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2′,3′-Dideoxycytidine: Regulation of Its Metabolism and Anti-retroviral Potency by Natural Pyrimidine Nucleosides and by Inhibitors of Pyrimidine Nucleotide Synthesis

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SUMMARY

The antiretroviral action of 2′,3′-dideoxycytidine (ddCyd) depends on its intracellular conversion to the 5′-triphosphate metabolite ddCTP. The effect of natural pyrimidines and pyrimidine nucleosides, as well as of a number of inhibitors of pyrimidine nucleotide synthesis (i.e., N-(phosphonacetyl)-L-aspartate, 6-azauridine, pyrazofurin, 3-deazauridine, and hydroxyurea) on the metabolism of the potent anti-human immunodeficiency virus drug ddCyd has been investigated in human and murine cell lines. Deoxycytidine (dCyd) and cytidine (Cyd) effectively blocked the intracellular phosphorylation of ddCyd: dCyd by competition with ddCyd for 2′-deoxycytidine kinase, and Cyd probably by competition with the higher nucleoside mono- and diphosphate kinases. These conclusions are supported by the observations that (i) the cytostatic effects of ddCyd against human Molt/4F cells are significantly reversed by dCyd; (ii) the antiviral effects of ddCyd against human immunodeficiency virus-infected human ATH8 cells are reversed by dCyd and Cyd; (iii) phosphorylated metabolites of ddCyd could not be detected in a 2′-deoxycytidine kinase-deficient murine leukemia (L1210)/araC cell line; and (iv) ddCyd lacked any cytostatic effect against this araC-resistant L1210 cell line. In contrast to dCyd and Cyd, thymidine (dThd) stimulated formation of phosphorylated ddCyd metabolites. The degree of this stimulation proved dependent on preincubation time and dThd concentration. There was a correlation between the increased ddCTP levels upon preincubation of the cells with dThd, and decreased dCyd-5′-triphosphate pools, presumably caused by inhibition of cytidine-5′-diphosphate reductase by dThd-5′-triphosphate. In an attempt to discover compounds other than dThd that are able to stimulate ddCTP formation, a number of inhibitors of pyrimidine nucleotide metabolism were also studied. Under our experimental conditions, 3-deazauridine and hydroxyurea proved equally as effective as dThd in stimulating ddCyd phosphorylation. Finally, we could demonstrate that dThd significantly enhanced the protective effect of ddCyd against human immunodeficiency virus-infected ATH8 cells.

Attention has recently been directed toward the use of 2′,3′-dideoxyribonucleosides as potential antiretroviral agents (1, 2). Among this class of nucleoside analogues, ddCyd has proved to be the most potent *in vitro* inhibitor of human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS). At a concentration of 0.5 μM, ddCyd totally protected ATH8 cells (a human T-cell clone, selected for its rapid growth and extreme sensitivity to the cytolytic effects of HTLV-III_B) against the cytopathic effects of HIV (1). This nucleoside also inhibited the infectivity and replication of HIV as assessed by viral DNA formation, RNA expression, and production of the virus-coded P24 protein in susceptible human T-cells after exposure to HIV virions *in vitro* (1, 2).

The major antiviral effect of ddCyd is thought to derive from its inhibition of the retroviral DNA polymerase (reverse transcriptase) (3, 4) and/or DNA chain terminating activity (5). To achieve such inhibition, ddCyd must first be phosphorylated by dCyd kinase (dCK) (4, 6, 7) and further anabolized to the 5′-triphosphate (3). However, because ddCyd proved to have a much lower affinity for dCK than its physiologic substrate dCyd (4, 7), low intracellular levels of ddCTP relative to dCyd-5′-triphosphate (dCTP) may prevent ddCyd from achieving its optimal antiviral effect. In an attempt to investigate the feasibility of increasing intracellular ddCtP levels by manipulating dCyd and ddCyd metabolism, we have studied the potential of natural pyrimidine nucleosides and a series of inhibitors of

ABBREVIATIONS: Cyd, cytidine; dCyd, 2′-deoxycytidine; ddCyd, 2′,3′-dideoxycytidine; Urd, uridine; dUrd, 2′-deoxyuridine; dThd, thymidine; dTDP, dThd-5′-diphosphate; dTTP, dThd-5′-triphosphate; CMP, cytidine-5′-monophosphate; CDP, cytidine-5′-diphosphate; CTP, cytidine-5′-triphosphate; dCMP, dCyd-5′-monophosphate; dCDP, dCyd-5′-diphosphate; dCTP, dCyd-5′-triphosphate; ddCMP, ddCyd-5′-monophosphate, ddCDP, ddCyd-5′-diphosphate; ddCTP, ddCyd-5′-triphosphate; dCK, 2′-deoxycytidine kinase; DPBS, Dulbecco's phosphate buffered saline; HIV, human immunodeficiency virus; PALA, N-(phosphonacetyl)-L-aspartate; HTLV-III, human T-lymphotropic virus, type III; AIDS, acquired immunodeficiency syndrome.

pyrimidine nucleotide synthesis known to have the ability to influence pyrimidine biosynthetic pathways (i.e., 3-deazauridine, hydroxyurea, 6-azauridine, *N*-(phosphonacetyl)-L-aspartate (PALA), and pyrazofurin) to stimulate phosphorylation of ddCyd by murine leukemia L1210 and human lymphocyte ATH8 cells.

We found that dThd, 3-deazauridine, and hydroxyurea significantly stimulate phosphorylation of ddCyd to its 5′-mono-, di- and triphosphate derivatives and that dThd significantly enhances the antiviral effects of ddCyd in HTLV-III/HIV-infected ATH8 cells *in vitro* with no parallel increase in the toxicity of ddCyd. Our observations may be of value in current attempts to develop an effective combination chemotherapy for the control of AIDS.

Materials and Methods

Cells

Murine leukemia L1210 cells were grown in RPMI-1630 medium (Quality Biologicals Inc., Gaithersburg, MD) supplemented with 16.7% (v/v) inactivated fetal calf serum and 2 mM L-glutamine. The L1210/araC subline has been selected from the parental L1210 cells for its ability to grow in the presence of araC (1 μg/ml). This mutant cell line is deficient in deoxycytidine kinase (8).

