0026-895X/03/6301-105–110$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:105–110, 2003

Vol. 63, No. 1
1868/1030559
Printed in U.S.A.

Assessment of the Effect of Phosphorylated Metabolites of Anti-Human Immunodeficiency Virus and Anti-Hepatitis B Virus Pyrimidine Analogs on the Behavior of Human Deoxycytidylate Deaminase

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Received May 22, 2002; accepted September 25, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Deoxycytidylate deaminase, catalyzing the conversion of dCMP to dUMP, is an important enzyme in the de novo synthesis of thymidine nucleotides. It also may be involved in the action, as well as the metabolism of anticancer agents. Recently, several L- and D-configuration pyrimidine deoxynucleoside analogs were found to be potent antiviral and antitumor agents. Their interaction with dCMP deaminase as a monophosphate or a triphosphate metabolite is not clear. These include D-nucleoside analogs such as β-D-2′,3′-dideoxycytidine (ddC), β-2′-fluoro-5-methyl-arabinofuranosyluracil (FMAU), 3′-azido-2′,3′-dideoxythymidine (AZT), and 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) as well as L-nucleoside analogs such as β-L-dioxolane-cytidine (L-OddC), β-L-2′,3′-dideoxy-3′-thiacytidine, β-L-2′,3′-dideoxy-5′-fluoro-3′-thia-cytidine (L-FSddC), β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine, and L-FMAU. None of the L-deoxycytidine analog monophosphates act as substrates or inhibitors. Among these pyrimidine deoxynucleoside analog monophosphates, D-FMAU monophosphate (MP) is the most potent competitive inhibitor, whereas L-FMAUMP has no inhibitory activity. Interestingly, AZTMP and D4TMP also have potent inhibitory activities on dCMP deaminase. Among the dCTP and TTP analogs examined, D- and L-FMAUTP were the most potent inhibitors and had the same extent of inhibitory effect. These results suggest that a chiral specificity for the substrate-binding site may exist, but there is no chiral specificity for the regulator-binding site. This is also supported by the observation that L-OddC and L-FSddC have inhibitory activities as triphosphates but not as monophosphates. None of the D- and L-dCTP analogs activated dCMP deaminase as dCTP. The biological activities of AZT and D4T could be partially attributable to their inhibitory activity against dCMP deaminase by their phosphorylated metabolites, whereas that of ddC and the L-deoxycytidine analogs may not involve dCMP deaminase directly.

For de novo synthesis of TMP in cells, deoxycytidylate deaminase (dCMP deaminase; EC 3.5.4.12), catalyzing the conversion of dCMP to dUMP, is a key enzyme (Reichard, 1988). This enzyme is believed to play an important role in providing a balanced supply of dCTP and TTP for DNA synthesis. The enzymatic interconversions of the pyrimidine deoxyribonucleotides are shown in Fig. 1. It is an allosteric enzyme that can be activated by dCTP and inhibited by TTP (Maley and Maley, 1972). It was also demonstrated that this enzyme could catabolize the monophosphates of cytarabine (Jamieson et al., 1987) and gemcitabine (Heinemann et al., 1992), which are anticancer drugs. In recent years, several pyrimidine deoxynucleoside analogs were found to be useful in clinic for the treatment of HIV and HBV infections, as well as for cancers. AZT, a thymidine analog, was the first approved drug for the treatment of AIDS, but its use in patients has been hampered by its hematological and delayed toxicity (Richman et al., 1987; Hirsh, 1988; Surbone et al., 1988; Chen et al., 1991). Previous studies have suggested that AZT could be phosphorylated stepwise to AZTTP, with its 5′-monophosphate metabolite being the major metabolite within cells (Matthes et al., 1987; Balzarini et al., 1988; Frick et al., 1988; Balzarini et al., 1989; Ho and Hitchcock, 1989; Sommadossi et al., 1989; Fridland et al., 1990). High intracellular AZTMP levels may lead to inhibition of TMP kinase and TMP synthase, which in turn may result in a reduction of the TTP pool to facilitate its activity at the DNA polymer-

This work was supported by National Institutes of Health grants CA63477 and AI38204.

