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Cellular Pharmacology of 2′,3′-Dideoxy-2′,3′-Didehydrothymidine, a Nucleoside Analog Active against Human Immunodeficiency Virus

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2′,3′-Dideoxy-2′,3′-didehydrothymidine (D4T) is a thymidine nucleoside analog which has potent anti-human immunodeficiency virus activity in vitro. We have studied its metabolism in cells to assist in determining its mechanism of action. D4T is metabolized in cells to the mono-, di-, and triphosphate nucleotides. Our data suggest that the initial conversion to the monophosphate is catalyzed by thymidine kinase. This enzyme has an affinity for D4T 600-fold lower than for thymidine and catalyzes the rate-limiting step in production of the triphosphate. Nevertheless, intracellular concentrations of the triphosphate approximately equal to the reported K_i for human immunodeficiency virus reverse transcriptase are attained with extracellular concentrations of free drug as low as 0.05 μM. The pattern of phosphorylation is different from that of 3′-azido-3′-deoxythymidine (AZT), which has an affinity for thymidine kinase equivalent to that of thymidine and is easily phosphorylated. The rate-limiting step in formation of AZT triphosphate is conversion of mono- to diphosphate, and thus the monophosphate accumulates. On removal of D4T or AZT from the media, both triphosphates have an intracellular half-life of about 200 min, and this rate ultimately controls the rate of elimination of the drugs from cells. The differences in metabolism of D4T and AZT observed in vitro may be responsible for the differences in toxicity seen in vitro and in vivo and support the exploration of the clinical utility of D4T as an anti-human immunodeficiency virus agent.

Acquired immunodeficiency syndrome is caused by infection with human immunodeficiency virus (HIV) (5, 12, 15). This is a retrovirus, the genome of which is single-stranded RNA. To establish infection within a cell, the virus integrates a genomic DNA copy (provirus) into the host DNA (24). The production of this double-stranded DNA from the RNA is catalyzed in the cell cytoplasm by the viral reverse transcriptase, an enzyme that is carried with the RNA in the virion particle. Because this process is essential and unique to the virus, the inhibition of the reverse transcription process which produces the provirus is an important target for chemotherapy.

One compound which is active against HIV at this target is 3′-azido-3′-deoxythymidine (AZT; zidovudine) (11, 18, 19, 25, 26). AZT is the only drug currently approved for the therapy of acquired immunodeficiency syndrome. Its benefits are substantial in terms of antiviral effect as well as general health improvements (9, 20, 27). However, this drug is not without limitations. The major problem associated with the use of AZT is dose-limiting myelosuppression, which leads to anemia and neutropenia (22). These toxicities often require reduction of the dose or discontinuation of the drug.

The mechanisms of action of AZT and various other nucleoside analogs which lack a 3′-hydroxyl group may share a number of features. In cells, these compounds act as alternate substrates for kinases which activate them to triphosphates (11). These triphosphates then compete for binding to the HIV reverse transcriptase with the normal nucleoside triphosphates required for DNA synthesis (11, 18, 25, 26). This inhibits the rate of synthesis of the viral DNA. In addition, these unnatural nucleoside triphosphates can act as alternate substrates for reverse transcriptase and be incorporated into the growing DNA chain instead of the natural one (25). This event terminates the elongation process because there is no 3′-hydroxyl group to which the next nucleotide can be attached. The absolute activity of these nucleoside analogs is therefore a complex function of the ability of the compound to enter cells and be phosphorylated, the efficiency of the inhibition of reverse transcription by the corresponding triphosphates (both inhibition of the enzyme and incorporation to terminate chain elongation), and the ability of the drug and its metabolites to modulate the concentration of the natural competing nucleotide triphosphate.

2′,3′-Dideoxy-2′,3′-didehydrothymidine (D4T) is another nucleoside analog lacking the 3′-OH group, and it has been shown to have in vitro anti-HIV activity similar to that of AZT (2, 3, 13, 16, 17, 23). However, its toxicity in vitro to human bone marrow cells is less than that of AZT (17). Since there are no currently available predictive animal models for evaluation of anti-HIV agents, an analysis of the biochemistry of the drug is helpful to reinforce the potential for clinical utility. Thus, we have investigated the cellular pharmacology of D4T. This paper shows some significant differences between its metabolism and that of AZT.

