preincubation of HL-60 or K-562 cells for 24 hr with either dThd or dGuo causes a 2- to 4-fold increase in the incorporation of \[^{3}H\]dCyd into acid-insoluble material (Chart 2). Studies in our laboratory

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Chart 1. Growth inhibition of in vitro soft agar cloning of cultured human leukemic cells K-562 (A) and HL-60 (B) by dCyd, dAdo, dThd, and dGuo. Data shown are compiled from 2 to 4 separate experiments. One hundred % control equals 275 colonies/7500 cells plated (37% cloning efficiency) for K-562 and 1255 colonies/7500 cells plated (16.7% cloning efficiency) for HL-60. S.E. < 15%.
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have shown linear incorporation of [3H]dCyd into acid-insoluble material of K-562 or HL-60 cells throughout the 60-min pulse. Analysis of the acid-insoluble fraction by the method of Schmidt and Thannhauser (32) reveals greater than 97% of acid-insoluble radioactivity within the DNA fraction (data not shown).

The stimulatory effect of added dThd or dGuo on the incorporation of [3H]dCyd into acid-insoluble material is apparent at 10 μM for K-562 cells and at 50 μM for HL-60 cells. dAdo fails to enhance incorporation of [3H]dCyd into acid-insoluble material. In fact, 10 μM dAdo significantly inhibits cellular incorporation of [3H]dCyd into acid-insoluble material in K-562 cells. No enhancement of incorporation of labeled dThd, dAdo, or dGuo into acid-insoluble material is observed after preincubation with any other deoxynucleoside (data not shown). However, preincubation with 50 μM dAdo causes a >50% decrease in incorporation of [3H]dGuo into acid-insoluble material, and preincubation with 50 μM dGuo causes a >50% decrease in incorporation of [3H]dAdo into acid-insoluble material.

Partial Reversal by dCyd of dGuo Inhibition of HL-60 Cloning. Previously, we reported that dCyd partially rescued soft agar cloning of HL-60 cells from growth inhibition caused by dThd and completely reversed the growth inhibition caused by dThd in HL-60 cells in liquid suspension culture (1). This protective effect of dCyd from dThd growth inhibition has been reported by others for several different cultured cell lines (10, 11, 26, 28). dCyd (1000 μM) raises the IC50 of dGuo for HL-60 cells from 80 to 230 μM, approximately a 3-fold increase (Chart 3).

Effects of Deoxynucleosides on Intracellular Pools of dCTP and dGTP. We studied the effects of a 16-hr incubation with 750 μM nucleoside on the intracellular pools of dCTP and dGTP in HL-60 cells (Table 1). In cultures derived from late-passage (p-71) HL-60 cells, dThd decreases intracellular dCTP pool size to 26% of control; dGuo decreases intracellular dCTP to 19% of control. The 750 μM concentration of deoxynucleoside used was one-sixth of the IC50 of dThd and 10 times the IC50 of dGuo for HL-60 (p-71) cells (Chart 1B).

Nucleotide pool studies were also performed on HL-60 cell cultures derived from cells cryopreserved after the tenth passage (p-10) in tissue culture. The doubling time of these early-passage (p-10) HL-60 cells is 50 to 60 hr, compared to 48 hr for HL-60 (p-71) cells. HL-60 (p-10) cells are less sensitive to reduction of dCTP pools by deoxyribonucleoside than are HL-60 (p-71) cells. dAdo diminishes HL-60 (p-10) cellular dCTP...
poolsto only 35% of control, dThd reduces HL-60 (p-10) dCTP pools to 61% of control, and dAdo reduces HL-60 (p-10) dCTP pools to 39% of control. As seen with the late-passage (p-71) cultures, dGuo is most potent in lowering intracellular dCTP pools.

In both early- and late-passage HL-60 cell cultures, intracellular dGTP pool sizes are doubled by dThd, are not affected by dAdo, and are increased 23- to 35-fold by dGuo.

Similar reduction of dCTP pools caused by deoxyribonucleosides are observed in K-562 cells (Table 1). dThd, dGuo, and dAdo decrease K-562 intracellular dCTP pools to 46, 35, and 67% of control, respectively. K-562 intracellular pools of dGTP are slightly lowered by dAdo, quadrupled by dThd, and increased 395-fold by dGuo.