Human ATH8 cells represent an immortalized helper/inducer T cell clone obtained by cloning a normal tetanus-toxoid-specific T-cell line in the presence of lethally irradiated HTLV-I-producing MJ tumor cells (9). Clone ATH8 was selected for this study on the basis of its rapid growth and high sensitivity to the cytopathic effect of HIV (HTLV-III_B). HTLV-III_B was derived from a pool of American patients with AIDS (10). The cells were grown in RPMI-1640 medium, supplemented with 15% (v/v) inactivated fetal calf serum, 15% (v/v) interleukin-2 (lectin-depleted) (Cellular Products, Buffalo, NY), 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 units of penicillin and 50 μg of streptomycin per ml.

Radiochemicals

[5-^{3}H]ddCyd (specific radioactivity 6 and 5.5 Ci/mmol; radiochemical purity greater than 99%) was obtained from Moravek Biochemicals (Brea, CA).

Chemicals

Uracil, cytosine, uridine, cytidine, 2′-deoxyuridine, 2′-deoxycytidine, and thymidine were purchased from Sigma Chemical Corporation (St. Louis, MO). 2′,3′-Dideoxycytidine, 2′,3′-dideoxycytidine-5′-diphosphate, and 2′,3′-dideoxycytidine-5′-triphosphate were from Pharmacia–P.L. Biochemicals (Piscataway, NJ). 2′,3′-Dideoxycytidine-5′-monophosphate was synthesized as described previously (6). All other reagents used were of the highest quality obtainable.

Effect of Natural Pyrimidines and Pyrimidine Nucleosides on the Metabolism of [^{3}H]ddCyd in L1210 cells

L1210 cells were seeded at 2×10^5 cells/ml into culture flasks containing 10 ml of cell suspension and allowed to grow for 12–14 hours. Then, cells (approximately 4×10^5/ml) were exposed to 1 μM [^{3}H]ddCyd alone (control) or in the presence of varying concentrations (1–100 μM) of uracil, cytosine, uridine, cytidine, 2′-deoxyuridine, 2′-deoxycytidine, or thymidine. After 4.5 hr, the incubation was terminated and cells were subsequently washed three times with cold phosphate-buffered saline to remove most of the radiolabeled nucleoside. Intracellular metabolites of ddCyd were extracted from the cell pellet with 10% trichloroacetic acid; the trichloroacetic acid-soluble fraction of the cell lysate was separated from the trichloroacetic acid-insoluble fraction by centrifugation at 12,400 × *g* for 2 min and was neutralized with tri-n-octylamine in freon (1:4). This cell extract was analyzed by ion exchange high performance liquid chromatography using a radial compression column of Partisil-10 SAX with a gradient of ammonium phosphate as previously described (6).

Metabolism of [^{3}H]ddCyd after Preincubation of L1210 or ATH8 cells with Thymidine

Ten-milliliter cultures of L1210 or ATH8 cells (seeded at 2×10^5 cells/ml) were preincubated with different concentrations of dTHd (20 μM, 100 μM, 500 μM) for 2, 5, or 12 hr before 1.0 μM [^{3}H]ddCyd was added for an additional 4.5 hrs. At the end of the incubation period, cells were harvested and intracellular metabolites of [^{3}H]ddCyd were determined as described above.

Effect of Inhibitors of Pyrimidine Nucleotide Synthesis on the Anabolism of [^{3}H]ddCyd

L1210 cells (2×10^5 cells/ml, 10 ml) were preincubated for 12 hr at 37° with the following inhibitors of pyrimidine nucleotide synthesis: PALA (0.30 and 3.0 μg/ml); 6-azauridine (0.26 and 2.6 μg/ml), pyrazofurin (0.028 and 0.28 μg/ml); 3-deazauridine (0.18 and 1.8 μg/ml); hydroxyurea (5.4 and 54 μg/ml); and dThd (13 and 130 μg/ml). The concentrations represent the 50% inhibitory concentration for cell proliferation (IC_{50}) and a concentration 10 times the IC_{50}, respectively. After the preincubation, 1 μM [^{3}H]ddCyd was added for an additional 4.5 hr, and intracellular metabolites were determined as described above.

Determination of Intracellular dCTP Pools in L1210 Extracts

A radioimmunoassay capable of quantitating dCTP in femtomole amounts in cell extracts has been developed by Piall *et al.* (11) and applied to our L1210 cell extracts.

Briefly, L1210 cell extracts were prepared as described in the previous section and treated by boronate affinity chromatography. The boronate affinity gel was made following the methods of Inman and Dintzis (12) and of Gehrke *et al.* (13). Extracts were applied to the column in 0.5 volume of 3 M CH_3COONH_4, pH 8.9.

The radioimmunoassay was performed directly on the fractions after adjustment of the pH to 6.2 with acetic acid. A constant amount of [^{3}H]dCTP was added to each assay tube; dCTP antibody, which was derived from a rabbit in response to a conjugate of dCTP-albumin, was added to assay tubes containing different sample dilutions and incubated for 1 hr on ice, after which antibody-bound and free ligand were separated by the addition of dextran-coated charcoal. After centrifugation, aliquots of the supernatant were counted for radioactivity. The dCTP antibody cross-reacts with CTP, dCDP, and 2′-deoxycytidine-5′-monophosphate (dCMP) to the extent of 2.7%, 7.1%, and 2.1%, respectively (11). Cross-reactivity with ddCTP was ca. 50%, but it did not significantly interfere with dCTP determination under our experimental conditions (low ddCTP levels versus high dCTP pools).

Effect of L1210 Cell Cycle on the Intracellular Metabolism of [^{3}H]ddCyd

One μM [^{3}H]ddCyd was added 15, 25, 36, and 44 hr after seeding the L1210 cells in culture flasks at an initial concentration of 3×10^5 cells/ml; thereafter, cells were incubated for an additional 4.5 hr. The different initiation times for ddCyd incubation extended over the exponential growth phase (15 and 25 hr) as well as the stationary growth phase (44 hr) of the cells.

Inhibition by ddCyd of the Cytopathic Effect of HIV against ATH8 Cells

ATH8 cells were pretreated with polybrene at 2 μg/ml for 30 min at 37°. Cells were then pelleted, suspended in fresh RPMI-1640 culture medium containing 15% fetal calf serum, 15% interleukin-2 (v/v), 50 μM β-mercaptoethanol, 4 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin, and infected with 3000 virus particles/cell for

60 min at 37°. After infection, cells were reconstituted in culture medium and seeded in culture tubes at 2 ml/tube in the presence or absence (controls) of ddCyd (0.5, 0.3, 0.1, 0.075, 0.05 μM). After incubation for 6–8 days at 37°C, the number of viable cells was counted in a hemocytometer under the microscope by the trypan blue exclusion method and compared with controls (noninfected cells that were incubated at the same concentrations of the compounds as the virus-infected cells).

