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Lack of Enantiospecificity of Human 2′-Deoxycytidine Kinase: Relevance for the Activation of β-L-Deoxycytidine Analogs as Antineoplastic and Antiviral Agents

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SUMMARY

We demonstrate that human 2′-deoxycytidine kinase (dCK) is a nonenantioselective enzyme because it phosphorylates β-D-2′-deoxycytidine (D-dCyd), the natural substrate, and β-L-2′-deoxycytidine (L-dCyd), its enantiomer, with the same efficiency. Kinetic studies showed that L-dCyd is a competitive inhibitor of the phosphorylation of D-dCyd with a K_i value of 0.12 μM, which is lower than the K_m value for D-dCyd (1.2 μM). Chemical modifications of either the base or the pentose ring strongly decrease the inhibitory potency of L-dCyd. L-dCyd is resistant to cytidine deaminase and competes in cell cultures with the natural D-dCyd as substrate for dCK, thus reducing the incorporation of exogenous [^{3}H]dCyd into DNA. L-dCyd had no effect on the pool of dTTP deriving from the salvage or from the *de novo* synthesis, does not inhibit short term RNA and protein syntheses, and shows little or no cytotoxicity. Our results indicate a catalytic similarity between human dCK and herpetic thymidine kinases, enzymes that also lack stereospecificity. This functional analogy underlines the potential role of dCK as activator of L-deoxycytidine analogs as antiviral and antineoplastic agents and lends support to the hypothesis that herpesvirus thymidine kinase might have evolved from a captured cellular dCK gene, developing the ability to phosphorylate thymidine and retaining that to phosphorylate deoxycytidine.

Direct conversions of a base into a ribonucleoside monophosphate by a phosphoribosyltransferase and of a nucleoside (ribonucleosides or deoxyribonucleosides) into a nucleoside monophosphate by a nucleoside kinase are the two principal salvage reactions of nucleotide synthesis. In particular, nucleoside kinases participate in the balance between the efflux of nucleosides from the cell and their uptake from the extracellular medium, which is regulated by the need of dNTPs for DNA synthesis. The distribution of nucleoside kinases in cells and tissues is highly variable. Although *Escherichia coli* has only one kinase for thymidine, protozoan parasites, which are unable to synthesize purine and/or pyrimidine nucleotides *de novo*, may rely entirely on the salvage of ribonucleosides and deoxyribonucleosides from the environment (1–3). Mammalian cells possess kinases for all deoxynucleosides (3). Of particular interest among them is dCK, which catalyzes the phosphorylation of dCyd to dCMP in the presence of a nucleoside 5′-triphosphate as phosphate donor (4). This enzyme supplies dCTP not only for DNA synthesis, specially in cells (e.g., lymphocytes) or tissues (e.g., thymus and spleen) with more active nucleotide salvage pathways but also for the biosynthesis of membrane lipids (5).

dCK has been isolated and purified from many sources and is characterized by a considerably low selectivity for both the substrate and the phosphate donor. It can phosphorylate not only dCyd but also dAdo, dGuo (6), and several pyrimidine and purine deoxyribonucleoside analogs modified in both the base and the sugar ring (e.g., ddCyd, 1-β-D-arabinofuranosylcytosine, 9-β-D-arabinofuranosyladenine, and 2-chloro-2′-de-

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ABBREVIATIONS: dCK, 2′-deoxycytidine kinase; dCyd, deoxycytidine; D-dCyd, β-D-2′-deoxycytidine; L-dCyd, β-L-2′-deoxycytidine; L-Cyd, β-L-cytidine; TK, thymidine kinase; CD, cytidine deaminase; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; ddCyd, 2′,3′-dideoxycytidine; α-L-dCyd, α-L-2′-deoxycytidine; α-D-dCyd, α-D-2′-deoxycytidine; L-F-ddCyd, 2′,3′-dideoxy-β-L-5-fluoro-cytidine; D-F-ddCyd, 2′,3′-dideoxy-β-D-5-fluoro-cytidine; IdU, 5-iodo-2′-deoxy-L-uridine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; dThy, deoxythymidine; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

oxyadenosine) (7), using various nucleoside triphosphates as phosphate donors. For these properties, dCK greatly differs from the other cellular deoxyribonucleoside kinases and resembles the herpesvirus TK, which efficiently catalyze the phosphorylation not only of thymidine but also of dCyd as well as of a number of pyrimidine and purine analogs that are poor substrates or are not substrates for the cytosolic TK (3).