ABBREVIATIONS: HIV, human immunodeficiency virus; HBV, human hepatitis B virus; AZT, 3′-azido-2′,3′-dideoxythymidine; gemcitabine (dFdC), β-D-2′,2′-difluorodeoxycytidine; TP, triphosphate; MP, monophosphate; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; ddC, β-D-2′,3′-dideoxycytidine; FMAU, β-2′-fluoro-5-methyl-arabinofuranosyluracil; L-SddC, β-L-2′,3′-dideoxy-3′-thiacytidine; L-FSddC, β-L-2′,3′-dideoxy-5′-fluoro-3′-thia-cytidine; L-Fd4C, β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine; L-OddC, β-L-dioxolane-cytidine; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; araC, 1-β-D-arabinofuranosylcytosine.

ase level. D4T is another thymidine analog that has been approved as an anti-HIV drug (De Clercq, 2001). It is also phosphorylated in cells with D4TTP as a major metabolite (Balzarini et al., 1989; Ho and Hitchcock, 1989; Marongiu et al., 1990; Zhu et al., 1991). Previous studies have shown that its toxicity is less than AZT both in vitro and in vivo (Balzarini et al., 1989; Zhu et al., 1991). ddC is a potent anti-HIV deoxycytidine analog that is also phosphorylated in cells (Balzarini et al., 1988). FMAU was found to have potent activities against herpes viruses and HBV (Kong et al., 1988; Fourel et al., 1992). The clinical studies were discontinued because of toxicity (Abbruzzese et al., 1989). All these compounds are in the D-configuration, which is the natural configuration of nucleosides in cells. L-SddC (3TC; lamivudine), is the first L-configuration nucleoside analog shown to have anti-HIV and -HBV activities (Doong et al., 1991; Chang et al., 1992; Schinazi et al., 1992) and is currently used in the treatment of HIV and HBV infection. L-FSddC (FTC) and L-Fd4C were shown to be potent anti-HBV and -HIV compounds (Bridges and Cheng, 1995; Cheng, 2001). Both of these compounds are under clinical trials. L-OddC was found to have potent anti-HIV and -HBV activity (Bridges and Cheng, 1995). It is also potent against tumor cell growth in cultures and in animals (Grove et al., 1995). It is currently under clinical studies for its effectiveness against leukemia and solid tumors (Kadhim et al., 1997; Moore et al., 1997; Giles et al., 2001). L-FMAU, a thymidine analog, was found to have only anti-HBV activity, not anti-HIV activity, in cell cultures (Chu et al., 1995). It is also under clinical trials for the treatment of HBV (Cheng, 2001). All of these L-nucleoside analogs can be phosphorylated stepwise to triphosphate metabolites, although the enzymes involved and kinetics could be different (Krishnan et al., 2002; Liou et al., 2002).

Because dCMP deaminase could play an important role in deoxypyrimidine analog metabolism and all of these compounds could be phosphorylated in cells, we assessed the possible interactions of this enzyme with these nucleoside analogs. In this report, we have described the effects of these anti-HIV and -HBV agents on partially purified dCMP deaminase from HepG2 cells, with respect to its interaction with monophosphate and triphosphate metabolites of these pyrimidine deoxynucleoside analogs.

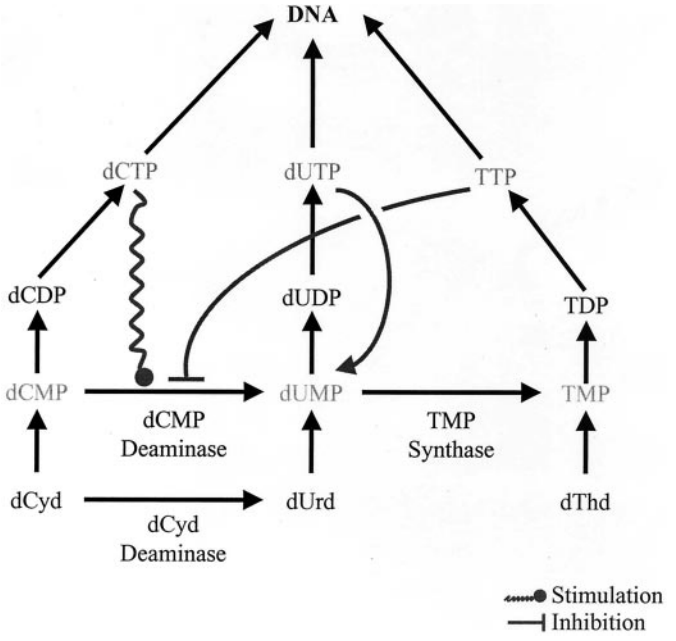


Fig. 1. Pyrimidine deoxyribonucleotide interconversion in mammalian cells.

