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Effect of deoxycytidine on the in vitro response of human leukemia cells to inhibitors of de novo pyrimidine biosynthesis*

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Summary. The effect of high concentrations of exogenous dCyd on the growth inhibitory properties of several inhibitors of de novo pyrimidine biosynthesis (dThd, 3-DAU, PALA, PF) was examined in three cultured human leukemic cell lines (HL-60, K-562, KG-1), and a dCyd kinase-deficient, Ara-C-resistant variant (HL-60/Ara-C). In the presence of dCyd concentrations (10^{-3} *M*), far exceeding normal human plasma levels (0.5 to 4.0×10^{6} *M*), substantial but partial reversal of pyrimidine antagonist-mediated growth inhibition and restoration of intracellular dCTP levels was noted in all cell types except HL-60/Ara-C. When high concentrations of dCyd (10^{-3} *M*) were combined with low levels of uridine or cytidine (10^{-5} *M*), full restoration of growth was observed in sensitive cell lines. When exposed to supraphysiologic concentrations of dCyd, HL-60/Ara-C cells were more sensitive to the growth inhibitory effects of pyrimidine antagonists than parent HL-60 cells; this phenomenon was maximal at 10^{-4} *M* dCyd and was not observed in the presence of dCyd concentrations of 10^{-6} *M* or lower. These studies suggest that in the presence of low concentrations of uridine or cytidine, perturbations in intracellular dCTP pools may play a critical role in determining the in vitro antiproliferative response of human leukemic myeloid cells to diverse inhibitors of de novo pyrimidine biosynthesis. They also raise the possibility that modulation of exogenous dCyd concentrations may improve the therapeutic efficacy of pyrimidine antagonists toward certain salvage pathway-deficient, drug-resistant leukemic cells.

Introduction

The naturally occurring nucleoside dCyd is present in human plasma at concentrations of $0.5–4.0 \times 10^{-6}$ *M* [10]. It is transported across cell membranes by a carrier-mediated facilitated diffusion mechanism which it shares with other nucleosides such as dThd and uridine [38]. After phosphorylation by the pyrimidine salvage pathway enzyme dCyd kinase to dCMP [21], it is ultimately converted to its triphosphate derivative, dCTP, which serves as a substrate for the enzyme DNA polymerase [2]. Opposing these processes are the degradative enzymes cytidine and deoxycytidylate deaminase, which convert dCyd and dCMP to deoxyuridine (dUrd) and deoxyuridylate (dUMP) respectively [7, 33]. Since a balanced supply of deoxyribonucleotide triphosphates is necessary for continuing DNA synthesis and cell proliferation [35, 39], interruption of the synthesis of any of these metabolites, and dCTP in particular, can result in lethal effects. For example, the nucleoside dThd, whose triphosphate derivative dTTP is a potent inhibitor of mammalian ribonucleotide reductase [45], has been shown to block dCTP synthesis in both animal and human tumor cells in culture, leading to antiproliferative effects [27, 44]. Based in part upon these findings, high-dose thymidine infusions have been administered to patients with leukemia and lymphoma, and some responses have been noted [9, 26].

During the last several years, a number of inhibitors of de novo pyrimidine biosynthesis have entered clinical trials in man. Such agents include PF, an inhibitor of orotidylate decarboxylase [40], PALA, an inhibitor of aspartate transcarbamylase [41], and 3-DAU, an inhibitor of CTP synthetase [32]. The antiproliferative effects of these agents have been attributed to depletion of intracellular pyrimidine ribonucleoside triphosphate (UTP and CTP) pools and interference with RNA synthesis [19, 36]. This belief is supported by studies demonstrating that exogenous Urd can restore pyrimidine pools and reverse the inhibitory effects of PALA and PF toward several malignant cell lines both in vitro as well as in vivo [6, 20, 22, 23, 37]. However, additional studies have shown that PF, PALA, and 3-DAU also interfere with formation of dCTP (and hence DNA replication) in both murine and human tumor cells [4, 16, 30, 36, 37], presumably by blocking pyrimidine biosynthesis proximal to the conversion of CDP to dCDP by ribonucleotide reductase [21]. Since administration of exogenous dCyd is capable of reversing the in vitro antiproliferative

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Abbreviations. dCyd, deoxycytidine; PALA, *N*-(phosphonacetyl-L-aspartate); PF, pyrazofurin; 3-DAU, 3-deazauridine; Ara-C, 1-β-D-arabinofuranosylcytosine; dCTP, deoxycytidine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; dThd, deoxythymidine; dGTP, deoxyguanosine triphosphate; dUrd, deoxyuridine; dUMP, deoxyuridylate; dTTP, deoxythymidine triphosphate; HPLC, high-pressure liquid chromatography; 5-FU, 5-fluorouracil

effects of PALA, PF, and 3-DAU to varying degrees in several tumor cell lines [29, 30, 38] it is likely that these pyrimidine antagonists act by interfering with RNA synthesis, DNA synthesis, or a combination of the two. The present studies were undertaken to characterize the effect of supraphysiologic concentrations of dCyd on the antiproliferative responses of several continuously cultured human leukemia cell lines to inhibitors of de novo pyrimidine biosynthesis. A further aim was to determine whether modulation of exogenous dCyd concentrations might improve the selectivity of these agents toward a dCyd kinase-deficient, Ara-C-resistant variant recently isolated by our group [1].