Interestingly, the comparison of the amino acid sequence of 15 higher vertebrate herpesvirus TKs with the sequence of the human dCK has revealed that four or five of six conserved sites of the herpesvirus TKs are related to human dCK, suggesting that herpesvirus TKs evolved from a captured cellular dCK gene (8).

Recently, we demonstrated that contrary to human cytosolic TK, herpes simplex virus type 1 TK phosphorylates both β-D- and β-L-enantiomers of not only thymidine (9, 10) but also thymidine analogs such as IdU and (*E*)-5-(2-bromovinyl)-2′-deoxy-L-uridine (11), and that the lack of stereospecificity could be a common feature of herpes simplex virus TKs (12). Because of this finding and the possible evolution of herpesvirus TKs from cellular dCK, we investigated the degree of enantioselectivity of human dCK. Indeed, it has been reported that both enantiomers of BCH-189 (2′,3′-dideoxy-3′-thiacytidine) and of its derivative 2′,3′-dideoxy-5-fluoro-3′-thiacytidine are substrates for human dCK with a surprising preference for the L-enantiomers (13). However, kinetic parameters showed that both enantiomers of BCH-189 and 2′,3′-dideoxy-5-fluoro-3′-thiacytidine are phosphorylated with a catalytic efficiency 80–200-fold lower than that observed with D-dCyd, the natural substrate (13). Furthermore, the utilization of enantiomeric analogs does not prove the absolute lack of selectivity of an enzyme: in fact, the usually unattached enantiomer of the natural substrate, if modified, may be able to take up a position that is catalytically effective despite its unnatural configuration (14). Thus, we investigated the ability of human dCK to phosphorylate *in vitro* and in cell culture L-dCyd, the enantiomer of the natural substrate, as well as some other nucleoside analogs. Also, we studied the effects of L-dCyd on macromolecular syntheses and on cellular viability. Our results indicate that human dCK is a nonenantiospecific enzyme; for this reason, it can function as activator of L-dCyd analogs potentially efficient against neoplasias such as lymphocytic leukemia, low-grade lymphomas, and hairy cell leukemia (15), all of which have very active salvage nucleotide pathways, or against viruses such as human immunodeficiency virus and hepatitis B virus (16–22).

Materials and Methods

Chemicals. Commercial reagents and solvents of analytical grade were used unless otherwise stated. [^{3}H]dThy (25 Ci/mmol), [^{3}H]2′dCyd (18–30 Ci/mmol), [^{3}H]uridine (27 Ci/mmol), [^{3}H]leucine (59 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). [^{3}H]dAdo (7 Ci/mmol) and [^{3}H]dGuo (13 Ci/mmol) were from ICN Biomedicals (Zoetermeer, the Netherlands). Unlabeled nucleosides and nucleotides were from Boehringer-Mannheim Biochemica (Mannheim, Germany).

Evaluated compounds. L-dCyd (9, 23), 1(β-L-xylofuranosyl)-cytosine (24), α-L-dCyd (25), α-D-dCyd (26), L-ddCyd, and L-F-ddCyd (27) were synthesized as previously described. D-F-ddCyd was graciously supplied by Dr. Victor Marquez (National Institutes of Health, Bethesda, MD).

Purification of human dCK from HeLa cells. HeLa cells (20 g) resuspended in 100 ml of 10 mM bis-Tris, pH 6.5, 1 mM DTT, and 0.5 mM PMSF were allowed to swell on ice for 20 min and then homogenized with a potter for 10 periods of 30 sec in ice. The homogenate was centrifuged at 700 × *g* for 10 min to precipitate the nuclei, and the supernatant was collected. Nuclei were then washed in 100 ml of 50 mM bis-Tris, pH 6.5, 1 mM DTT, 0.5 mM PMSF, and 0.25 M sucrose; homogenized with a potter; and precipitated again by centrifugation at 700 × *g* for 10 min. The supernatant was combined with the previous supernatant and centrifuged at 8500 × *g* for 10 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 1 hr; filtered through an S-Sepharose (column volume, 20 ml) equilibrated with 30 mM bis-Tris, pH 6.5, 1 mM DTT, 0.5 mM PMSF, and 0.125 M sucrose; and then adsorbed on a DEAE-Sepharose (column volume, 20 ml) equilibrated with the same buffer. The column was eluted with a linear gradient of 0–1 M KCl in 30 mM bis-Tris, pH 6.5, 1 mM DTT, 0.5 mM PMSF, and 0.125 M sucrose at the flow rate of 5 ml/min. dCK activity eluted as a single sharp peak at 0.2 M KCl. Active fractions were pooled, and aliquots of the enzyme were maintained in liquid nitrogen until use. The final preparation had an enzymatic activity of 5000 units/mg.