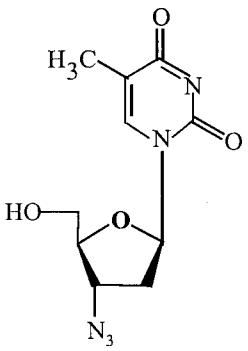
Materials and Methods

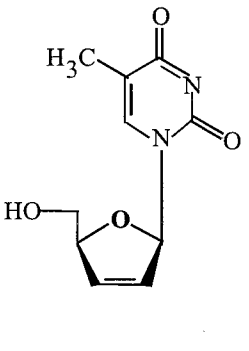
Synthesis and Purification of Nucleoside Analog Monophosphates and Triphosphates. Monophosphate and triphosphates of nucleoside analogs were synthesized and purified according to the procedure published by Ruth and Cheng (1981) with minor modifications. Briefly, 20 mg of nucleoside was stirred in trimethyl phosphate (10 μl/mg of nucleoside) at −10°C. Phosphorus oxychloride ($POCl_3$; 0.9 Eq) was added, the reaction was allowed to stir for 30 min, and a second 0.8 Eq of $POCl_3$ was added. At intervals, 0.5-μl aliquots of the reaction mixture were treated with excesses of aqueous potassium hydroxide and assayed by analytic anion exchange HPLC using a Whatman (Clifton, NJ) PXS 10/25 SAX column. After maximal formation of the intermediate nucleoside phosphodichloridate was observed, the reaction was slowly added to the excess tris(tributylammonium)pyrophosphate (5–8 Eq) in dimethylformamide (3–4 volumes relative to original reaction). The reaction was assayed for triphosphate formation at frequent intervals by anion-exchange HPLC chromatography. When formation of triphosphate appeared maximal, the reaction was neutralized with cold excess aqueous potassium hydroxide. The products (monophosphate and triphosphate) were purified by column chromatography on Sephadex DEAE A-25 (Whatman), eluted with different concentrations of KCl. Fractions containing appropriately pure monophosphate and triphosphate (95–99% by HPLC) were combined, lyophilized, and desalted.

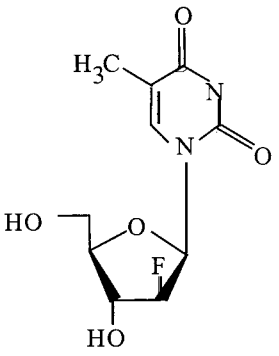
Purification of dCMP Deaminase from HepG2 Cells. HepG2 cells were lysed by repeated freeze-thawing in a lysis buffer [10 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 5 mM NaF, 15 mM $MgCl_2$, 20 mM KCl, and 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. The lysate was centrifuged at 17,000 X g for 20 min. The crude extract was then subjected to purification on a Blue Sepharose CL6B column (Amersham Biosciences Inc., Piscataway, NJ). The elution buffer contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM KCl, 5 mM dithiothreitol, 5 mM NaF, and 1× protease inhibitor cocktail. All the other buffers for elution were prepared in an elution buffer. After the passage of crude extract through the column, 45 ml of the elution buffer was passed through the column to remove poorly bound proteins. This was followed by the elution with 45 ml of 5 mM 3-phosphoglycerate to remove phosphoglycerate kinase, 45 ml of elution buffer, and 60 ml of 0 to 5 mM ADP gradient. The dCMP deaminase activity was eluted out of column with activity peaked at 2 to 4 mM ADP eluate. The protein concentration of original lysate and fraction was determined using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). The specific activity of combined peaks was determined to be 60-fold more than the original lysate and the recovery rate was calculated to be more than 100%.