In the preincubation experiments, cells were exposed to 100 μM or 50 μM dThd for 24 hr before virus infection and addition of ddCyd.

Cell Cycle Analysis of L1210 Cells Preincubated with dThd

Cell cycle analysis of L1210 cells pretreated with dThd was carried out by the method of Crissman *et al.* (14). Briefly, logarithmically growing L1210 cells were seeded at 4×10^5 cells/ml in culture medium and treated with 0, 20, 100, or 500 μM dThd for 15 hr. At the end of the incubation period, 10^6 cells were drawn from each sample and washed twice with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (DPBS). To the cell pellets were added 0.3 ml of cold DPBS containing 10% glucose, and while gently shaking, 0.9 ml of cold absolute ethanol was added dropwise. Samples were stored refrigerated. After decanting the ethanol, the fixed cells were suspended in 0.8 ml DPBS and each sample was divided into two equal volumes (ca. 0.4 ml). To each volume was added 50 μl of propidium iodide (370 μg/ml) (Sigma), and after incubation for 2–3 min at room temperature, samples were incubated with 20 μl of ribonuclease A (10,000 units/ml) (Sigma) for at least 20–30 min before analysis on a FACS 440 (Becton-Dickinson, Sunnyvale, CA) flow cytometry system. At least 5×10^4 cells were analyzed for each measurement. Propidium iodide was excited with 250 mW of 488 nm light from an argon laser (Coherent Innova 90-5, Palo Alto, CA). Fluorescence was measured using a 580 LP filter. Percentages of cells in the $G_0 + G_1$ and S phases of the cell cycle were calculated using the fitting routines included in the program COTFIT provided by Becton-Dickinson on the Consort 40 data analysis system.

Results

Effect of L1210 Cell Growth Rate on the Anabolism of [^{3}H]ddCyd

At different time points during L1210 cell growth, [^{3}H]ddCyd (1 μM) was added for a 4.5-hr incubation period to evaluate the relationship between L1210 cell growth and the phosphorylation of ddCyd. As demonstrated in Table 1, the phosphorylation of ddCyd to its 5′-phosphate derivatives was most extensive during the initial exponential growth phase (15 hr after seeding the cells (first time point)). The longer the cells were incubated, the more their phosphorylating capacity declined. At 44 hr after seeding of the cells, the total amount of ddCyd 5′-phosphates was only 17% of that measured at the first time point. Irrespective of the cell growth rate, the 5′-triphosphate metabolite of ddCyd was always more abundant than its 5′-diphosphate, whereas the 5′-monophosphate level was invariably the lowest. It should be stressed that the first two time points (15 and 25 hr) reflect the exponential growth phase of the cells, the third time point (34 hr) the end of the exponential growth phase, whereas at the fourth time point, cell growth rate had entered the stationary phase.

TABLE 1

Effect of the state of growth of L1210 cells on the metabolism of [^{3}H]ddCyd[a]

Time after seeding	Cell density ($\times 10^{-6}$/ml)	[*methyl*-^{3}H]dThd-incorporated	Intracellular concentration of [^{3}H]ddCyd metabolites		
			ddCMP	ddCDP	ddCTP
		pmole/10^6 cells	*nM*	*nM*	*nM*
15 hr	4.9	2.19	200	210	310
25 hr	8.2	3.34	100	160	260
34 hr	11.8	1.74	21	86	160
44 hr	12.4	1.72	14	47	53

[a] At the time points indicated, cell suspensions were divided. One half of the cells were incubated with 1 μM [^{3}H]ddCyd for 4.5 hr before the determination of ddCyd anabolites. The other half were incubated with 1 μM [*methyl*-^{3}H]dThd for 2 hr before the determination of TCA-insoluble radiolabel. The data represent the mean values for two experiments; the range of individual values was within 10% of the indicated values.

Effect of Natural Pyrimidines and Pyrimidine Nucleosides on the Metabolism of [^{3}H]ddCyd in L1210 Cells

In all experiments described, nucleosides were added simultaneously with [^{3}H]ddCyd to the cells ~12–15 hr after seeding.

Of all pyrimidines (i.e., Ura and Cyt) and pyrimidine nucleosides (i.e., dThd, dUrd, dCyd, Urd, Cyd) evaluated, only dCyd and Cyd significantly inhibited intracellular phosphorylation of ddCyd, dCyd being most effective (Table 2); at equimolar concentrations (1 μM), dCyd inhibited ddCyd phosphorylation by 60% and at a 10-fold higher concentration (10 μM), dCyd decreased ddCyd anabolism at least 85–91%. Neither Urd, dUrd, uracil, nor cytosine exerted any inhibitory effect on ddCyd metabolism.

In contrast, 100 μM dThd stimulated the formation of ddCDP and ddCTP from 1 μM ddCyd ~3-fold (Table 2).

Cytostatic Activity of ddCyd against Several Murine and Human Tumor Cell Lines

The cytostatic effects of ddCyd against several representative murine and human cell lines were determined together with the potential ability of natural nucleosides to reverse the inhibitory effects of ddCyd on cell proliferation (Tables 3 and 4).

ddCyd showed little inhibitor effect against murine lymphoblast leukemia (L1210), lymphoblast/macrophage (P388), and mammary carcinoma (FM3A) cell proliferation, the IC_{50} being >100 μM. However, sensitivity of human lymphoid cells (both B-cells (Raji) and T-cells (Molt/4F and ATH8)) to the cytostatic effects of ddCyd was much higher than that of the murine cells and the human nonlymphoid cells (IC_{50}: 12–35 μM).