dCK kinase assay. dCK was assayed with the use of a radiochemical method that measures the formation of dCMP from labeled dCyd. The enzyme was incubated at 37° in 25 μl of a mixture containing 30 mM HEPES-K^+, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM DTT, and 1 μM [^{3}H]dCyd (2200 cpm/pmol). The reaction was terminated by spotting 20 μl of the incubation mixture onto a 25-mm DEAE paper disk (DE-81 paper; Whatman). The disk was washed twice in an excess of 1 mM ammonium formate, pH 3.6, to remove unconverted nucleoside; once in distilled water; and finally in ethanol. Radioactive dCMP was estimated by scintillation counting in 1 ml of Betamax scintillating fluid (ICN Biomedicals). One unit is defined as the amount of enzyme catalyzing the formation of 1 pmol of dCMP in 1 hr at 37°.

When [^{3}H]dGuo or [^{3}H]dAdo was used as substrates in the dCK assay, the enzyme was incubated in the previous buffer containing 450 μM [^{3}H]dGuo (130 cpm/pmol) or 210 μM [^{3}H]dAdo (380 cpm/pmol).

When [γ-^{32}P]ATP was used as the phosphate donor, the enzyme was incubated in 30 mM HEPES-K^+, pH 7.5, 2 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (125 cpm/pmol), 0.5 mM DTT, and a 40 μM concentration of the compound to test. At the end of the incubation at 37°, each sample was added with 2 μl of a solution containing dCyd and dCMP (10 mM concentration each) as markers. Samples were then heated 5 min at 80° and centrifuged at 10,000 rpm for 10 min, and 20 μl of the supernatant was injected for HPLC.

HPLC separation of nucleosides and nucleotides. The ionic exchange chromatography method using the BioRad (Hercules, CA) 100 MAPS preparative system was used to separate nucleosides from nucleotides. A 4.6-mm × 25-cm TSKgel DEAE-2SW column (TosoHaas, Stuttgart, Germany) was used at room temperature under the following conditions: injection volume, 20 μl; detection, UV 260 nm; eluents, buffer A (CH_3CN/0.1 M potassium phosphate buffer, pH 3.0; 20:80), and buffer B (CH_3CN/0.6 M potassium phosphate buffer, pH 3.0; 20:80). Gradient conditions were 30-min linear gradient from buffer A to buffer B. The flow rate was 1 ml/min. Thirty fractions (fraction volume, 1 ml) were collected and counted in a β-counter.

CD assay. Human CD activity was determined with a direct spectrophotometric assay based on the decrease in absorbance at 282 nm when the dCyd is deaminated (28). The standard reaction mixture (1 ml) contained 100 mM Tris·HCl, pH 7.5, without or with a 0.13 mM concentration of the compound to test. The reaction was started by the addition of HeLa cell extract and incubated at 37°, and the absorbance was directly determined during the incubation in a Kontron spectrophotometer (Zürich, Switzerland).

The HeLa S3 extract was prepared as described for the purification of dCK up to the ultracentrifugation step.

Determination of the effect of L-dCyd on the phosphorylation of D-dCyd by human dCK and on its subsequent uptake into DNA in cell cultures. HeLa cells growing at 37° in 5% $C0_2$ in DMEM containing 10% FCS were trypsinized using a 0.1% trypsin-EDTA solution, seeded onto 24-well plates at a concentration of 32,000 cells/ml (500 μl/well) in DMEM with FCS, and incubated for 25 hr. Then, [^{3}H]dCyd (30 μCi/ml) and varying concentrations of L-dCyd were added. Incubation was continued for 6 hr. At the end of the incubation period, the medium was removed from each well, and the cells were washed with phosphate-buffered saline and trypsinized (150 μl of 0.1% trypsin EDTA/well). To each well, 150 μl of distilled water was added, and 150-μl samples were removed and spotted onto 25-mm GF/F (Whatman) filters. The filters were then washed three times in 5% (w/v) trichloroacetic acid for 10 min and twice in ethanol and dried. Acid-insoluble material was counted in 1 ml of Betamax (ICN Biomedicals) fluid in a Beckman β-counter (Beckman Instruments, Palo Alto, CA).