Materials and methods

Cells. HL-60, KG-1, and K-562 cells are derived from the original lines, as previously described [14, 25, 31]. They are maintained in suspension culture in RPMI 1640 medium supplemented with 1% nonessential amino acids and 10% fetal calf serum (GIBCO, Grand Island, NY USA) in a 37 °C, 5% CO_2, controlled atmosphere incubator. Levels of dCyd in complete medium are less than 10^{-7} *M*, as determined by HPLC. Cells are passed twice weeky and routinely examined for mycoplasma contamination. The characteristics of a dCyd kinase-deficient, highly Ara-C-resistant HL-60 cell subline have been reported previously [1]. These cells are maintained in the same manner as parent cells, except that medium contains 10^{-6}*M* Ara-C which is replenished twice weekly.

Drugs and chemicals. dThd, dCyd, 3-DAU, Ara-C, dCTP, dThd, cytidine, uridine, dGTP, CTP, and UTP were purchased from Sigma Chemicals (St. Louis, Mo., USA). PALA was furnished by Dr. David Cooney, Drug Development Branch, National Cancer Institute (Bethesda, MD). PF was purchased from Cal Biochem (La Jolla, Calif., USA). [^{3}H]dGTP (30–40 Ci/mmol) was purchased from Amersham Radiochemicals (Springfield, Ill. USA). Lyophilized noncovalent double-stranded copolymer of deoxypolyinosinate-deoxypolycytidylate was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis., USA). *M. luteus* DNA polymerase (2.7.7.7) (sp. act. 8 u/mg protein) was purchased from Sigma Chemicals. Drugs were stored as dry powders at −20 °C and reconstituted in RPMI media immediately prior to use. The solutions were filter-sterilized utilizing 22-μm filter discs (Millipore Corporation, Cambridge, Mass., USA).

Pyrimidine nucleoside triphosphate determinations. Approximately 2.5×10^7 logarithmically growing cells were seeded in 75-cm^2 tissue culture flasks (Corning, Corning, NY USA), to which were added the designated concentration of dThd, 3-DAU, PALA, or PF along with 10^{-3}*M* dCyd. The flasks were incubated for 18 h in a 37°, 5% CO_2, fully humidified incubator. At the end of this period, the cell suspension was centrifuged at 400 *g* for 6 min at 4 °C. The cell pellets were washed twice with cold Dulbecco's PBS and precipitated with cold 0.5 *M* $HC10_4$. The details of this procedure have been reported elsewhere [15]. After neutralization with KOH, the acid-soluble extracts were subjected to HPLC analysis utilizing an Altex model 332 HPLC apparatus (Altex Scientific, Inc., Berkeley, Calif., USA) and a Partisil SAX column (25 cm × 10 mm I. D., Whatman, Inc., Clifton, NJ, USA). An isocratic 0.225 *M* Na_2HPO_4 buffer system was used with a flow rate of 2.0 ml/min. Absorbance at 254 nm was quantitated with the aid of an Altex Model RC1A integrator, and UTP and CTP peaks identified by coelution with known standards and peak height ratios at 254 and 280 nm. UTP and CTP levels for each experimental condition were expressed as pmol/10^6 cells.

Deoxycytidine triphosphate determinations. Intracellular levels of dCTP in leukemic cells exposed to pyrimidine antagonists in conjunction with dCyd were determined utilizing a modification of the DNA polymerase assay of Solter and Handschumacher. The details of this procedure have been described previously [24]. Approximately 2.5×10^{-7} logarithmically growing cells were incubated with each pyrimidine antagonist with and without 10^{-3} *M* dCyd for 18 h. To avoid loss of deoxyribonucleotides through washing, methanol extracts of the cell pellets were obtained according to the method of Trysted [43]. Values for each condition were expressed as pmol dCTP/10^6 cells.

Growth studies. The effect of exogenous dCyd on the response of continuously cultured human leukemic cells to dThd, PF, PALA, or 3-DAU was assessed utilizing a suspension culture growth method which has been described in detail previously [15]. Cells were seeded at an initial density of 10^5 cells/ml in the presence of varying concentrations of dCyd, and aliquots were removed at the end of 72 h incubation in a 37 °C, 5%, CO_2, fully humidified incubator. HPLC analysis of medium at the end of this period revealed a less than 10% decline in the initial dCyd concentration for both unpertubed and drug-treated cells exposed to 10^{-3} dCyd. Under these conditions, control cells continue to grow exponentially, undergoing approximately four doublings and reaching a cell density of 1.5×10^6 cells/ml. In some experiments, medium was used containing 10^{-6}–10^{-5} *M* uridine or cytidine. The IC_{50} for each pyrimidine antagonist, defined as that concentration producing a 50% reduction in suspension culture growth, was obtained by extrapolation from dose-response curves as previously described [8].