It is worthwhile noting that ddCyd proved ineffective against a dCK-deficient L1210 cell line (IC_{50} > 2370 μM), whereas its inhibitory effect against a thymidine kinase-deficient L1210 cell line was comparable to that against the parental cell line

TABLE 2

Effect of pyrimidines and pyrimidine nucleosides on the phosphorylation of [^{3}H]ddCyd by L1210 cells[a]

Compound	Intracellular concentration (nM) of	
	ddCDP	ddCTP
None (control)	40	100
Thymidine 100 μM	110	270
2′-Deoxyuridine 100 μM	36	110
dCyd 10 μM	7	3
dCyd 1 μM	15	41
Urd 100 μM	54	120
Cyd 100 μM	5	10
Uracil 50 μM	46	130
Cyt 100 μM	36	90

[a] Test compounds were added to the cells simultaneously with 1 μM [^{3}H]ddCyd, and incubations were continued for 4.5 hr before extraction of ddCyd metabolites (see Materials and Methods). The data represent the mean values for two separate experiments; the range of individual values was within 25% of the indicated values. Levels of ddCMP were not determined.

TABLE 3

Cytostatic effect of ddCyd against murine and human cell lines[a]

Cell line	50%-inhibitory concentration for ddCyd (μM)
Murine lymphoblast/macrophage P388	>100
Murine lymphoblast/leukemia L1210	320
Murine lymphoblast/leukemia L1210/BdUrd (thymidine kinase-deficient)	298
Murine lymphoblast/leukemia L1210/araC (dCK-deficient)	>2370
Murine mammary carcinoma FM3A	>2370
Human B-lymphoblast Raji	12
Human T-lymphoblast Molt/4F	17
Human T-lymphocyte ATH8	35
Human adenosquamous carcinoma NCI-H125	>100
Human bronchioloalveolar carcinoma NCI-H322	>100
Human large cell carcinoma NCI-H460	>100

[a] The origin and cultivation of these cell lines and the methodology for the determination of the cytostatic effects of drugs against these lines has been described previously (8, 10, 15). The data represent the mean values from two to four separate experiments; the range of individual values was within 30% of the indicated values.

TABLE 4

Effect of natural pyrimidine nucleosides on the cytostatic activity of ddCyd against the human Molt/4F cell line[a]

Nucleoside	50%-inhibitory concentration for ddCyd (μM)
None	17
Thymidine (20 μM)	17
2′-Deoxyuridine (550 μM)	30
dCyd (2200 μM)	>2370
Urd (510 μM)	13
Cyd (2060 μM)	620

[a] Nucleosides were added at subtoxic concentrations. The data represent the mean values from two to four separate experiments; the range of individual values was within 30% of the indicated values.

(~300 μM). Neither dThd nor Urd was able to reverse the cytostatic effect of ddCyd against Molt/4F cells. However, cytidine decreased the inhibitory activity of ddCyd against Molt/4F cell proliferation at least 70-fold, whereas addition of dCyd totally abolished any inhibitory effect of ddCyd (IC_{50} > 2370 μM) (Table 4).

Effect of Preincubation and Initial Extracellular Concentration of dThd on the Formation of ddCTP from ddCyd and on the dCTP Levels in L1210 Cells

The fact that dThd was able to stimulate the formation of phosphorylated metabolites from ddCyd when added together with [^{3}H]ddCyd to the cells (Table 2) led us to investigate this effect in greater detail.

Several concentrations of dThd (20 μM, 100 μM, 500 μM) and preincubation times (12 hr, 5 hr, 2 hr) before addition of [^{3}H]ddCyd were investigated. As shown in Fig. 1, at every dThd concentration tested, longer preincubation times with dThd (up to 12 hr) were invariably more effective in stimulating ddCyd anabolism. The levels of ddCTP were at least 2.5 to 3-fold higher after 12 hr preincubation than after 2 hr. This stimulation is also much more intensive at the highest dThd concentration (500 μM) than at the lowest tested (20 μM) (about 3-fold after 12 hr preincubation).

In contrast to the stimulation of ddCyd metabolism upon preincubation of the cells with dThd, dCTP levels dropped considerably (Fig. 1). Intracellular dCTP levels of untreated L1210 cells were about 14 μM. Addition of 20 μM dThd, 2, 5, or 12 hr before [^{3}H]ddCyd treatment decreased dCTP levels 2- to 4-fold. Pretreatment with 100 μM and 500 μM dThd further decreased intracellular dCTP levels at least 20-fold. In the case of 5 hr and 12 hr preincubation, 500 μM dThd did not further enhance the drop in dCTP levels compared with 100 μM dThd, although ddCTP levels were further increased.

Stimulatory Effect of dThd on the Intracellular Phosphorylation of Increasing Concentrations of Initial Extracellular [^{3}H]ddCyd

We evaluated the effect of dThd on the intracellular phosphorylation of ddCyd as the initial concentrations of the latter compound were increased. Cells were preincubated with 100 μM dThd before [^{3}H]ddCyd was added at concentrations ranging from 1 μM to 10 mM. In these experiments, formation of intracellular ddCTP levels was stimulated 5-fold when 1 or 10 μM ddCyd was given to the cells in combination with dThd (Table 5). However, at increasing initial ddCyd concentrations, stimulation decreased proportionally, until, at the highest ddCyd concentration, levels of ddCTP attained in dThd-treated cells were only 2-fold those in untreated cells. The rate of ddCTP synthesis was linear up to 100 μM initial ddCyd concentration. Almost identical observations were made in the case of the formation of ddCDP: optimal stimulation of phosphorylation in the presence of dThd occurred up to 10 μM initial ddCyd concentration. Linearity of ddCDP synthesis occurred up to 1 mM (instead of 100 μM for ddCTP). Intracellular ddCDP and ddCTP levels were present at almost equimolar concentrations at an initial ddCyd concentration of 2.5 mM, whereas the ddCDP levels were even higher than those for ddCTP at 10 mM initial ddCyd.

Effect of dThd on Cell Cycle Distribution of L1210 Cells

The effect of increasing concentrations of dThd (i.e., 20, 100, and 500 μM) on the cell cycle distribution of L1210 cells was determined (Table 6, Fig. 2). At dThd concentrations up to 20 μM, no change in cell cycle distribution was observed. At 500 μM dThd, i.e., at a concentration that severely inhibited L1210 cell proliferation, L1210 cells accumulated in the $G_0 + G_1$ phase.