MATERIALS AND METHODS

[*methyl*-^{3}H]D4T (20 mCi/mmol; 1 mCi/ml in ethanol [radiochemical purity, ≥98.5%]) and [2-^{14}C]AZT (56 mCi/mmol; 0.1 mCi/ml in ethanol [radiochemical purity, ≥99.0%]) were purchased from Moravek Biochemicals. Before use, [^{3}H]D4T was diluted where appropriate with unlabeled D4T and the ethanol was removed under a stream of nitrogen. Medium for growth of cells was from GIBCO Laboratories and consisted of RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, 1% sodium pyruvate, and 1% nonessential amino acids. Alkaline phosphatase from *Escherichia coli* was purchased from Sigma Chemical Co.

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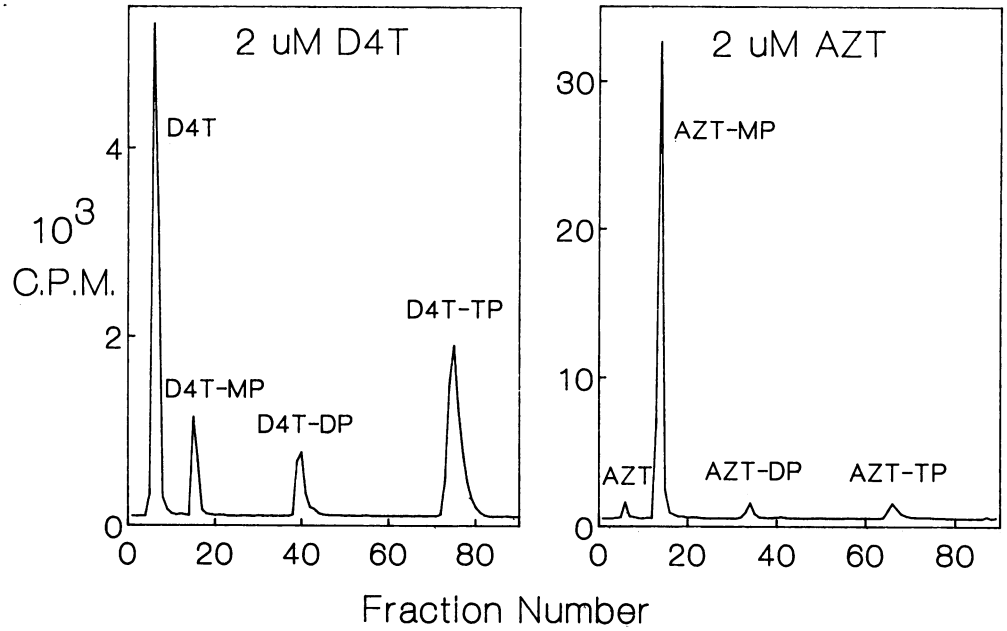

FIG. 1. Comparison of phosphorylation of 2 μM D4T with 2 μM AZT in CEM cells. Cells were incubated for 24 h with labeled drug and then processed to quantitate intracellular metabolites.

Cellular metabolism studies. Experimental points were derived from cultures of 9 ml of CEM cells at 5×10^5 cells per ml in medium in a T-75 flask. These were mixed with 1 ml containing (usually) 30 μCi (20 nmol) of [^{3}H]D4T. After incubation at 37°C in a 5% CO_2 atmosphere for the time indicated (usually 24 h), cells were pelleted by centrifugation (1,800 × *g*, 5 min, 4°C) and washed once with phosphate-buffered saline. The cells were extracted twice by vortexing with 1 ml of 60% methanol (or ethanol) and, after 30 min in an ice bath, centrifugation as described above. Combined extracts were dried under vacuum (Savant Speed-Vac), and the samples were stored at −20°C until analyzed. Metabolites were separated by high-performance liquid chromatography and quantitated by radioactivity. Concentrations in the cells were determined by using the method of Furman et al. (11). When the effects of other, unlabeled, nucleosides (thymidine, deoxycytidine, dideoxyadenosine, and dideoxyinosine) were studied, the nucleosides were present at a 20 μM final concentration and added 3 h prior to addition of labeled D4T. When the effect of AZT on D4T phosphorylation (and vice versa) was studied, both compounds were added simultaneously at a 2 μM final concentration of each.