Determination of DNA, RNA and protein synthesis in cell cultures. HeLa cells growing in DMEM containing 10% FCS at 37° in suspension were centrifuged, resuspended in DMEM without calf serum at a density of 10^6 cells/ml, and further incubated for 30 min at 37° before the addition of [^{3}H]dThy or [^{3}H]uridine or [^{3}H]leucine to a concentration of 25, 36, and 36 μCi/ml, respectively. Incubation was then continued, and 0.08-ml samples were removed at 0, 15, 30, 45, and 60 min and spotted onto 25-mm GF/C (Whatman) filters. The trichloroacetic acid-insoluble material was then determined as described above.

Measurement of cell viability: dye exclusion assay. Molt-4 cells grown in RPMI-1640 medium containing 10% FCS were seeded onto 96-well microplates at a concentration of 5000 cells/ml (150 μl/well). After 15 hr at 37° in 5% CO_2, 50 μl/well of serial dilutions of the compound to test was added. The microplate, which was sealed with Saran Wrap to limit evaporation of the medium and diffusion of metabolic carbon dioxide, was then incubated for 7 days under the above conditions. Then, 50 μl of the cell suspension was removed from the well and mixed with 50 μl of tryptan blue solution (0.5% in phosphate-buffered saline). Both viable cells (not stained) and dead cells (blue stained) were then counted. Each point was done measured in triplicate, and the number of cells counted in each well is the average of four determinations.

Results

Some kinetic properties of purified human dCK. The purified dCK that we used did not catalyze the phosphorylation of cytidine, adenosine, guanosine, thymidine, or uridine, indicating the absence of ribonucleoside kinases and TK. Furthermore, no phosphatase activity was present as judged by the stability of dNTPs during standard assay conditions. When the kinetic behavior was studied in detail with regard to different phosphate acceptors and two phosphate donors (ATP and UTP), we found that in presence of ATP, the apparent K_m values were 1.2, 210, and 450 μM for dCyd, dAdo, and dGuo, respectively (Table 1). Also, the V_{max} values were much higher for the purine nucleosides compared with dCyd (Table 1), which is in agreement with the literature (6).

When UTP was the phosphate donor, the apparent K_m value for dCyd was 0.2 μM; however, the experiments were performed with ATP, which most likely serves as phosphate donor *in vivo*.

L-dCyd inhibits the phosphorylation of D-dCyd by human dCK. First, we studied the effect of L-dCyd on the phosphorylation of [^{3}H]dCyd by human dCK. Under our assay conditions, in which [^{3}H]dCyd is present at 1.2 μM, a concentration corresponding to the apparent K_m value when ATP is the phosphate donor, L-dCyd inhibits the dCK activity with a IC_{50} value of 0.24 μM. When the mechanism of inhibition of L-dCyd was studied, we obtained results (Fig. 1) that indicate a purely competitive inhibition with respect to D-dCyd, with a K_i value of 0.12 μM. A similar pattern of competitive inhibition by L-dCyd was observed when UTP was the phosphate donor, and the K_i value was 0.48 μM (data not shown).

L-dCyd also inhibited the phosphorylation of the other two natural substrates of dCK (D-dAdo and D-dGuo; Table 2), whereas other L-nucleosides, such as L-dAdo, L-dGuo, L-dThy, L-deoxyuridine, and its derivatives L-IDU and L-(*E*)-5-(2-bromovinyl)-2′-deoxy-L-uridine were not inhibitory up to the highest tested concentration, 500 μM (data not shown).

Effect of L-dCyd derivatives modified in the base or in the sugar ring on the phosphorylation of D-dCyd by human dCK. Several L-dCyd analogs, such as L-ddCyd, L-F-ddCyd, 1(β-L-xylofuranosyl)-cytosine, and α-L-dCyd, inhibited the phosphorylation of [^{3}H]dCyd by human dCK much less than L-dCyd (Table 3). Thus, modifications in either the base or the pentose ring decrease the effectiveness of inhibition of human dCK. It is interesting to observe that L-ddCyd, L-F-ddCyd, and α-L-dCyd are more inhibitory toward dCK activity than their D-enantiomer. This result is consistent with the reported observation that L-2′,3′-dideoxy-3′-thiacytidine and its derivative L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine are better substrates for dCK than their corresponding D-enantiomers (13).