Evaluation of Several Inhibitors of Pyrimidine Nucleotide Synthesis for their Ability to Stimulate ddCyd Phosphorylation

Several drugs that are known to selectively inhibit different steps in the *de novo* synthesis of pyrimidine nucleotides (Fig. 3) have been investigated for their ability to stimulate ddCyd phosphorylation. All compounds were added to the cells at their 50% inhibitory dose for cell proliferation and at a 10-fold higher dose for 12 hr, after which preincubation period, 1 μM [^{3}H]ddCyd was added for an additional 4.5 hr. Under these experimental conditions, 3-deazauridine and hydroxyurea stimulated ddCyd phosphorylation with an efficiency comparable to dThd (Table 7). Pyrazofurin had a moderate stimulatory effect, and PALA barely enhanced the levels of ddCyd 5′-phosphate metabolites. 6-Azauridine showed only marginal, if any, stimulatory capacity. Irrespective of the compound tested, final ddCTP levels were always higher than ddCDP levels (Table 7).

Enhancement of the Anti-HIV effect of ddCyd by Preincubation of ATH8 Cells with dThd

The ability of dThd to increase the protective effect of ddCyd against HIV-infected ATH8 cells has been studied. At 0.5 μM,

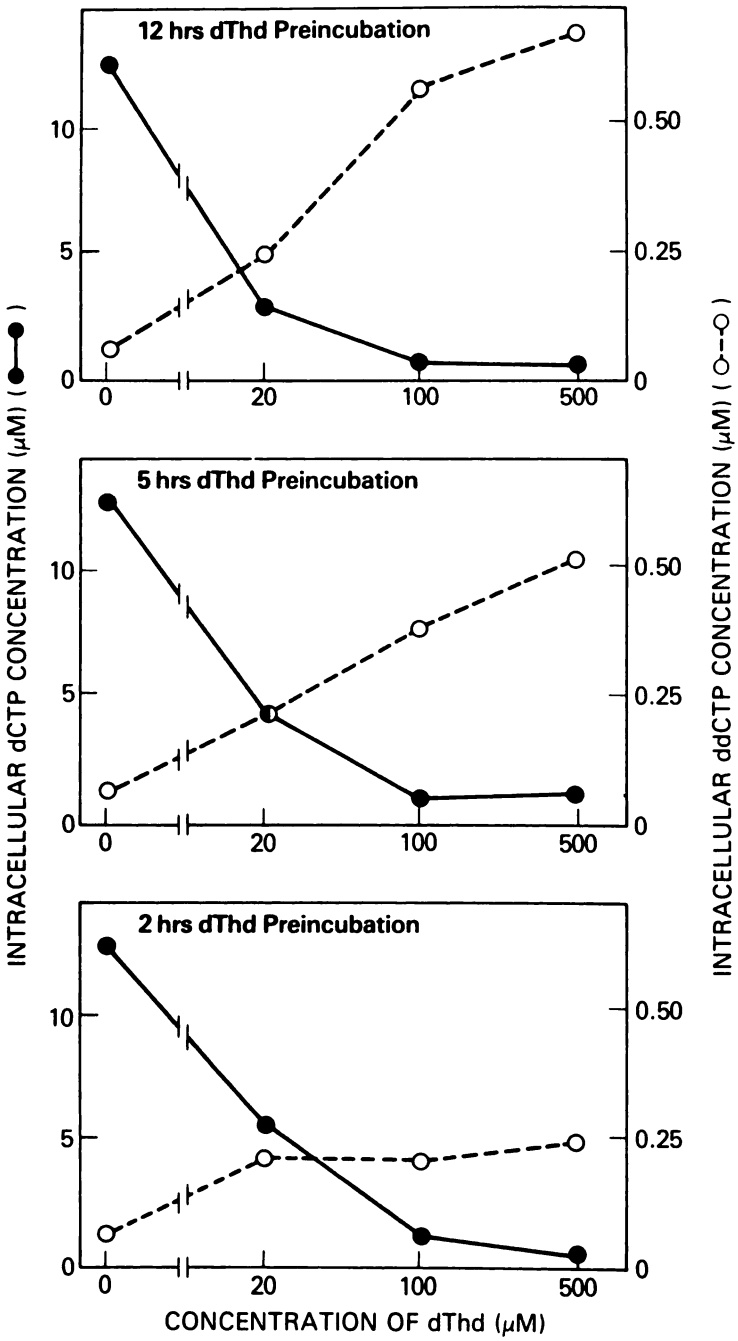

Fig. 1. Effect of dThd on intracellular dCTP and ddCTP levels in L1210 cells. L1210 cells were seeded at 4×10^5 cells/ml and incubated for 2, 5, or 12 hr with 0,20, 100, or 500 μM dThd before the addition of 1 μM [^{3}H]ddCyd. After an additional incubation for 4.5 hr, cells were centrifuged, washed, and further processed for the determination of the intracellular pools of dCTP (●) and ddCTP (○) as described in Materials and Methods. Data shown are the means of a duplicate analysis. The range of the individual values was within 20% of the indicated values.

ddCyd exerts a complete inhibitory effect against the cytopathogenicity induced by HIV upon infection of ATH8 cells. At lower concentrations, the protective effect was only partial, becoming marginal or insignificant at 0.05 μM (Fig. 4A). However, when the cells were pretreated with 50 μM dThd for 24 hr before infection with HIV, a concentration of 0.3 μM ddCyd was totally protective and 0.05 μM ddCyd still exerted 20% protection to the cells compared with ~5% in untreated cells (Fig 4B). The potentiation of the antiviral effect of ddCyd by dThd was still more pronounced when cells were pretreated with 100 μM dThd: total protection of viability of the cells was achieved at 0.10 μM ddCyd, whereas at 0.05 μM, about 30% of the ATH8 cells were still viable in virus-infected cell cultures (Fig. 4C).

Combination experiments of dThd (50 μM and 100 μM) with high ddCyd concentrations (up to 50 μM) were carried out to check if toxicity of ddCyd against ATH8 cells was also increased (Table 8). We found an enhanced cytostatic effect for the combination of dThd (100 μM) with ddCyd, but only at the higher ddCyd concentration (50 μM), which was itself cytostatic for the cells. Inhibition of cell proliferation increased from 35% (at a single dose of 50 μM ddCyd) to 60% (at 50 μM ddCyd plus 100 μM dThd). Combination of 20 μM ddCyd with dThd did not increase the cytostatic effects against ATH8 cells. It should be noted that the slightly increased toxicity achieved with ddCyd, in combination with dThd, occurred at ddCyd concentrations that were at least 100-fold higher than those that were able to protect the cells from virus-induced cytopathogenicity.