The data shown in this paper allow the comparison of equimolar concentrations of dThd, dGuo, and dAdo on dCTP and dGTP intracellular pools. We are currently studying the effects of equipotent (i.e., IC50) concentrations of nucleoside on dCTP and dGTP pool sizes.

Adenosine Deaminase Assay. The effect of added dAdo may be attenuated by the presence of relatively high activity of adenosine deaminase (EC 3.5.4.4) in the tissue culture medium. We measured the activity of adenosine deaminase in Roswell Park Memorial Institute Medium 1640 containing 20% heat-inactivated fetal calf serum by using the method of Gustin and Kemp (13). We find that 44% of added adenosine (750 μM) is degraded after 3 hr and 88% is degraded after 8 hr at 37°.

DISCUSSION

Our studies have shown that, in addition to dThd, the naturally occurring nucleosides dAdo and dGuo are capable of inhibiting the growth of human leukemic cells in culture and have equal or greater potency than does dThd. Like dThd, the mechanism of growth inhibition by dAdo and dGuo appears to involve reduction in the intracellular pools of dCTP.

The effect of dGuo and dThd in lowering the endogenous intracellular pools of dCTP is reflected by the enhancement of incorporation of [3H]dCyd into acid-insoluble material caused by preincubation of cells with these nucleosides, since the isotope dilution effect of endogenous dCTP on the specific activity of [3H]dCTP (derived from added [3H]dCyd) is lessened. Also, dCyd kinase, the rate-limiting step in the cellular incorporation of dCyd into DNA (salvage pathway) is subject to feedback inhibition by dCTP (23, 29). Thus, an agent which decreases the amount of dCTP derived from de novo synthesis should stimulate the incorporation of added [3H]dCyd into DNA. Although dGuo caused a greater diminution in dCTP pools than did dThd, dThd caused a greater stimulation of [3H]dCyd incorporation than did dGuo. This suggests that dGuo may interfere with the biochemical processes involved in the incorporation of dCyd into DNA, in addition to diminution of de novo-derived dCTP pools.

Maximal enhancement of incorporation of [3H]dCyd into acid-insoluble material of K-562 cells caused by dThd or dGuo occurs at concentrations approximately equivalent to the IC50 for cell growth inhibition observed for these nucleosides. Thus, it is likely that the additional decrease in [3H]dCyd incorporation at concentrations of dThd or dGuo higher than the IC50 is due to inhibition of cell growth. In contrast, enhanced incorporation of [3H]dCyd in HL-60 cells caused by dThd is maximal at a concentration only one-tenth that of the IC50 of dThd for these cells. A possible explanation for this lowering of [3H]dCyd incorporation at higher (but noninhibitory) concentrations of dThd is that of competition by dThd for cellular transport and/or phosphorylation of dCyd. Plagemann et al. (28, 29) have identified in Novikoff hepatoma cells a single, high-Km facilitated diffusion system capable of transporting all nucleosides. dThd competes with dCyd for transport by this system with apparent K of approximately 120 μM (29). This may also explain the competitive behavior we observe between dAdo and dGuo for incorporation of their respective radioisotopically labeled analogs into acid-insoluble material. Phosphorylation of dCyd is the rate-limiting event in the pathway of incorporation of dCyd into DNA (29). Human dCyd kinase from leukemic blast cells exists in 2 isoenzymic forms, mitochondrial and cytoplasmic (6). However, only the mitochondrial enzyme is inhibited by dThd or dCyd. The contribution, if any, of the mitochondrial enzyme to the salvage pathway for dCTP used in cellular DNA synthesis is presently unknown.

The inhibition of [3H]dCyd incorporation into HL-60 and K-562 cell acid-insoluble material by dThd is paradoxical, since we have demonstrated that dAdo causes a significant reduction in the endogenous intracellular pools of dCTP in HL-60 cells. The expected result would be a stimulation of [3H]dCyd incorporation after incubation with dAdo, as was found following incubation with dThd or dGuo. To explain this, we must speculate that high concentrations of dAdo compete for cellular uptake or phosphorylation of dCyd in HL-60 cells or that dAdo inhibits DNA synthesis by other mechanisms.