Human dCK phosphorylates both enantiomers of dCyd to their corresponding monophosphates with the same efficiency. To understand whether L-dCyd is a competing substrate or simply a nonsubstrate inhibitor of the enzyme, we incubated dCK with either the L- or the D-enantiomer of dCyd in the presence of [γ-^{32}P]ATP, and the resolution of the reaction products was performed by HPLC (Fig. 2). The amounts of D- or L-dCMP produced in 1 hr were similar: dCK phosphorylated 60 and 55 pmol of D- and L-dCyd, respectively, showing that human dCK is a nonenantioselective enzyme. The lack of labeled L-dCyd did not allow determination of the phosphorylation kinetic parameters. However, the similar amount of the monophosphate products

TABLE 1
Kinetics parameters for the phosphorylation of D-nucleosides by human dCK

Substrate	Phosphate donor	K_m	V_{max}	V_{max}/K_m
		μM	nmol/hr/mg	
D-dCyd	ATP	1.2	5.7	4.80
D-dCyd	UTP	0.2	1.4	7.00
dGuo	ATP	450.0	58.0	0.13
dAdo	ATP	210.0	50.0	0.24

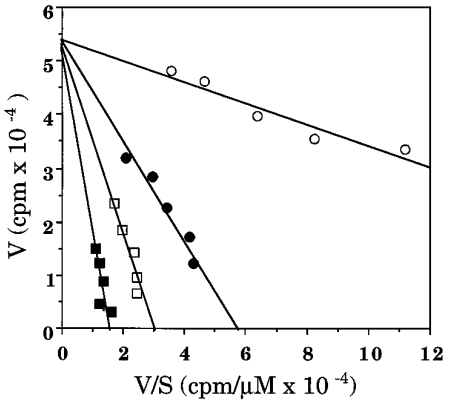

Fig. 1. Hofstee plot of the effect of L-dCyd on the activity of human dCK in the presence of increasing concentrations of the natural substrate [^{3}H]dCyd. The enzyme was assayed as described in Materials and Methods with variation of the concentration of L-dCyd: ○, 0 μM; ●, 1 μM; □, 2 μM, and ■, 4 μM. Each point was determined in triplicate.

TABLE 2

Inhibition of the catalytic activity of dCK by L-dCyd

Inhibitor	Substrate	IC_{50}	K_i
		μM	
L-dCyd	D-dCyd	0.24	0.12
L-dCyd	D-dAdo	0.18	N.D.
L-dCyd	D-dGuo	0.16	N.D.
D-dCyd	D-dAdo	0.60	N.D.
D-dCyd	D-dGuo	0.78	N.D.

N.D., not determined.

TABLE 3

Inhibitory effect of L-dCyd analogs on the phosphorylation of [^{3}H]-D-dCyd by purified human dCK

Compound	IC_{50}
	μM*
L-dCyd	0.24
L-ddCyd	154
L-F-ddCyd	156
L-xyloCyd	867
α-L-dCyd	98
D-ddCyd	810
α-D-DCyd	376
D-F-ddCyd	634

* Different concentrations of the compounds were tested in presence of 1 μM [^{3}H]-D-dCyd in the assay conditions described in Materials and Methods. IC_{50} was determined by plotting the reciprocal of enzymatic velocity (1/V) versus the compound concentrations (I).

observed with both enantiomers in the HPLC analysis suggests a comparable efficiency of phosphorylation.

Furthermore, we observed that the phosphorylation of [^{3}H]dGuo and [^{3}H]dAdo is inhibited by both D- and L-dCyd (Table 2). The clear-cut competition between the pyrimidine (D- and L-dCyd) and purine nucleosides indicates a common nucleoside acceptor binding domain for pyrimidines and purines on the human dCK.

L-dCyd is resistant to human CD. CD catalyzes the deamination of Cyd, dCyd, and several cytidine analogs used as antiviral or anticancer drugs and thus often reduces their pharmacological activity. *In vivo* studies have shown marked tissue and species differences in the extent of deamination of (d)Cyd and (d)Cyd analogs and higher levels of deaminase activity in human malignant tumors (28). *In vitro*, kinetic differences have been observed in CD purified from human or

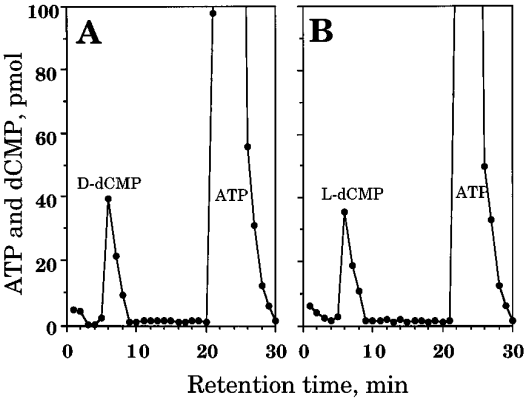

Fig. 2. HPLC elution profile of the products of reactions of human dCK incubated with (A) [γ-^{32}P]ATP and D-dCyd or (B) L-dCyd.