Discussion

Metabolism of ddCyd is clearly dependent on the cell proliferation rate. The most intensive conversion of ddCyd to its

TABLE 5

Effect of thymidine on the intracellular anabolism of graduated concentrations of tritiated ddCyd

Initial exogenous ddCyd concentration (μM)	Intracellular levels (μM) of ddCDP			ddCTP		
	no dThd (−)	with dThd (+)	ratio +/−*	no dThd (−)	with dThd (+)	ratio +/−*
1	0.06	0.19	3.2	0.11	0.52	4.7
10	0.36	1.8	5.0	0.96	5.1	5.3
100	4.5	16.9	3.8	9.5	29.1	2.4
1,000	55	121	2.2	68	129	1.8
2,500	115	224	2.0	120	225	1.8
10,000	357	630	1.8	297	567	1.9

* Ratio of phosphorylated ddCyd metabolites in the presence of 100 μM dThd (+) to phosphorylated ddCyd metabolites in the absence of dThd (−). Levels of ddCMP were not determined.

TABLE 6

Effects of dThd on the cell cycle distribution of L1210 cells*

dThd concentration	Cell number per ml after dThd incubation	% of cells in	
		$G_0 + G_1$ phase	S phase
0	830,000	28.6	71.4
20	732,000	25.8	74.2
100	516,000	28.0	72.0
500	436,000	53.9	46.1

* Initial cell number was 350,000 cells/ml. Cells were incubated with the indicated concentrations of dThd for 15 hr. Cell cycle analysis was carried out as described in Materials and Methods. Data represent a single experiment. In a second experiment, similar results were obtained within a 10% range.

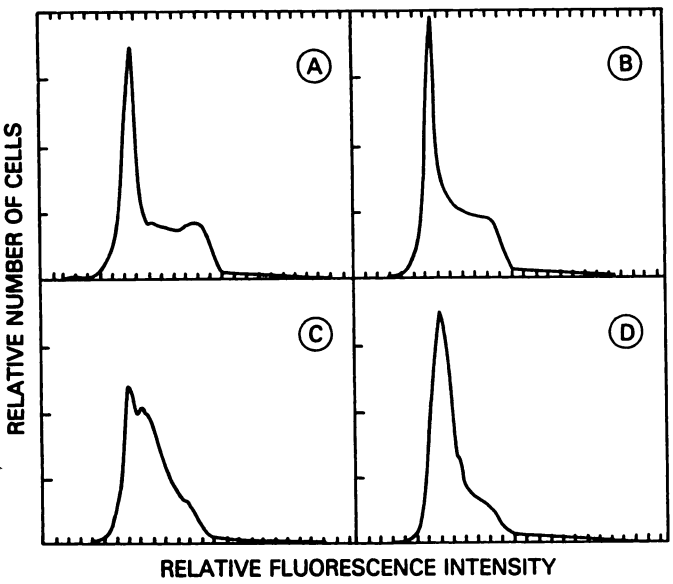

Fig. 2. Cell cycle analysis of L1210 cells preincubated with dThd. Cell cycle analysis of L1210 cells pretreated with dThd was carried out using a FACS 440 flow cytometry system. At least 5×10^4 cells were analyzed for each sample. Percentage of cells in $G_0 + G_1$ and S-phase were calculated and shown in Table 6. Cells were incubated with 0 μM dThd (control) (*Panel A*), 20 μM dThd (*Panel B*), 100 μM dThd (*Panel C*), and 500 μM dThd (*Panel D*) before analysis. Data represent a single experiment. In a second experiment, similar results were obtained within a 10% range.

corresponding 5′-mono-, di- and triphosphate derivatives occurs in the early exponential growth phase and proved to be 5- to 15-fold higher than at the beginning of the stationary growth phase (Table 1). Because dCK activity, measured in dialyzed L1210 cell-free extracts, does not vary significantly during cell

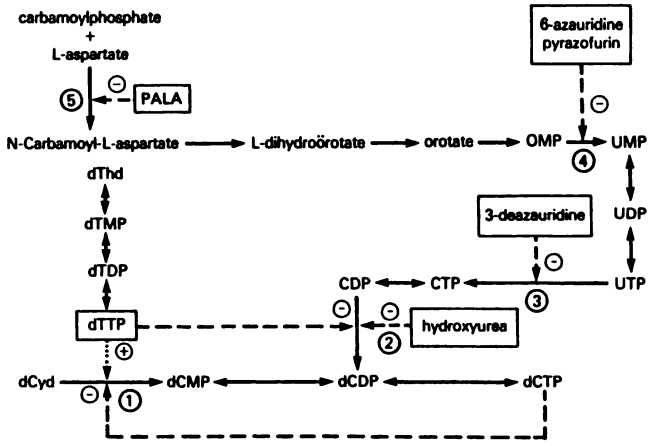

Fig. 3. Sites of action of inhibitors of pyrimidine nucleotide biosynthesis. Key enzymes: (*1*) deoxycytidine kinase; (*2*) ribonucleotide reductase; (*3*) CTP synthetase; (*4*) OMP decarboxylase; (*5*) aspartate transcarbamoylase.

TABLE 7

Effect of several inhibitors of pyrimidine nucleotide metabolism on the anabolism of [^{3}H]ddCyd in L1210 cells*

Inhibitor	Initial concentration	Intracellular levels of ddCDP	ddCTP	dCTP
	μg/ml	nM		% of control
None (control)		100	150	100
PALA	3.00	170	240	72
	0.30	150	230	
6-Azauridine	2.60	70	160	29
	0.26	110	200	
Pyrazofurin	0.280	150	290	3
	0.028	220	430	
3-Deazauridine	1.80	420	810	3
	0.18	580	880	
Hydroxyurea	54.0	430	820	12
	5.4	350	600	
dThd	130	540	730	6
	13	670	900	

* Cell cultures were preincubated with inhibitor for 14 hr before 1 μM [^{3}H]ddCyd was added. The assumed target enzymes for inhibition are L-aspartate transcarbamoylase (ATCase) (PALA) (16–18); OMP decarboxylase (6-azauridine (19, 20), and pyrazofurin (21, 22)); CTP synthetase (3-deazauridine) (23, 24); NDP reductase (hydroxyurea) (25, 26); dThd kinase, NDP reductase, and dCMP deaminase (dThd, active as its 5′triphosphate) (27, 29). The lowest initial concentration for all inhibitors corresponds to their IC_{50} for L1210 cell proliferation. The highest initial concentration corresponds to 10× this IC_{50} value. ddCMP levels were not determined. dCTP levels were determined in triplicate.