Results

The effects of increasing exogenous dCyd concentrations on the response of HL-60, KG-1, K-561, and HL-60/Ara-C cells to each pyrimidine antagonist are illustrated in Fig. 1. When cells were exposed to 5×10^{-4} *M* dThd, 5×10^{-4} *M* PALA, 5×10^{-7} *M* PF, or 5×10^{-6} *M* 3-DAU, growth inhibition was generally greater than 80% and in some instances (e.g., K-562 cells exposed to PALA, PF, 3-DAU) greater than 90%. Coadministration of dCyd at concentrations approximating the lower limits of physiologic levels (10^{-7} *M*) did not restore growth to pyrimidine antagonist-treated cells. Small increments in growth were observed in HL-60, KG-1, and K-562 cells when the dCyd concentration was increased to 10^{-6} *M*, and further protection was noted when the concentration exceeded normal plasma levels (10^{-5} *M*). For example, the growth of KG-1 cells exposed to PF was 50% of control in the presence of 10^{-5} *M* dCyd vs 14% in its absence. Similarly, administration of 10^{-5} *M* dCyd to HL-60 cells exposed to 3-DAU restored growth to 29% of control values vs 12% in

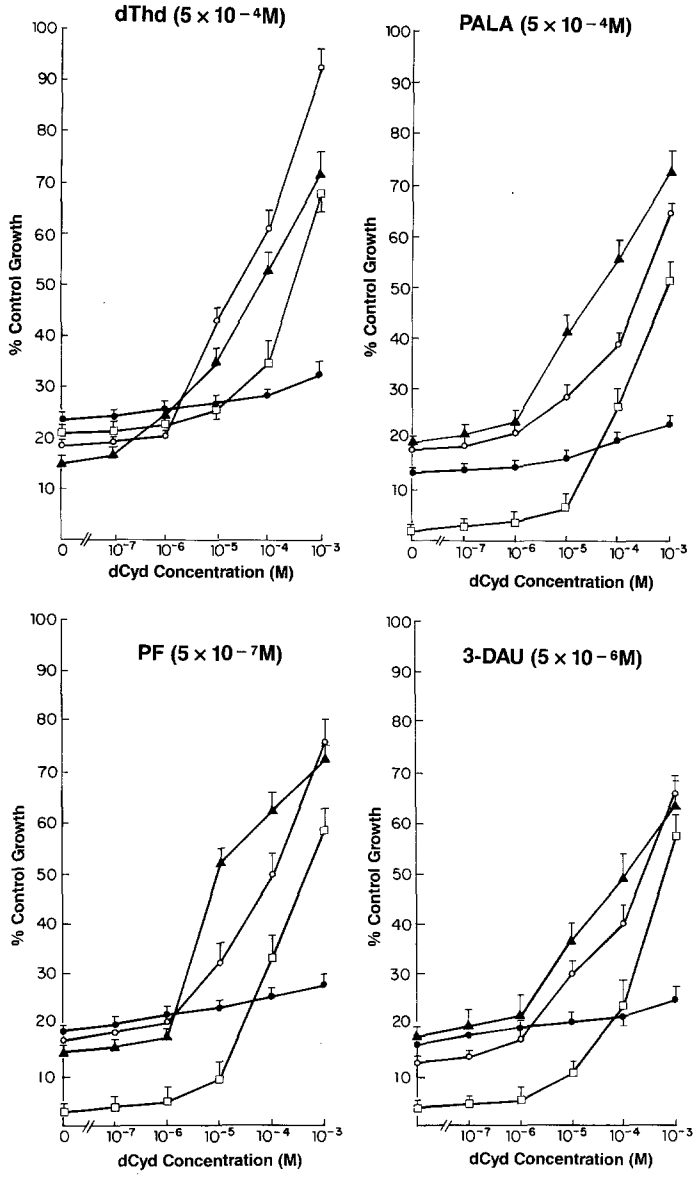

Fig. 1. The effect of increasing dCyd concentration on pyrimidine antagonist-mediated inhibition of suspension culture growth was assessed in the human leukemic cell lines HL-60, KG-1, K-562, and the dCyd kinase-deficient variant HL-60/Ara-C. Cells were grown in the continuous presence of 5×10^{-4} *M* dThd, 5×10^{-4} *M* PALA, 5×10^{-7} *M* PF, or 5×10^{-6} *M* 3-DAU in conjunction with dCyd (10^{-7}–10^{-3} *M*). Growth was assessed at the end of 72 h incubation. Values represent the means for at least four separate experiments performed in duplicate ± 1 SD. ○——○ HL-60; ●——● HL-60/Ara-C; □——□ K-562; ◀——▶ KG-1

the absence of dCyd. In the presence of 10^{-4} *M* dCyd, marked protection from pyrimidine antagonist-mediated growth inhibition was observed for all sensitive cell lines. The degree of protection ranged from 23% of control growth for K-562 cells exposed to 3-DAU to 60% for KG-1 cells exposed to PF and HL-60 cells exposed to dThd. Maximal growth protection was observed at a dCyd concentration of 10^{-3} *M* and ranged from 52% for K-562 cells exposed to PALA to 92% for HL-60 cells exposed to dThd.

In contrast to these sensitive cell lines, dCyd was relatively ineffective in restoring growth to HL-60/Ara-C cells exposed to any of the pyrimidine antagonists. For example, HL-60/Ara-C cells exposed to PALA experienced only a 9% increase in growth in the presence of 10^{-3} *M* dCyd (23% vs 14%). The degree of protection was less for other pyrimidine antagonist and lower dCyd concentrations.

In separate studies, administration of dCyd alone at concentrations as high as 10^{-3} *M* did not enhance the growth of any of the leukemic cell lines (data not shown). In addition, as pyrimidine antagonist concentrations were increased, the capacity of dCyd to restore growth in sensitive cell lines tended to decline. However, coadministration of 10^{-3} *M* dCyd continued to permit approximately 50% of control cell growth under these conditions in all cell lines except HL-60/Ara-C (not shown).