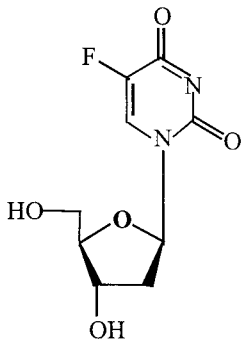
Enzyme Assays. The assay was a modification of a previously described method that used radiolabeled substrates (Maley and Maley, 1960). The dCMP reaction mixture contained, in a volume of 75 μl, 50 mM MES, pH 7.5, 2 mM dithiothreitol, 2 mM $MgCl_2$, 25 μg/ml bovine serum albumin, 0.5 mM EDTA, 20 mM NaF, and the additives. Ten microliters of enzyme preparation was used in each reaction. The reaction was terminated by the addition of 50 μl of 1.2 M trichloroacetic acid. One unit of dCMP deaminase is defined as the amount of the enzyme that catalyzes the formation of 1 nanomole of dUMP from dCMP per minute at 37°C under standard assay conditions.

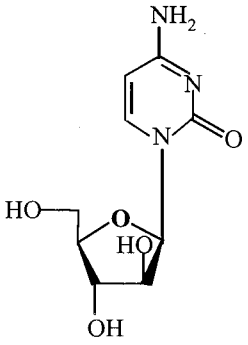
When substrates were used for which radioactive material was not available, the enzymatic reaction was terminated by adding 1.2 M trichloroacetic acid, then extracted with trioctylamine/trichlorotrifluoroethane (55:45, v/v) twice. The substrate and product were sep-

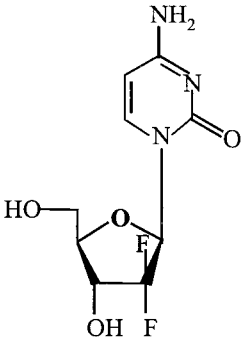


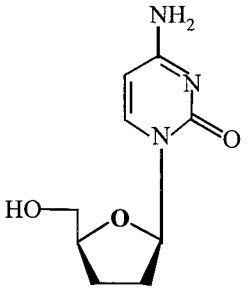


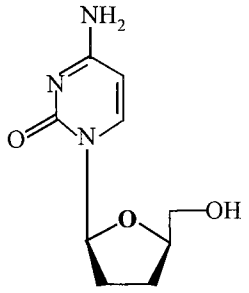


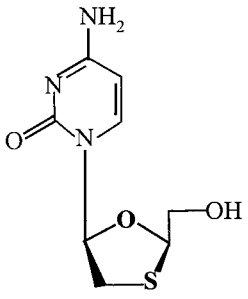


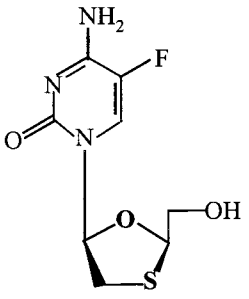


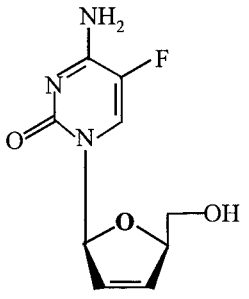


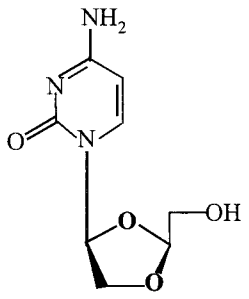












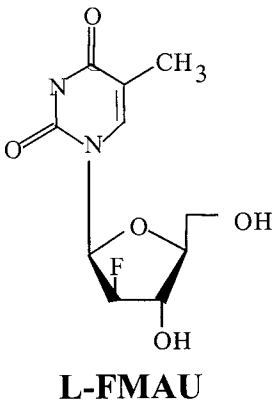


Fig. 2. The structures of pyrimidine analogs used in this study

arated by HPLC with a SAX anion exchange column (Whatman), and a potassium phosphate buffer gradient was applied as described previously (Mancini and Cheng, 1983). UV absorption peaks were integrated and the ratios were determined.