Excess exogenous purine deoxynucleosides inhibit DNA synthesis, primarily by cellular conversion via purine nucleoside-nucleotide kinases to deoxyribonucleoside triphosphates, which then cause feedback inhibition of key enzymes in purine and pyrimidine deoxynucleotide biosynthesis (3, 4, 20, 22, 24, 28, 38). The mechanism of lymphocytotoxicity in inherited combined immunodeficiency disease (deficiency of adenosine deaminase or purine nucleoside phosphorylase) is thought to involve intracellular accumulation of the triphosphate forms of the nucleoside substrates for these enzymes. Gudas et al. (12) have shown that of the nucleoside substrates of purine nucleoside phosphorylase, dGuo is the most potent inhibitor of T-cell lymphoma (S-49) cell growth in culture. They demonstrated that dGuo causes a substantial reduction in intracellular dCTP, as well as dTTP pools, and that dGuo cytotoxicity was partially reversed by the addition of dCyd. Inhibition of growth of L5178Y cells by dGuo has been reported by Theiss et al. (38). These investigators also observed partial protection from dGuo toxicity by dCyd but attributed the mechanism of dGuo toxicity to depletion of thymidylate pools. They attributed the protective effect of dCyd to the eventual metabolism of dCyd to thymidylate via the dCMP deaminase pathway.

Carson et al. (3, 4) have demonstrated a high sensitivity of cultured T-lymphoid cells to growth inhibition by dAdo which correlates with the intracellular accumulation of dATP. A mechanism of toxicity via inhibition of ribonucleotide reductase was proposed. Lowe et al. (20) have shown by flow cytometric analysis that dAdo, like dThd (14), causes accumulation of cells in the G1-S phase of the cell cycle. Further studies by Carson et al. (5) have shown that the IC50 for dAdo decreases to 70 μM for K-562 cells and 10 μM for HL-60 cells when the

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adename disaminase inhibitor deoxycoformycin (5 μM) is added to the tissue culture medium (Roswell Park Memorial Institute Medium 1640, supplemented with 10% heat-inactivated fetal calf serum). The addition of the adenosine deaminase inhibitor makes the potency of dAdo slightly greater than that we observe for dGuo used alone. Work in our laboratory has confirmed that relatively high levels of adenosine deaminase activity are present in our cell culture medium. On this basis, we feel that our observed IC50 values for dAdo would probably be similar to those obtained by Carson et al. (5) had we added deoxycoformycin to our culture medium. Our data, like those of Carson et al. (5), show that K-562 cells are more resistant to dAdo toxicity than are HL-60 cells; Carson et al. (5) attribute this resistance to the higher dAMP-dephosphorylating activity of K-562 cells.

We feel that, in HL-60 cells, dGuo, dAdo, and dThd primarily inhibit cell growth by cellular conversion to deoxynucleoside triphosphates, with subsequent reduction of dCTP pools essential to DNA synthesis by inhibition of ribonucleotide reductase. The enhanced potency of dGuo (and dAdo in K-562), as compared to dThd, may be the result of lowering of both dCTP and dTTP intracellular pools. Since dCyd could only partially reverse dThd and dGuo growth inhibition, we feel that, at concentrations in excess of the IC50 for nucleoside plus dCyd, other mechanisms of cell growth inhibition are occurring. These may include DNA polymerase (α) inhibition as proposed by Steinberg et al. (37) or competition for nucleotide or nucleoside kinases.

dThd has potential as an adjunct to chemotherapeutic agents by rendering cells more susceptible to killing by certain cytotoxic drugs, by virtue of lowering of dCTP pools and arresting cells in the G1-S phase of the cell cycle. Our present work demonstrates similar potential of the naturally occurring nucleosides dAdo and dGuo as chemotherapeutic adjuncts, and dGuo has the added benefit of increased potency.

ACKNOWLEDGMENTS

We wish to thank Howard Rosen, Clifford Salinger, and Scott Rifkin for their technical assistance and Helen Chiewicki and Barbara Dresel for typing this manuscript.

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*Cancer Res* 1981;41:4493-4498.

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