growth progression (30), it appears unlikely that the decline in ddCyd phosphorylating capacity of the cells at longer incubation times can be explained by decreased amounts of intracellular dCK. However, because dCK is subject to a complex interaction with a number of nucleosides and nucleotides, dCTP, CDP, uridine-5′-disphosphate, dThd-5′-diphosphate, dCMP, and dCDP being inhibitors, and dThd-5′-triphosphate (dTTP), uridine-5′-triphosphate, and dUrd-5′-triphosphate being activators of the enzyme (31, 32), it is possible that dCK becomes more inhibited during progression of the growth state of the cells by changing concentrations of these latter compounds: this in turn may explain the decreased levels of phosphorylated metabolites of ddCyd in late exponential phase. A similar phenomenon has been observed by Durham and Ives for araC phosphorylation (30). These investigators found that

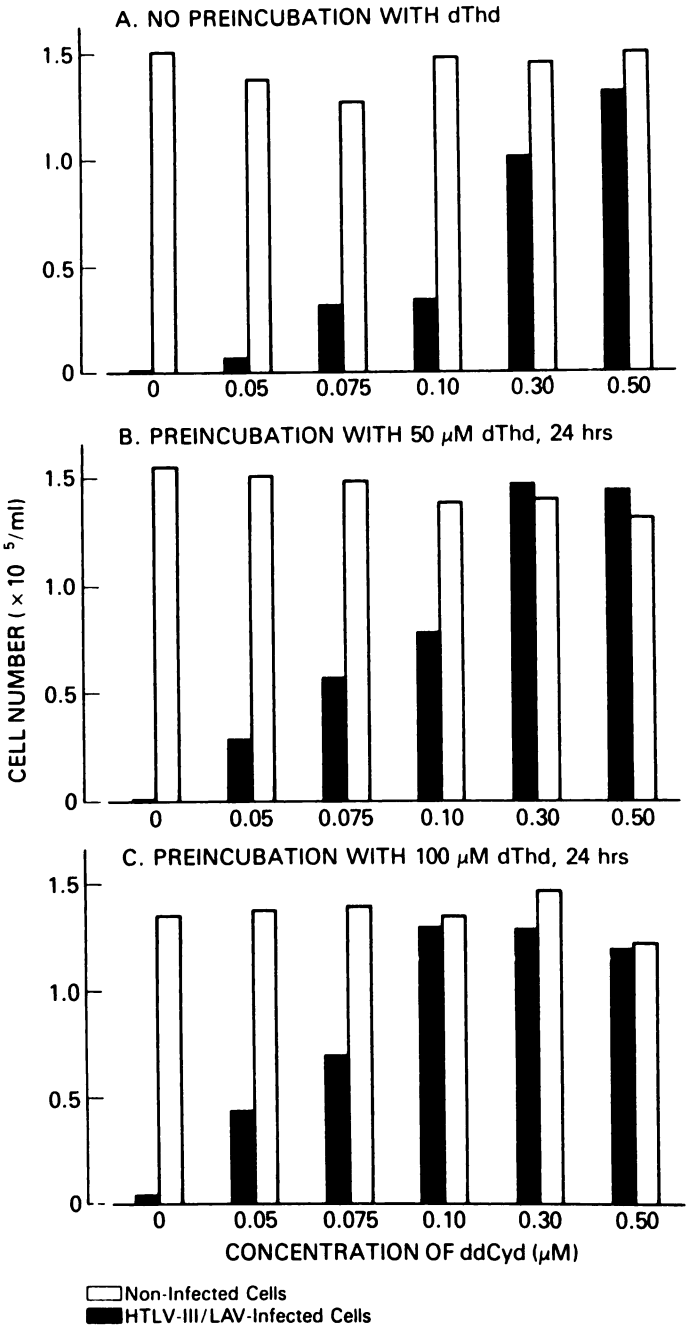

Fig. 4. Effect of dThd on the antiviral effect of ddCyd in HTLV-III/HIV-infected ATH8 cells. ATH8 cells (10^5/ml) were preincubated with 0, 50, or 100 μM dThd for 24 hr and subsequently infected with HTLV-III_B (3000 virus particles per cell). Varying concentrations of ddCyd were then added, and the cell cultures were further incubated at 37°. Mock-infected cell cultures containing similar concentrations of ddCyd were run in parallel. At day 7 postinfection, cell numbers of virus-infected cultures or mock-infected cultures were determined. Data shown are mean values from three separate experiments: the range of individual values was within 30% of the indicated values.

the dCyd/araC phosphorylation ratio decreased considerably upon dialysis of the cell extracts, and that this decrease was probably caused by the removal of nucleotides in the crude cell extracts that preferentially inhibited the phosphorylation of araC.

The formation of phosphorylated metabolites from ddCyd in murine leukemia L1210 cells is partially or nearly completely

TABLE 8

Effect of the combination of dThd with ddCyd on ATH8 cell growth[a]

Initial concentration of dThd during preincubation	Number of ATH8 cells ($\times 10^{-5}$/ml) in the presence of varying concentrations of ddCyd				
	0 μM	2 μM	5 μM	20 μM	50 μM
0 μM	1.77	1.80	1.91	1.62	1.19
50 μM	1.82	1.80	1.78	1.44	1.03
100 μM	1.80	1.75	1.68	1.68	0.73

[a] ATH8 cell numbers were determined as described in Materials and Methods. The data represent mean values from three separate experiments; the range of individual values was within 25% of the indicated values.

inhibited by dCyd and Cyd (Table 2). These results are compatible with the observations that (i) no phosphorylated metabolites of ddCyd could be detected in dCK-deficient P388/araAC and L1210/araC cells (6), and no cytostatic effects against L1210/araC cells by ddCyd were observed in contrast to the parental L1210 cells (Table 3); (ii) the cytostatic effects of ddCyd against human Molt/4F cells are dramatically reversed by Cyd and dCyd (Table 4); and (iii) the antiviral effects of ddCyd in ATH8 cells are partially reversed by the addition of dCyd and Cyd, dCyd being more effective (Ref. 2, and Balzarini and Broder, unpublished observations). One contributing factor to the decreased formation of phosphorylated ddCyd derivatives in the presence of dCyd may be the competition of dCyd with ddCyd for the catalytic site of deoxycytidine kinase, producing a decrease in the rate of phosphorylation of ddCyd (4, 7). Additional inhibition may occur after the conversion of dCyd to dCTP, because dCTP is a potent feedback inhibitor of dCK (31, 32). These two mechanisms, acting in concert, appear sufficient to explain the reversal of the cytostatic effect of ddCyd by dCyd in L1210 and human Molt/4F cells. A third mechanism that probably contributes to the reversal of the *antiviral* effect of ddCyd by dCyd in HIV-infected ATH8 cells is the competition of the natural substrate dCTP with ddCTP for the viral reverse transcriptase (3), thus inhibiting the incorporation of the latter compound as a viral DNA chain terminator (5).