To determine whether low concentrations of uridine (or cytidine) might alter the response of pyrimidine antagonist-treated cells to supraphysiologic concentrations of dCyd, cells were exposed to PALA, PF, or 3-DAU in conjunction with 10^{-6} *M* or 10^{-5} *M* uridine (or cytidine) along with 10^{-5}–10^{-3} *M* dCyd. The results are shown in Table 1. Addition of 10^{-5} *M* uridine to the medium had only a minor effect on restoring growth to any of the human leukemic cells exposed to PALA or PF. Similarly, coadministration of 10^{-5} *M* cytidine was only partially capable of restoring growth to 3-DAU treated cells. As cells were exposed to increasing concentration of dCyd in conjunction with uridine or cytidine, growth progressively increased for HL-60, K-562, and KG-1. For pyrimidine antagonist-treated cells exposed to 10^{-5} *M* uridine or cytidine in conjunction with the highest dCyd concentration (10^{-3} *M*), growth was equivalent to that of untreated control cells.

In contrast to the sensitive cell lines, under the same conditions pyrimidine antagonist-treated HL-60/Ara-C cells demonstrated little increment in growth as the dCyd concentration was increased from 10^{-5} *M* to 10^{-3} *M*. For example, coadministration of 10^{-3} *M* dCyd to cells grown in the presence of 10^{-5} *M* uridine or cytidine resulted in the same degree of protection from PALA, PF, or 3-DAU-mediated growth inhibition as uridine or cytidine administered alone.

In order to compare the effects of increasing concentrations of dCyd on the sensitivity of HL-60 vs HL-60/Ara-C cells to dThd, PALA, PF, and 3-DAU, an IC_{50} for each agent was determined in the presence of dCyd concentrations ranging from 10^{-7} *M* to 10^{-3} *M* (Fig. 2). When dCyd was administered at concentrations approximating the lower limits of normal plasma levels (10^{-7} *M*), the IC_{50} for each drug was similar for HL-60 and HL-60/Ara-C. The same result was observed for a dCyd concentration of 10^{-6} *M*. As the concentration of dCyd exceeded maximal physiologic levels, the sensitivity of HL-60/Ara-C compared to parent HL-60 cells increased, reaching a plateau at 10^{-4} *M* and increasing only slightly thereafter. DCyd had the greatest effect in conjunction with dThd, where HL-60/Ara-C were approximately 2 logs more sensitive than parent cells, and the smallest effect in conjunction with PALA, where HL-60/Ara-C slightly less than 1 log more sensitive than in HL-60 cells at equivalent dCyd concentrations. Coadministration of 10^{-4} *M* dCyd in conjunction with PF and 3-DAU had an intermediate effect on differences in growth inhibition between HL-60/Ara-C and HL-60 cells.

To determine whether supraphysiologic concentrations of dCyd might be exerting their protective effect on cells

Table 1. Suspension culture growth of leukemic cells

	Control growth (%)								
dCyd concentration (*M*)	0			10^{-4}			10^{-3}		
Urd (Cyd) concentration (*M*)	0	10^{-6}	10^{-5}	0	10^{-6}	10^{-5}	0	10^{-6}	10^{-5}
Condition									
HL-60									
PF	18	19	28	45	51	77	71	79	93
PALA	19	20	33	40	47	82	67	76	95
3-DAU	16	(21)	(41)	41	(49)	(78)	67	(75)	(96)
K-562									
PF	4	5	10	34	38	53	58	63	94
PALA	5	5	10	26	44	59	54	70	90
3-DAU	5	(10)	(21)	23	(36)	(46)	56	(64)	(96)
KG-1									
PF	23	29	24	45	50	76	64	70	98
PALA	23	23	30	52	55	69	69	76	99
3-DAU	25	(26)	(22)	45	56	(74)	60	(74)	(93)
HL-60/Ara-C									
PF	17	19	25	18	20	23	22	24	26
PALA	19	20	27	20	23	25	21	24	27
3-DAU	18	(19)	(32)	20	(36)	(36)	25	(28)	(39)

Logarithmically growing cells were seeded at an initial cell density of 10^5 cells/ml in tissue culture flasks containing PF (5×10^{-7} *M*), PALA (5×10^{-4} *M*), or 3-DAU (5×10^{-6} *M*) in the presence or absence of 10^{-6} *M* or 10^{-5} *M* uridine (Urd) or cytidine (Cyd) along with increasing concentrations of dCyd. At the end of 72 h incubation in a 37°, 5% CO_2 incubator, aliquots of the cell suspension were removed for cell density determinations. Values for each condition represent the increase in cell growth relative to untreated control cells. Figures in parentheses for 3-DAU-treated cells correspond to 10^{-6} *M* and 10^{-5} *M* cytidine; all other figures correspond to 10^{-6} *M* and 10^{-5} *M* uridine. Values represent the means for at least three separate experiments performed in duplicate

by preventing exhaustion of intracellular pyrimidine nucleoside triphosphate pools, levels of UTP and CTP were assayed in HL-60 cells exposed to each pyrimidine antagonist in conjunction with 10^{-3} *M* dCyd for 18 h (Table 2). This dCyd concentration was selected because it was associated with maximal protection from pyrimidine antagonist-mediated growth suppression. Exposure of cells to 5×10^{-4} *M* dThd for 18 h resulted in 10% reductions in intracellular UTP and CTP pools, but these values were not significantly different from controls ($p > 0.05$).