High-performance liquid chromatography methods. Separation of nucleotides was performed on a 25-cm Whatman Partisil 10 SAX column by using a Waters high-performance liquid chromatography system containing two pumps, an autoinjector, and a data station. The mobile phase contained two components (15 mM potassium phosphate [pH 3.5] [component A] and 700 mM potassium phosphate [pH 3.5] [component B]) and was pumped at 1 ml/min. Dried samples were suspended in 180 μl of water, and 150 μl was injected. The buffer gradient was then applied, starting at zero time with 100% component A and increasing linearly to 100% component B over 55 min. Then, 100% component B was pumped for 10 min. The eluate was fractionated at 1-min intervals (1 ml), and 0.8-ml samples were mixed with 4.5 ml of scintillation cocktail (Packard Inst-Gel XF) and counted (Beckman LS 6800). Typical chromatograms are shown in Fig. 1. Assignment of peaks of radiolabel to free drug and mono- and triphosphate was done by comparison to retention times with authentic samples produced chemically (17, 26). The assignment of diphosphate was assumed from its position between mono- and triphosphate.

Additional characterization of the di- and triphosphates was performed by enzymatic dephosphorylation. Material from the peaks was collected, and the majority of phosphate buffer was removed by precipitation with 60% methanol. After the supernatant was dried in vacuo, the material was further desalted on DEAE-Sephadex by using ammonium bicarbonate to elute the di- or triphosphate. After being dried again, the material was treated with alkaline phosphatase at 24°C for 30 min. Separation of alkaline phosphatase-digested nucleotides was performed on a RAININ 5-μm C-18 Short-one column (0.46 by 15 cm). The mobile phase consisted of 50% methanol (component A) and 25 mM KH_2PO_4 (component B) and was pumped at 1 ml/min. A gradient from 0% component A at zero time to 30% component A at 20 min was established. Under these conditions, the following retention times were observed for standards: TMP, 5.5 min; thymine, 7.3 min; thymidine, 13.3 min; and D4T, 18.8 min. All detectable radiolabel from the enzyme-treated di- and triphosphates was associated with the D4T peak.

Enzymological methods. Thymidine kinase was purified from CEM cell pellets by using the method of Chen and Prusoff (7). It was assayed by using [^{14}C]thymidine (53 mCi/mmol; Dupont, NEN Research Products) by a procedure described previously (6). Inhibition by AZT or D4T was measured at 2.5, 5, 10, and 20 μM thymidine. For AZT, concentrations used were 0, 5, 10, and 20 μM, whereas for D4T they were 0, 2,500, 5,000, and 10,000 μM. K_m and K_i values were derived by fitting the rate data to the equation $v = (V_{max} \cdot S)/\{S + K_m [1 + (I/K_i)]\}$ with an SAS program, where S and I are concentrations of the substrate and inhibitor, respectively.

TABLE 1. Effect of time of exposure to 2 μM D4T on production of phosphorylated forms by CEM cells[a]

Time of exposure (h)	% of total			
	Free drug	Monophosphate	Diphosphate	Triphosphate
3	59.0	8.7	4.4	27.9
6	50.1	9.1	6.7	34.1
24	40.0	9.2	13.7	37.1
48	49.6	10.9	6.3	33.2

[a] Cells were incubated with labeled D4T at a 2 μM final concentration. At the times indicated, cells were processed to quantitate the intracellular forms of D4T.

RESULTS

Because the anti-HIV activity of D4T has been reported in CEM cells (17), they were selected to study the phosphorylation of this compound. Initially, we studied the effect of time of exposure to 2 μM D4T on the intracellular metabolites (Table 1). It can be seen that by 6 h of exposure to D4T, the pools of the phosphorylated derivatives were in equilibrium and the majority of the triphosphate pool concentration finally achieved was already present even at 3 h of exposure. For convenience, further experiments were performed by using 24-h incubation with the drug. It has been reported that the phosphorylation pattern of AZT is different in different cell lines (4); therefore, we compared the phosphorylation of 2 μM AZT with that of D4T. Figure 1 shows that at 2 μM AZT, the major product (84%) in cells was the monophosphate and that di- and triphosphates accounted for only 6 and 7% of the total label. These are similar to the results reported with other human lymphocytic cell lines, for example H-9 cells (11) and ATH8 and Molt/4F cells (4). By contrast, the phosphorylation of 2 μM D4T in CEM cells was quite different (Fig. 1). Unphosphorylated compound accounted for 47% of the total intracellular radioactivity, and triphosphate accounted for 34%. These differences suggest that the rate-limiting reaction in formation of the triphosphates is different; with AZT it is conversion of mono- to diphosphate, whereas with D4T it is conversion of free drug to monophosphate. The results from experiments using different concentrations are shown in Table 2. Over a 400-fold increase in external concentrations of D4T (0.05 to 20 μM), the intracellular concentrations of total phosphorylated forms increased about 120-fold and the ratio of mono- to di- to triphosphates remained approximately the same. By contrast, over a 1,000-fold increase in external AZT concentration (0.02 to 20 μM), the concentrations of di- and triphosphate increased only about 4-fold, whereas the monophosphate concentration increased 150-fold.