Results

Substrate Behavior of dCMP Analogs. The kinetic properties of partially purified dCMP deaminase were re-examined. The concentration velocity relationship for the activation of dCMP deaminase by dCTP at increasing concentrations of dCMP was determined (data not shown). In the absence of dCTP, dCMP concentration and velocity relationship follows a sigmoid curve. The concentration of dCMP required to give maximum velocity was determined to be 1 mM. In the presence of 10 μM dCTP, the dose-response curve was hyperbolic, and the K_m value of dCMP was 22 μM; therefore, 4 μM dCTP will be sufficient to give maximum activity. This is consistent with reports published by others (Maley and Maley, 1972; Ellims et al., 1981; Mancini and Cheng, 1983; Maley et al., 1993). Pyrimidine analog monophosphates were examined as substrates of dCMP deaminase at 150 μM in the presence of 10 μM dCTP. The structures of pyrimidine analogs used are shown in Fig. 2. As reported previously (Mancini and Cheng, 1983; Jamieson et al., 1987; Heinemann et al., 1992), dFdCMP was a good substrate and araCMP was a fair substrate for this enzyme compared with dCMP (Table 1). We were unable to detect any deaminated product of ddCMP and L-configuration deoxycytidine analog monophosphates. We thus conclude that the ddCMP and L-dCMP analogs examined are not substrates of dCMP deaminase.

Effect of Pyrimidine Analog Monophosphates on dCMP Deamination. Because dCMP deaminase is an important enzyme for TMP synthesis, these antiviral and anticancer pyrimidine analog monophosphates were examined for the possibility of inhibitory effects on dCMP deamination. We chose 10 μM dCTP and 50 μM dCMP as a standard assay condition based on the above-described study. Except for dFdCMP, all of the dCMP analogs examined had no apparent effect on dCMP deamination, whereas all of the D-configuration dUMP and TMP analogs had inhibitory effects on dCMP deamination (Table 2). It is interesting to note that L-FMAUMP, unlike D-FMAUMP, has no inhibitory effects, suggesting a chiral specificity of dCMP deaminase. We further explored the inhibition by dUMP and TMP analogs by determining their K_i values. The inhibition curves of AZTMP and D4TMP at different concentration of dCMP are shown in Fig. 3 as examples. These analogs were determined as competitive inhibitors with respect to dCMP, using the method described previously by Cheng and Prusoff (1973). The K_i values were calculated and are shown in Table 2. Among these dUMP and TMP analogs, D-FMAUMP was the most potent inhibitor. AZTMP and D4TMP were also potent. Interestingly, 5FdUMP is a more potent inhibitor than dUMP.

Effect of Pyrimidine Analog Triphosphates on dCMP Deaminase. dCMP deaminase is an allosteric enzyme that can be activated by dCTP and inhibited by TTP. Thus, it is important to examine whether these pyrimidine analog triphosphates have any effect on the dCMP deaminase. As shown in Table 3, neither the D- nor the L-configuration dCTP analogs examined could activate dCMP deaminase at the concentration of 20 or 40 μM (data not shown) in the absence of dCTP. Thus, L-configuration dCTP analogs could not substitute for dCTP in terms of activating dCMP deaminase. We then explored whether these pyrimidine analog triphosphates had effects on the dCMP deaminase activation by dCTP; 2 μM dCTP was chosen because of the observation that dCMP deaminase exerts 60 to 80% activity at the optimal condition (>4 μM dCTP). Under these conditions, we could detect both inhibition and activation by these analog triphosphates. As presented in Table 4, among the dCTP analogs, L-OddCTP and L-FSddCTP caused 30% inhibition; none of the other analogs had an obvious effect. Among dUTP and TTP analogs, both D- and L-FMAUTP caused 40 to 45% inhibition on dCMP deaminase; dUTP caused 15% inhibition. dCMP deaminase is not inhibited by AZTTP and D4TTP, which are TTP analogs, although TTP is a very potent inhibitor.

TABLE 1

Relative rate of deamination of deoxycytidine analog monophosphates by dCMP deaminase

The activity of dCMP deaminase used was 0.83 U in the presence of 10 μM dCTP. Rates were normalized as percentages of dCMP deamination. Values are presented as mean ± S.D. of three independent experiments.