Coadministration of 10^{-3} *M* dCyd also did not produce statistically significant differences in UTP or CTP

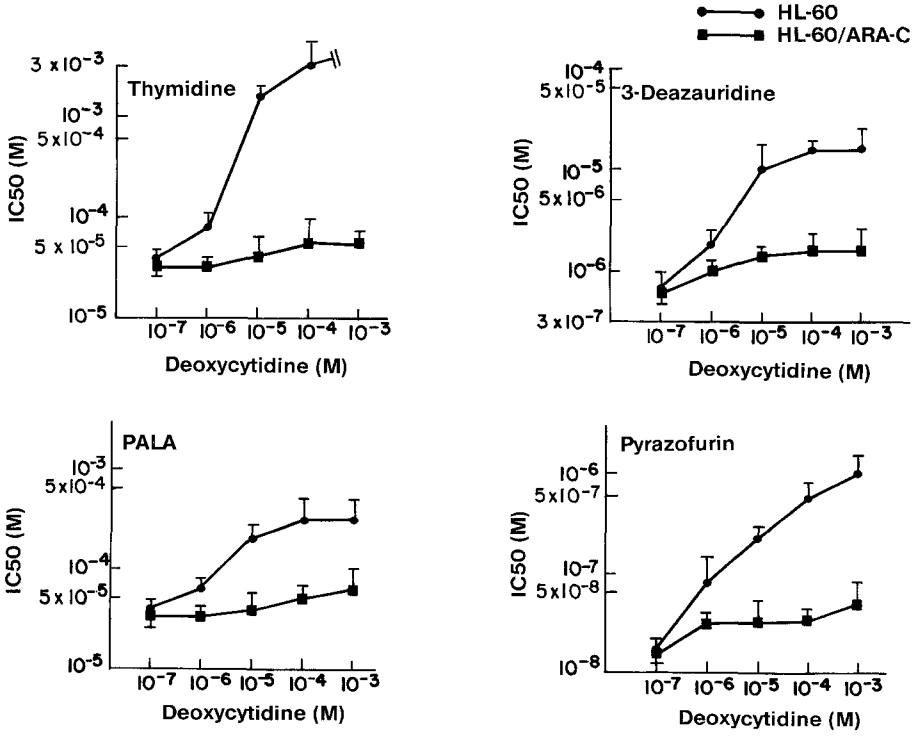

Fig. 2. The effect of dCyd concentration on the IC_{50} of various pyrimidine antagonists was compared in HL-60 and HL-60/Ara-C. The IC_{50} for each agent represents the drug concentration producing a 50% reduction in the 72-h suspension culture growth for each cell type. Values represent the means for at least three separate experiments performed in duplicate ±1 SD. Concentrations on the abscissa are expressed in logarithmic scale

Table 2. UTP and CTP pools in HL-60 cells

Condition	UTP (pmol/10^6 cells)	CTP (pmol/10^6 cells)
Control	947±110	546±130
dThd (5×10^{-4} *M*)	842± 88	484± 69
dThd + dCyd (10^{-3} *M*)	903±120	524± 75
PF (5×10^{-7} *M*)	186± 53	112± 42
PF + dCyd (10^{-3} *M*)	201± 43	124± 32
3-DAU (5×10^{-6} *M*)	846± 68	120± 44
3-DAU + dCyd (10^{-3} *M*)	878± 72	131± 38
PALA (5×10^{-4} *M*)	278± 59	153± 62
PALA + dCyd (10^{-3} *M*)	259± 68	163± 71

Logarithmically growing HL-60 cells were incubated for 18 h with pyrimidine antagonists at the indicated concentrations in the presence or absence of 10^{-3} *M* dCyd. Neutralized acid-soluble extracts were subjected to HPLC analysis and intracellular levels of UTP and CTP determined. Values represent the means of at least two separate experiments performed in duplicate ± 1 SD

levels. 3-DAU (5×10^{-6} *M*) resulted in a nearly 75% reduction in intracellular CTP pool levels and a smaller decrement in UTP pools. CTP levels were not restored to control values by coadministration of dCyd. PALA (5×10^{-4} *M*) resulted in 70% reductions and PF (5×10^{-7} *M*) resulted in 80% reductions in intracellular UTP and CTP levels. These effects were also not reversed by coadministration of 10^{-3} *M* dCyd.

Perturbations in intracellular dCTP levels in pyrimidine antagonist-treated HL-60, HL-60/Ara-C, KG-1, and K-562 cells exposed to 10^{-3} *M* dCyd for 18 h are shown in Table 3. Exposure of all cell types to dThd (5×10^{-4} *M*) produced the greatest reduction in dCTP levels (e.g., 75%–85%). Coadministration of 10^{-3} *M* dCyd resulted in dCTP levels which exceeded control values in HL-60, KG-1, and K-562. In contrast, dCyd was not capable of restoring dCTP concentrations to control levels in dThd-treated HL-60/Ara-C cells. In addition, coadministration of dCyd concentrations which were associated with minor degrees of reversal of dThd-mediated growth inhibition (e.g., 10^{-6} *M* and 5×10^{-6} *M*) resulted in minimal dCTP pool restoration in these cells. A similar pattern of response was observed for the pyrimidine antagonists 3-DAU, PF, and PALA, where reduction in dCTP levels ranged from 74% (3-DAU) to 62% (PALA) and in all cases was greater than 50%. Coadministration of 10^{-3} *M* dCyd in conjunction with each pyrimidine antagonist resulted in dCTP concentrations exceeding values for unperturbed HL-60, KG-1, and K-562 cells. However, 10^{-3} *M* dCyd did not reverse the effects of 3-DAU, PALA, or PF on dCTP pools in HL-60/Ara-C. In additional experiments, the effect of supraphysiologic concentrations of uridine on dCTP pool perturbations was examined in HL-60 cells exposed to pyrimidine antagonists. This uridine concentration has previously been shown to reverse the inhibitory effects of PALA and PF in these cells [31]. Coadministration of 5×10^{-5} *M* uridine in conjunction with 5×10^{-4} *M* PALA or 5×10^{-7} *M* PF restored dCTP levels to within 80% of control values (not shown).