canine liver (29). After intraperitoneal injections of radioactive L-Cyd to mice, Jurovcik and Holy (30) isolated from liver homogenate the corresponding uracil derivative, suggesting that L-cytidine is partly deaminated *in vivo*. However, Chang *et al*. (18) found that L-2′,3′-dideoxy-3′-thiacytidine is resistant to CD. To examine the behavior of L-dCyd relative to D-dCyd toward human CD, we incubated either D- or L-dCyd and, for comparison, D- or L-Cyd with HeLa extract following in a spectrophotometer the decrease of absorbance at 282 nm of the deaminated substrate. Fig. 3 shows that unlike L-Cyd, L-dCyd is almost fully resistant to deamination.

L-dCyd competes with dCyd as substrate for dCK in cell culture experiments, thus reducing the incorporation of dCTP into DNA. To verify the ability of L-dCyd to compete with the natural enantiomer *in vivo,* we studied the kinetics of [^{3}H]dCyd incorporation into DNA of HeLa cells growing in presence of different concentrations of L-dCyd. Under these conditions, we observed that L-dCyd reduced the amount of dCMP derived from [^{3}H]dCyd that was incorporated into DNA, with an IC_{50} value of ~3 μM (Fig. 4). The incorporation of [^{3}H]dCyd into DNA was demonstrated by its sensitivity (>80%) to aphidicolin (3 μg/ml) during the labeling time (31). These experiments suggest that *in vivo*, L-dCyd also interacts with human dCK.

L-dCyd has no effect on the pool of dTTP derived from the salvage and from the *de novo* synthesis, and it does not inhibit short term RNA and protein syntheses. D-dCyd and dCMP can be deaminated to deoxyuridine

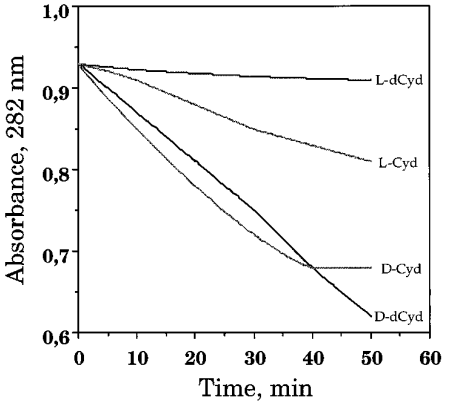

Fig. 3. Deamination of D-dCyd, L-dCyd, D-Cyd, and L-Cyd by HeLa S3 crude extract. The decrease of absorbance at 282 nm is a direct measurement of the enzymatic activity.

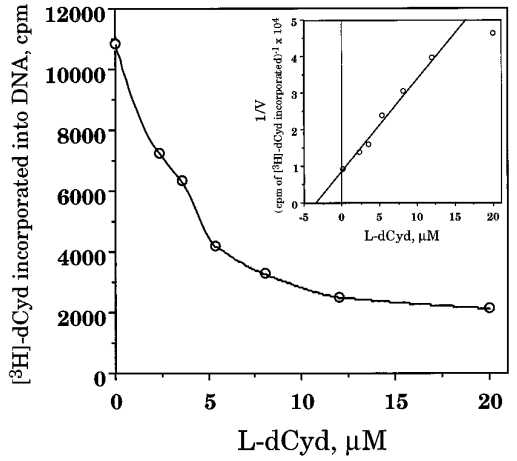

Fig. 4. Inhibition of acid-precipitable [^{3}H]dCyd by L-dCyd in HeLa cells. Cells were incubated for 6 hr in presence of both [^{3}H]dCyd and L-dCyd and then treated as described in Materials and Methods.

and dUMP, respectively, and further converted to dTMP, which dilutes the specific activity of [^{3}H]dThy when supplied as DNA precursor, thus affecting the labeling pattern of DNA. Under these conditions, in which the addition of D-dCyd affects, as expected, the labeling pattern of DNA, we observed that the L-enantiomer had no effect (Fig. 5), indicating that L-dCyd is unable to interfere with both the *de novo* and the salvage synthesis of dTTP. The lack of effect of L-dCyd on the salvage synthesis of dTTP was also confirmed when L-dCyd was added to cells growing in HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) where the *de novo* synthesis of dTMP is prevented (data not shown). Furthermore, up to 300 μM, the higher tested concentration, L-dCyd did not inhibit the incorporation of [^{3}H]uridine and [^{3}H]leucine (data not shown), indicating that under these conditions, L-dCyd had no effect on short term RNA and protein syntheses.