The fact that in the L1210/araC and P388/araAC cell lines that are deficient in dCK but contain normal levels of Cyd-Urd kinase, none of the phosphorylated metabolites of ddCyd could be detected, makes it unlikely that Cyd-Urd kinase can accept ddCyd as a substrate. Thus, the reversal of the cytostatic effects of ddCyd against Molt/4F cells by Cyd, and the decrease of phosphorylated ddCyd metabolites in the presence of Cyd cannot be explained as a competition phenomenon of ddCyd with Cyd at the level of Cyd-Urd kinase. However, because CMP-dCMP kinase and the nucleoside 5′-diphosphate kinase accept CMP and dCMP, and CDP and dCDP, respectively, as substrates, competition at these levels of phosphorylation with ddCMP and ddCDP may contribute to the reversal produced by Cyd. The formation of ddCTP was clearly stimulated by dThd (Table 2, Fig. 1). Because intracellular dCTP is a known feedback inhibitor of dCK (31, 33), the initial reduction of the dCTP pools caused by direct inhibition of CDP reductase by dTTP may be primarily responsible for the increased ddCyd phosphorylation at the lower dThd concentrations (20 μM and 100 μM). However, after exposure of the cells to increasing concentrations of dThd (i.e., 500 μM), dCTP pools seem to remain constant. If dCTP levels are the sole parameter in determining ddCyd anabolism, ddCyd phosphorylation might

also be expected to be constant. However, the greatest enhancement of ddCTP formation is observed at 500 μM, being significantly higher than that observed at 100 μM dThd (Fig. 1). Therefore, the increased ddCyd phosphorylation in the presence of almost constant dCTP levels but with increased dTTP pools may have other explanations. First, high dTTP concentrations may have a direct stimulatory effect on dCK and thus on ddCyd phosphorylation (32). Second, the increased levels of ddCTP at the higher dThd concentration might also be explained by cell synchronization. In attempts to detect the latter phenomenon, we were able to demonstrate significant synchronization of L1210 cells only at the highest dThd concentration tested (500 μM), whereas at the lowest dThd concentration (20 μM), no different distribution of the cells between $G_0 + G_1$ and S phase was obtained compared with control (Table 6, Fig. 2). The dThd concentration at which synchronization was detected is close to that reported by Xeros (33), who used up to 2 mM dThd for 24 hr to synchronize genetically heterogeneous Chang-appendix cells. Our data confirm our assumption that the increased phosphorylation of ddCyd to ddCTP in the presence of the lower dThd concentrations (20 and 100 μM) is mainly caused by decreased intracellular dCTP pools, whereas the additional phosphorylation observed in the presence of the highest dThd concentration (500 μM) may be explainable by a progressive synchronization of the cells in the early S phase with concomitant higher dCK activity.

The stimulatory capacity of dThd on the formation of phosphorylated ddCyd metabolites is optimal at an initial concentration of 1–10 μM ddCyd and shows a relative decrease at higher ddCyd concentrations; nevertheless, at a concentration as high as 10 mM ddCyd, dThd was still capable of increasing ddCDP and ddCTP levels 2-fold (Table 5). This smaller fractional enhancement of ddCyd phosphorylation in dThd treated cells over untreated cells at higher ddCyd concentrations is in accordance with the observations reported by Grant *et al.* (34) and Kinahan *et al.* (35), who found that dThd in combination with 5 μM araC resulted in a significantly decreased fractional enhancement of araC incorporation into DNA when compared with a dThd combination with 0.04 μM araC. Our data may reflect saturation by substrate of the phosphorylating enzymes and/or some feedback inhibitory mechanisms, presumably by ddCTP against dCK.

In this study, we have also investigated the potential of various compounds that inhibit different steps in the *de novo* synthesis of dCTP to increase ddCyd phosphorylation (Table 7, Fig. 3). It is most likely that the stimulatory effects of 3-deazauridine, pyrazofurin, and hydroxyurea on ddCyd anabolism are related to a decrease in dCTP production resulting from an inhibition of the CTP synthesis, *de novo* pyrimidine nucleotide synthesis, and ribonucleotide reductase, respectively. A search for the additional factor(s) that influence stimulation of phosphorylation of ddCyd is now in progress.

Finally, we demonstrated that preincubation of ATH8 cells with dThd increased the antiviral effect of ddCyd against HIV-infected ATH8 cells (Fig. 4). These observations have been confirmed for HIV-infected MT-4 cells (M. Baba, J. Balzarini, and E. DeClercq, unpublished observations). Thymidine added 1 day after viral infection no longer had any effect on the antiviral activity of ddCyd. These data may indicate that one has to deplete the intracellular dCTP pools before ddCyd administration to obtain the most effective potentiation of the antiviral effect of ddCyd by dThd. We pointed out that a 24-hr preincubation period of L1210 cells with 100 μM dThd before administration of 1 μM ddCyd resulted in a 3-fold increase of ddCDP and ddCTP levels. Because ddCTP competes with dCTP for DNA polymerase (either cellular (4) or viral (3)), reduction of the dCTP pools would be expected to favor increased competition of ddCTP at the level of DNA polymerase.

Under our experimental conditions, the dThd-induced increase in the concentration of ddCDP and ddCTP seems not to parallel the increase in apparent toxicity when L1210 or ATH8 cells are treated with the combination of ddCyd and dThd. This selective enhancement of antiviral over cytotoxic effect of ddCyd by dThd, which presumably reflects more efficient utilization of ddCTP by retroviral reverse transcriptase than by endogenous cellular DNA polymerases, may have considerable importance from a practical chemotherapeutic viewpoint.

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