Table 3. dCTP pools in leukemic cells

Condition	dCTP level (pmol dCTP/10^6 cells)			
	HL-60	HL-60/Ara-C	K-562	KG-1
Control	12.3±2.4	12.9±2.6	4.8±1.0	4.3±0.8
dCyd (10^{-3} *M*)	48.5±5.1	13.3±2.1	15.4±2.6	14.2±3.1
dThd (5×10^{-4} *M*)	2.0±0.7	2.4±0.9	1.1±0.6	0.9±0.4
+ dCyd (10^{-3} *M*)	29.7±5.8[a]	2.6±0.8	9.9±2.1	9.0±1.3
3-DAU (5×10^{-6} *M*)	3.4±1.6	3.6±1.4	1.4±0.5	1.2±0.3
+ dCyd (10^{-3} *M*)	31.4±5.4	3.8±1.6	11.4±2.3	10.0±2.1
PF (5×10^{-7} *M*)	3.8±1.4	4.0±1.5	1.6±0.6	1.4±0.4
+ dCyd (10^{-3} *M*)	33.1±6.2	4.2±1.6	9.4±2.3	8.6±1.9
PALA (5×10^{-4} *M*)	4.1±1.9	4.7±2.2	1.7±0.7	1.5±0.5
+ dCyd (10^{-3} *M*)	32.4±5.8	4.8±2.4	9.6±2.9	9.8±2.4

Logarithmically growing cells were incubated for 18 h in the presence of each pyrimidine antagonist at the designated concentration in the presence or absence of dCyd (10^{-3} *M*). Neutralized acid-soluble extracts were obtained and dCTP determinations performed utilizing the DNA polymerase assay. Values are expressed as pmol dCTP/10^6 cells and represent the means for at least two experiments performed in duplicate ± 1 SD

[a] Values for 10^{-6} *M* and 5×10^{-6} *M* dCyd are 2.4±0.5 and 3.8±1.2 pmol dCTP/10^6 cells, respectively

Discussion

Precedents exist for the use of naturally occurring nucleosides in cancer chemotherapy based on the premise that neoplastic cells may be less efficient in utilizing these compounds than normal tissues in circumventing toxicity associated with certain antimetabolites. For example, the nucleoside thymidine has been administered to "rescue" normal but not neoplastic cells from methotrexate-induced lethal effects [18]. In addition, preclinical studies have suggested that uridine may preferentially antagonize 5-FU incorporation into host cell RNA, leading to a net gain in therapeutic index for the 5-FU/uridine combination [34], and clinical studies in human have recently been initiated [28]. With respect to dCyd, in vitro studies have suggested that this nucleoside is inefficient in antagonizing Ara-CTP formation in human leukemic myeloblasts deficient in the enzyme dCyd kinase [17], and in vivo studies in mice inoculated with the murine leukemia L1210 have shown that administration of Ara-C in conjunction with dCyd leads to amelioration of host toxicity without loss of antileukemic effects, resulting in improved therapeutic efficacy for the combination [5]. The present studies demonstrate that supraphysiologic concentrations of dCyd may substantially modulate the in vitro antiproliferative effects of several inhibitors of de novo pyrimidine biosynthesis on cultured human leukemic cells, and raise the possibility that this phenomenon may be exploited with respect to certain types of pyrimidine salvage pathway-deficient, drug-resistant human leukemic cells.

Previous studies have demonstrated that inhibitors of de novo pyrimidine synthesis such as PF, PALA, and 3-DAU produce reductions in intracellular ribonucleotide pools, which in turn lead to depletion in intracellular deoxyribonucleotide levels, particularly dCTP [29, 30, 38]. They also suggest that exhaustion of the latter metabolite may represent the limiting factor for DNA replication and cell proliferation under some conditions [29, 30]. It is notable that reversal of pyrimidine antagonist-mediated growth inhibition occurred at dCyd concentrations far ex-

ceeding both normal plasma levels (5×10^{-7}–4×10^{-6} *M*) [10] as well as the Km of dCyd with respect to the enzyme dCyd kinase (10^{-5}–2×10^{-5} *M*) obtained from L1210 cells and human leukemic myeloblasts [9, 21]. Since the Km for carrier-mediated transport of nucleosides in mammalian cells is approximately 500 μ*M* [13], it is conceivable that high dCyd concentrations might antagonize the transport of nucleoside analogs such as 3-DAU. However, the perturbations in intracellular ribonucleotide pools seen in PF-, PALA-, or 3-DAU-treated cells exposed to 10^{-3} *M* dCyd argues against interference with pyrimidine antagonist transport or metabolism as a basis for restoration of cell growth. It is also noteworthy that coadministration of low levels of uridine (or cytidine) was necessary to restore growth fully to pyrimidine antagonist-treated cells exposed to high levels of dCyd. Previous studies have demonstrated that high (e.g., 50–100 μ*M*) concentrations of uridine are necessary to reverse PALA- or PF-mediated growth inhibition [6, 15, 37]. Our results suggest that in the presence of low levels of uridine, exhaustion of intracellular dCTP pools may represent the limiting factor for in vitro cell growth.