	Relative Rate (150 μM)
	%
dCMP	100
dFdCMP	14.9 ± 1.3
AraCMP	4.0 ± 0.8
ddCMP	<0.01
L-ddCMP	<0.01
L-OddCMP	<0.01
L-SddCMP	<0.01
L-FSddCMP	<0.01
L-Fd4CMP	<0.01

Discussion

dCMP deaminase is an important enzyme controlling the balance between the TTP and dCTP pools. It also plays an important role in the catabolism of gemcitabine and 1-β-D-

TABLE 2

Inhibition of dCMP deamination by pyrimidine analog monophosphates

dCMP deaminase (0.9 U) was used in this experiment. Substrate (dCMP) concentration was 50 μM. The reaction was performed in the presence of 10 μM dCTP. Rate was normalized as the percentage of dCMP deamination in the absence of additive. K_i was calculated using the equation $v = V_{max}S/K_m(1 + I/Ki) + S$. K_m was 22.2 μM. Values are presented as mean ± S.D. of three independent experiments.

Additive	Relative Rate (300 μM)	K_i
	%	μM
None	100	
dFdCMP	44.7 ± 2.0	
AraCMP	91.3 ± 0.5	
ddCMP	95.0 ± 7.2	
L-ddCMP	92.4 ± 12.3	
L-OddCMP	94.0 ± 1.2	
L-SddCMP	88.8 ± 3.2	
L-FSddCMP	81.7 ± 1.6	
L-Fd4CMP	94.7 ± 0.8	
dUMP	22.2 ± 4.7	25.7 ± 2.9
5FdUMP	13.5 ± 3.4	14.2 ± 2.4
TMP	3.9 ± 0.8	4.6 ± 0.6
AZTMP	17.9 ± 3.0	36.7 ± 4.2
D4TMP	15.3 ± 1.6	39.4 ± 2.5
D-FMAUMP	5.3 ± 0.4	10.3 ± 1.0
L-FMAUMP	105.3 ± 6.3	

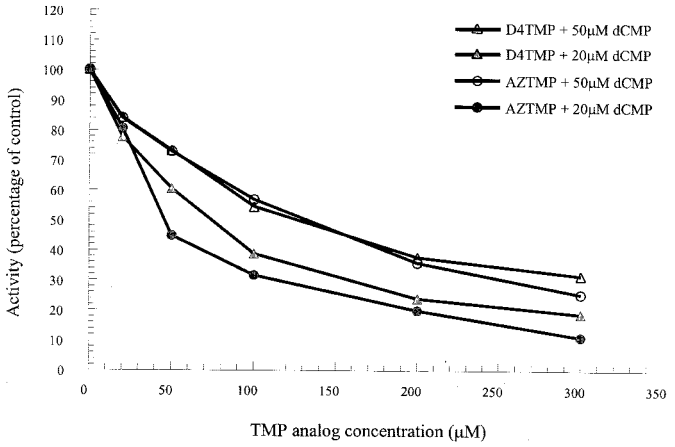


Fig. 3. Inhibition of dCMP deamination by AZTMP and D4TMP. The assays were performed at 50 or 20 μM dCMP as substrates in the presence of 10 μM dCTP. The assay solution contained the indicated concentration of AZTMP or D4TMP. The activities were normalized as percentage of none additive control. Each point represents the average of two independent experiments.

arabinofuranosylcytosine. Therefore, it could also play an important role in the action of recently discovered deoxypyrimidine analogs. AZT and D4T are anti-HIV drugs. Both are phosphorylated stepwise to triphosphate metabolites. In the case of AZT, AZTMP is the predominant metabolite. Cells exposed to AZT and D4T would decrease TTP level and increase dCTP level in cells (Frick et al., 1988; Ho and Hitchcock, 1989; Marongiu et al., 1990). It was already shown that AZTMP could inhibit thymidylate synthase and TMP kinase. The decrease of the TTP pool was attributed to these actions. AZTMP and D4TMP were shown to be good inhibitors of dCMP deaminase as described in this study, which raises a potential action site: dCMP deaminase, which catalyzes reactions one step before TMP synthase in decreasing the TTP pool. Previous reports revealed that AZTMP and D4TMP could accumulate to a high concentration in AZT- and D4T-treated cells; therefore, the inhibition we observed might be physiologically relevant. This inhibition could facilitate the action of AZT or D4T against HIV or cell growth by decreasing the de novo synthesis of TTP, a substrate for DNA synthesis.