L-dCyd does not affect cellular growth and viability. To evaluate the long term effects of L-dCyd on cellular growth and viability, we exposed lymphoblastoid T cells (MOLT-4) to various concentrations of the compound. Cell viability was measured by the dye exclusion assay described in Materials and Methods.

For comparison, we also determined the effect of the natural substrate D-dCyd as well as that of D-ddCyd, L-ddCyd, and D-IdU, which is known to affect cell growth and viability. As shown in Fig. 6, L-dCyd had no or little effect on MOLT-4 growth except at a very high concentration (4 mM); its action was comparable to that of the natural substrate D-dCyd. On the other hand, we observed that D-ddCyd, L-ddCyd, and D-IdU inhibited cell growth with IC_{50} values of 3, 0.8, and 4 μM, respectively.

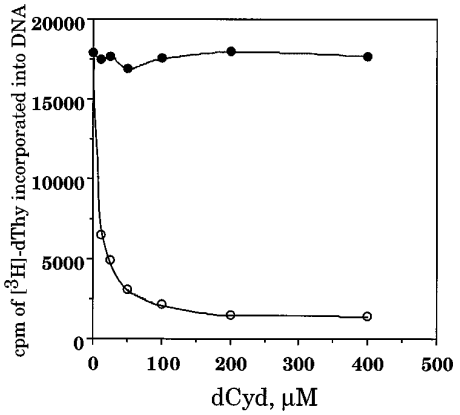

Fig. 5. Effect of (●) L-dCyd and (○) D-dCyd on dThy incorporation into DNA of HeLa cells.

Discussion

At any given time, the dNTPs in mammalian cells are far less than 1% of the amount needed for the synthesis of the entire genome. An inadequate supply of any of the dNTPs is lethal, whereas an oversupply is mutagenic. To provide the correct amount and balance of dNTPs, the cellular strategy is supported by an intricate regulation of the *de novo* and salvage pathways of nucleotide synthesis. The fine tuning of dNTP pool sizes needed for optimal and high-fidelity DNA replication also greatly relies on the balance between the efflux of precursor deoxyribonucleotides from the cell, mediated by deoxyribonucleotidases that convert them to deoxyribonucleosides, and their reabsorption and reconversion to deoxyribonucleotides through the phosphorylating action of deoxynucleoside kinases. Mammalian cells possess kinases for all deoxyribonucleosides, and their role is more relevant in cells (e.g., lymphocytes), tissues (e.g., thymus and spleen), or neoplasias (e.g., lymphocytic leukemia, low-grade lymphomas, hairy cell leukemia) with more active nucleotide salvage pathways. Among these kinases, of particular importance is dCK, which supplies dCTP for the biosynthesis of membrane lipids.

Furthermore, by discriminating between a correct and a

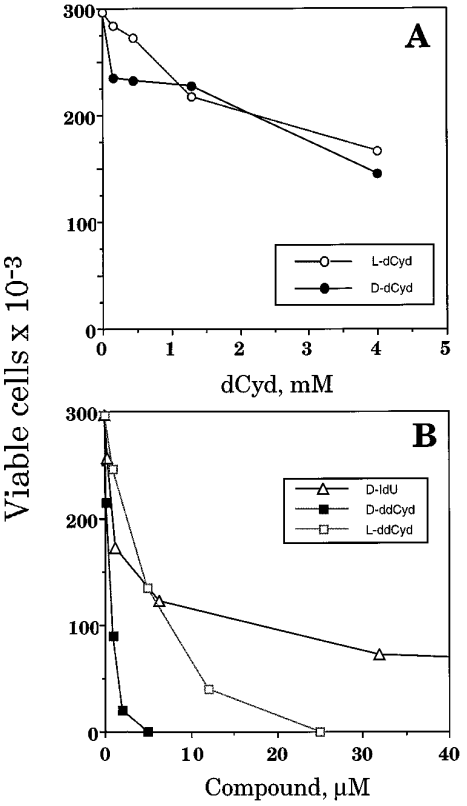

Fig. 6. Effect of different concentrations on MOLT-4 cell line growth after 7 days of exposure to the compounds.