The relative inability of dCyd to restore dCTP pools and reverse pyrimidine antagonist-mediated growth inhibition has implications for potentially selective chemotherapeutic approaches toward pyrimidine salvage pathway-deficient, drug-resistant cells such as HL-60/Ara-C. We have previously found that high concentrations of dCyd protect normal human bone marrow cells more than HL-60/Ara-C cells against the effects of high concentrations of dThd [1]; the present studies raise the possibility that similar effects might occur with PALA, PF, or 3-DAU. It should be recognized that while loss of the enzyme dCyd kinase is a common mechanism by which cultured leukemic cells develop resistance to Ara-C [11], an association with clinical Ara-C refractoriness has not been established [42]. In addition, information regarding the in vivo effects of dCyd with respect to pyrimidine antagonist-mediated cytotoxicity is limited, although previous studies have shown that endogenous dCyd levels may influence dThd efficacy in intact animals [12] and administration of dCyd may improve the therapeutic index of Ara-C in mice [5]. In view of the potential ability of dCyd to modulate the antitumor efficacy of several clinically available pyrimidine antagonists, animal studies designed to test the in vivo relevance of these in vitro findings are currently being implemented.

References

1. Bhalla K, Nayak R, Grant S (1984) Isolation on characterization of a deoxycytidine kinase deficient, human promyelocytic leukemic cell line highly resistant to 1-β-D-arabinofuranosylcytosine. Cancer Res 44: 5029.
2. Bjursell G, Reichard P (1973) Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. J Biol Chem 248: 3904.
3. Blumenreich MS, Woodcock TM, Andreef M, Hiddemann W, Chou T-C, Vae K, O'Hehir M, Clarkson B, Young C (1984) Effect of very high-dose thymidine infusions on leukemia and lymphoma patients. Cancer Res 44: 2203.
4. Brockman RW, Shaddix SC, Williams M, Nelson JA, Rose LM, Schabel FM Jr (1975) The mechanism of action of 3-deazauridine in tumor cells sensitive and resistant to arabinofuranosyl-cytosine. Ann NY Acad Sci 255: 501.
5. Buchman VM, Belyanchikova NK, Mkeidze DM, Litovchenko TA, Lichinitser MR, Barkhotkina MF, Svet-Moldavsky GJ (1979) 2'-deoxycytidine hydrochloride protection of mice against the lethal toxicity of cyhosine arabinoside. Cancer Chemother Pharmacol 3: 229.
6. Cadman E, Benz C (1980) Uridine and cytidine metabolism following inhibition of de novo pyrimidine synthesis by pyrazofurin. Biochimica Biophys Acta 609: 372.
7. Chabner BA, Johns DG, Coleman CN, Drake JC, Evans WN (1974) Purification and properties of cytidine deaminase from normal and leukemic granulocytes. J Clin Invest 53: 922.
8. Chou T-C, Burchenal JH, Schmid FA, Braun KA, Watanabe KA, Fox JF, Phillips FS (1982) Biochemical effects of 2'-fluoro-5-methyl 1-β-D-arabinofuranosyluracil and 2'-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine in mouse leukemic cells sensitive and resistant to 1-β-D-arabinofuranosylcytosine. Cancer Res 42: 3957.
9. Coleman CN, Stoller RG, Drake JC, Chabner BA (1975) Deoxycytidine kinase: properties of the enzyme from human leukemic granulocytes. Blood 46: 791.
10. Danhauser LL, Rustum YM (1980) Effect of thymidine on the toxicity, antitumor activity, and metabolism of 1-β-D-arabinofuranosylcytosine in rats bearing a chemically induced colon carcinoma. Cancer Res 40: 1274.
11. Drahovsky D, Kreis W (1970) Studies on drug resistance II kinase patterns in P815 neoplasms sensitive and resistant to 1-β-D-arabinofuranosylcytosine. Biochem Pharmacol 39: 538.
12. Egan EM, Sargent L, Roswsky A, Kufe DW (1981) Rescue of thymidine toxicity in L1210 ascites by elevated endogenous levels of deoxycytidine. Cancer Treat Rep 65: 853.
13. Eilam Y, Cabantchik ZI (1977) Nucleoside transport in mammalian cell membranes: a specific inhibitory mechanism of high affinity probes. J Cell Physiol 92: 185.
14. Gallegher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F, Gallo R (1979) Characterization of the continuous differentiating myeloid cell line from a patient with acute promyelocytic leukemia. Blood 54: 713.
15. Grant S, Bhalla K, Gleyzer M (1984) Effect of uridine on response of 5-azacytidine resistant human leukemic cells to inhibition of de novo pyrimidine synthesis. Cancer Res 44: 5505.
16. Grant S, Rauscher F, Cadman E (1982) Differential effect of *N*-(phosphonacetyl-L-aspartate) on 1-β-D-arabinofuranosylcytosine metabolism and cytotoxicity in human leukemia and normal bone marrow progenitors. Cancer Res 42: 4007.
17. Harris AL, Grahame DG (1981) Cytosine arabinoside triphosphate production in human leukemic myeloblasts: interaction with deoxycytidine. Cancer Chemother Pharmacol 5: 185.
18. Howell SB, Herbst K, Boss GR, Frei E II (1980) Thymidine requirements for the rescue of patients treated with high-dose methotrexate. Cancer Res 40: 1824–1829.
19. Jayaram HN, Cooney DA, Vistica DT, Kariya S, Johnson RK (1979) Mechanism of sensitivity or resistance of murine tumors to *N*-(phosphonacetyl-L-aspartate). Cancer Treat Rep 63: 1291.
20. Johnson RK (1977) Reversal of toxicity and antitumor activity of *N*-(phosphonacetyl)-L-aspartate by uridine or carbamyl-DL-aspartate in vivo. Biochem Pharmacol 26: 81.
21. Ives DH, Durham JP (1970) Deoxycytidine kinase III. Kinetics and allosteric regulation of the calf thymus enzyme. J Biol Chem 245: 2285.
22. Karle JM, Anderson LW, Cysyk RL (1984) Effect of plasma concentrations of uridine on pyrimidine biosynthesis in cultured L1210 cells. J Biol Chem 259: 67.
23. Karle JM, Anderson LW, Erlichman C, Cysyk RL (1980) Serum uridine levels in patients receiving *N*-(phosphonacetyl)-L-aspartate. Cancer Res 40: 2938.
24. Kinahan JJ, Otten M, Grindey GB (1979) Evaluation of ribonucleoside and deoxyribonucleoside triphosphate pools in cultured leukemia cells during exposure to methotrexate plus thymidine. Cancer Res 39: 3531.