In the past decade, L-nucleoside analogs have been recognized as a new class of antiviral and anticancer agents. The metabolism and actions of the L-nucleoside analogs discovered in this laboratory have been reported (Chang et al., 1992; Bridges and Cheng, 1995; Grove and Cheng, 1996; Zhu et al., 1998). Their interactions with dCMP deaminase as monophosphates or triphosphates had not been examined yet. In view of the important role of dCMP deaminase, partially purified dCMP deaminase was used to explore this question. The ddCMP and L-dCMP analogs studied were neither substrates nor inhibitors of dCMP deaminase. It was demonstrated that none of the L-nucleoside analogs examined were substrates or inhibitors of cytidine deaminase (Chang et al., 1992; Bridges and Cheng, 1995; Grove and Cheng, 1996; Zhu et al., 1998). Therefore, these compounds will not be metabolized in the same manner as 1-β-D-arabinofuranosylcytosine or gemcitabine. Their interactions with human dCMP deaminase were reported in this study. It is interesting to note that dCMP deaminase activity could be inhibited by L-OddCTP and L-FSddCTP by 30% at the concentration that was 30-fold greater than dCTP. The significance of this inhibition needs to be explored further.

It is intriguing to note that D-FMAUMP exerts good inhibitory activity, but L-FMAUMP does not. These data indicate that there is a chiral specificity for dCMP deaminase at the monophosphate binding site. On the other hand, both D- and L-FMAUTP exert the same extent of inhibition, suggesting that there is no chiral specificity for the regulatory triphosphate nucleotide-binding site. This is consistent with the notion that the structural requirement for substrate and activator are quite different. This notion is further supported by the observation that 5FdUMP is a more potent inhibitor than dUMP, whereas dUTP is a more potent inhibitor than 5FdUTP. L-FSddCTP is a more potent inhibitor than L-SddCTP, suggesting that even the regulator-binding mode, with respect to dTTP and dCTP, which competed with each other, are different. We were unable to demonstrate the inhibition of dCMP deaminase by dFdCTP as reported by others (Heinemann et al., 1992). This might be because of the lower concentration of dFdCTP used in the assay, different assay conditions, or enzyme preparation. In conclusion, the action against HIV or cell cytotoxicity caused by AZT or D4T may be

TABLE 3

Effect of deoxycytidine analog triphosphates on dCMP deaminase activity in the absence of dCTP

dCMP concentration was 50 μM; 0.9 U of enzyme was used for every reaction. The rate was normalized as the percentage of dCTP sample. Values are presented as mean of three independent experiments.

Triphosphate	Relative Rate (20 μM)
	%
dCTP	100
dFdCTP	<0.1
AraCTP	<0.1
ddCTP	<0.1
L-ddCTP	<0.1
L-SddCTP	<0.1
L-FSddCTP	<0.1
L-Fd4CTP	<0.1
L-FSddCTP	<0.1

TABLE 4

Effect of pyrimidine analog triphosphates on dCMP deaminase activity in the presence of dCTP

Reactions were performed at 50 μM dCMP as the substrate in the presence of 2 μM dCTP. One unit enzyme was used in every reaction. Rate was normalized as the percentage of none-additive sample. Values are presented as mean ± SD of there independent experiments. TTP concentration was 24 μM in this assay

Additive	Relative Rate (60 μM)
	%
None	100
dFdCTP	101.2 ± 2.9
AraCTP	102.3 ± 2.7
ddCTP	105.8 ± 1.5
L-ddCTP	105.1 ± 0.1
L-OddCTP	67.2 ± 7.2
L-SddCTP	104.6 ± 0.2
L-FSddCTP	72.6 ± 2.1
L-Fd4CTP	103.4 ± 1.1
dUTP	84.7 ± 0.6
5FdUTP	99.0 ± 2.0
TTP	2.0 ± 1.4
AZTTP	95.2 ± 0.9
D4TTP	102.4 ± 0.9
D-FMAUTP	54.5 ± 7.7
L-FMAUTP	64.8 ± 4.0

partially attributable to their impacts on dCMP deaminase, whereas that caused by ddC and the L-nucleoside analogs, with the exception of L-FMAU, are unlikely to involve dCMP deaminase directly.

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