modified nucleoside, kinases might be part of the several sequential metabolic processes required to reach a high fidelity of DNA synthesis not ensured by DNA polymerases alone, in particular, by those lacking the 3′-to-5′ exonuclease proofreading activity. Thus, cellular cytosolic TKs discriminates better against modified thymidine or deoxyuridine analogs than do cellular DNA polymerases against the corresponding modified deoxyribonucleoside triphosphates. In fact, cytosolic TK tolerates only 3′ modifications of the sugar ring and some nonbulky 5-substituents on the pyrimidine base, whereas DNA polymerases show a much higher tolerance versus deoxyribonucleoside triphosphates with bulky 5-substituents of the pyrimidine ring (3). On the contrary, a remarkable nonselective deoxynucleoside kinase is the TK encoded by herpesviruses, which efficiently catalyzes the phosphorylation not only of thymidine but also of dCyd as well as of a number of nucleoside analogs, most of which are poor substrates or are not substrates for cytosolic TK (3).

Recently, the comparison among 15 herpesvirus TK sequences revealed several highly conserved amino acid residues, which have been grouped into six sites. Interestingly, the analysis also revealed that although there is no apparent similarity between the sequences of herpesvirus TKs and cellular cytosolic TK, there is a clear analogy in four or five of the six conserved sites with a sequence of human 2′-dCyd kinase, which among cellular kinases, shows the least substrate selectivity, accepting several modifications of the sugar and the base.

This analysis suggested that the herpesvirus TKs might have evolved from a captured cellular dCK gene developing the ability to phosphorylate thymidine and retaining that to phosphorylate dCyd (8).

Recently, we showed that herpes simplex virus type 1 TK is a nonenantiospecific enzyme because it can efficiently phosphorylate the L-enantiomer of the natural substrate thymidine (9) and of thymidine analogs with the inverted configuration of the sugar ring (11). This finding and the abovementioned suggestion that herpesvirus TKs might have evolved from cellular dCK led us to address the issue of whether the sequence similarity between herpesvirus TKs and human dCK would lead to a functional analogy, in particular, in the ability to recognize and process the enantiomer of their natural substrates.

Indeed, in the current study, we demonstrated that there exists a similarity in the catalytic behavior of human dCK and some herpesvirus TKs, which supports the hypothesis of a considerable degree of genetic homology between them (8). In fact, cellular dCK also does not discriminate between the natural D-enantiomer and the unnatural L-enantiomer of dCyd, which are phosphorylated with the same efficiency: L-dCyd, despite its higher affinity for the active site of dCK (K_i = 0.12 μM versus K_m = 1.2 μM for D-dCyd), is phosphorylated *in vitro* with a final efficiency similar to that of D-dCyd, most probably due to an unfavorable mutual arrangement within the active site of the bound L-dCyd and the phosphate donor D-ATP.

Interestingly, we also observed that *in vivo*, L-dCyd interacts with human dCK, reducing by 50% the incorporation into DNA of dCMP derived from [^{3}H]dCyd at concentrations (3 μM) higher than those required to inhibit the enzyme *in vitro* (IC_{50} = 0.24 μM), probably depending on the intracellular pool of dCyd.

Furthermore, L-dCyd is not a substrate of human CD and does not affect [^{3}H]dThy incorporation into DNA. This contrasts with D-dCyd, which affects the pool of dTTP via deamination. This leads to the hypothesis that either L-dCMP is resistant to deamination or, if deaminated, the derived L-dUMP is not a substrate for thymidylate synthase.

Unlike L- and D-ddCyd, L-dCyd is not cytotoxic toward lymphoblastoid T cells and does not inhibit human immunodeficiency virus-1 replication up to 100 μM, the highest tested concentration.[1] The lack of both cytotoxicity and antiviral activity of L-dCyd might thus result from the nonconversion of L-dCMP to L-dTMP, from impaired conversion of L-dCMP to L-dCTP, or from the failure of L-dCTP to act, contrary to L-dTTP (32, 33) and some L-deoxyribonucleoside triphosphate analogs (18, 34–36), as substrate or inhibitor of cellular and viral DNA polymerases.[2] Finally, we found that modifications in either the base or the pentose ring of L-dCyd, such as those occurring in L-ddCyd and L-F-ddCyd, decrease the effectiveness of the analogs in inhibition of human dCK, but their affinity for the enzyme remains greater than that of the corresponding D- analogs. This agrees with the results relative to the *in vivo* metabolism of 2′,3′-dideoxy-3′-thiacytidine (18) and to its *in vitro* phosphorylation by human dCK (13) and underlines the potential role of dCK as activator of L-nucleoside enantiomers as antineoplastic and antiviral agents (16–22).

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