25. Koeffler HP, Golde DW (1978) Acute myelogenous leukemia: a human cell line responsive to colony stimulating activity. Science 200: 1153.
26. Kufe DW, Beardsley P, Karp D, Parker L, Rosowsky A, Canellos G, Frei E III (1980) High-dose thymidine infusions in patients with leukemia and lymphoma. Blood 55: 580.
27. Lee SS, Giovanella BC, Stehlin JS Jr (1977) Selective lethal effect of thymidine on human and mouse tumor cells. J Cell Phys 92: 401.
28. Leyva A, van Groennengen CJ, Kraal I, Gall H, Peters G, Tankelma J, Pinedo HM (1984) Phase I and pharmacokinetic studies of high-dose uridine intended for rescue from 5-fluorouracil toxocity. Cancer Res 44: 5928.
29. Lockshin A, Mendoza JT, Giovanella BC, Stehlein JS (1984) Cytotoxic and biochemical effects of thymidine and 3-deazauridine on human tumor cells. Cancer Res 44: 2534.
30. Low E, Kufe D (1981) Synergistic effects of inhibitors of de novo pyrimidine biosynthesis acivicin and *N*-(phosphonacetyl-L-aspartic acid). Cancer Res 41: 3419.
31. Lozzio CB, Lozzio BB (1975) Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45: 321.
32. McPartland RP, Wang MC, Bloch A, Weinfeld H (1974) Cytidine-5'-triphosphate synthetase as a target for inhibition by the antitumor agent 3-deazauridine. Cancer Res 34: 3107.
33. Maley GF, Maley F (1963) Nucleotide interconversions VIII. Thymidine sparing effect on the utilization of deoxycytidine by chick embryo mince. Biochem Biophys 68: 293.
34. Martin DS, Stolfi RL, Spiegelman S, Young CW (1982) High-dose 5-fluorouracil with delayed uridine rescue in mice. Cancer Res 40: 1274.
35. Moore EC, Hurlbert RB (1966) Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. J Biol Chem 241: 4802.
36. Moyer JD, Handschumacher RE (1979) Selective inhibition of pyrimidine synthesis and depletion of nucleotide pools by *N*-(phosphonacetyl)-L-aspartate. Cancer Res 39: 3089.
37. Plagemann PGW, Behrens M (1976) Inhibition of de novo pyrimidine nucleotide and DNA synthesis on growth of cultured Novikoff rat hepatoma cells and other cell lines by pyrazofurin (NSC 143095). Cancer Res 36: 3807.
38. Plagemann PGW, Marz R, Wohlhenter RM (1978) Transport and metabolism of deoxycytidine and 1-β-D-arabinofuranosylcytosine into cultured Novikoff hepatoma cells, relationship to phoshorylation and regulation of triphosphate synthesis. Cancer Res 38: 987.
39. Reichard P (1972) Control of deoxyribonucleotide synthesis in vitro and in vivo. Adv Enzyme Reg 10: 3.
40. Sweeney MJ, Davis FA, Gutowski GE, Hamill RL, Hoffman DH, Poore GA (1973) Experimental antitumor activity of pyrazomycin. Cancer Res 33: 2619.
41. Swyryd EA, Seaver SS, Stark G (1974) *N*-(phosphonacetyl-L-aspartate), a potent transition state analog inhibitor of aspartate transcarbamylase blocks proliferation of mammalian cells in culture. J Biol Chem 249: 6945.
42. Tattersall MNH, Ganeshaguru K, Hoffbrand AV (1974) Mechanism of resistance of human acute leukemia cells to cytosine arabinoside. Br J Haematol 27: 39.
43. Trysted G (1975) The pool size of deoxyadenosine 5'-triphosphate and deoxycytidine 5'-triphosphate in phytohemagglutinin stimulated and non-stimulated human lymphocytes. Exp Cell Res 91: 429.
44. Tsuboi K, Kwong L (1978) Antiproliferative agents and differential survival between normal and cancer cells. Cancer Res 38: 3745.
45. Xeros N (1962) Deoxyriboside control and synchronization of mitosis. Nature 194: 682.

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