TABLE 2. Concentrations of phosphorylated forms of D4T and AZT in CEM cells

Compound	Concn (μM)				
	In medium, free drug	In cells			
		Free drug	Monophosphate	Diphosphate	Triphosphate
D4T	0.05	0.044	0.012	0.020	0.064
	2.0	1.9	0.38	0.40	1.4
	20	13	2.6	2.0	6.8
AZT	0.02	0.33	5.1	1.8	3.1
	0.2	1.03	27	3.4	5.6
	2.0	4.6	110	5.4	8.4
	20	64	740	7.4	11

With D4T, the concentration of free drug inside the cell was similar to that outside, suggesting that transport is controlled by diffusion. The results for AZT, however, would at first glance suggest it was concentrated by the cell. We believe this to be artifactual and the result of a small degree (6% or less) of hydrolysis of the large amount of monophosphate in these extracts which may occur during preparation and analysis of the sample. Cells which do not metabolize these compounds provide more reliable data relating to their transport.

Since exposure to constant external drug concentrations rarely occurs in vivo, the effect of removing drug from the medium was investigated. Cells were incubated with 2 μM labeled D4T or AZT for 24 h and then washed, suspended in fresh medium, and sampled at intervals. The result is shown in Fig. 2. With D4T, the intracellular pools all reequilibrated rapidly and decayed at a constant rate (about a 200-min half-life). Interestingly, the pool size of D4T monophosphate relative to the diphosphate was about one-tenth that seen with excess external drug, suggesting that the monophosphate decay was initially faster but then became dependent on the flux from the di- and triphosphate pools. A similar effect was observed in the experiment with AZT, but because the monophosphate pool was initially much larger, it took longer to become dependent on other pools. The triphosphate of AZT, like that of D4T, decayed with a half-life of about 200 min.

The free drug measured in these extracts is also thought to arise from a small amount of hydrolysis as described above. Free drug inside the cell with no drug outside would be expected to diffuse out rapidly.

Our initial attempts to measure the interaction between D4T and thymidine kinase showed that D4T had a low affinity. Therefore, to explore which enzymes may be involved in D4T activation, we studied phosphorylation of D4T after pretreatment of cells with some other nucleosides and analogs (Table 3). Thymidine and deoxycytidine were chosen as natural nucleosides with defined activation enzymes (thymidine and deoxycytidine kinases, respectively). Dideoxyadenosine has been shown to be activated by both adenosine kinase and deoxycytidine kinase, whereas dideoxyinosine is thought to be activated by the reversal of a 5′ nucleotidase which dephosphorylates IMP (14). Cells were incubated for 3 h with unlabeled nucleosides at 20 μM concentrations, and then labeled D4T was added to a concentration of 2 μM for a further 24 h. As can be seen, dideoxyadenosine, dideoxyinosine, and deoxycytidine had no effect on either uptake or phosphorylation of D4T. Thymidine also did not affect the amount of free D4T present in the cells, suggesting it has no impact on D4T transport. However, it almost completely suppressed phosphorylation. This observation strongly implicated a role for thymidine kinase in activation of D4T.

Thymidine kinase was therefore purified from CEM cells and used to assess the interaction between this enzyme and D4T. Analysis of the kinetic data from different thymidine and D4T concentrations gave a K_m for thymidine of 6.6 μM and a K_i for D4T of 4,600 μM. A similar experiment in which D4T was replaced by AZT (at lower concentrations) gave a K_m for thymidine of 7.4 μM and a K_i for AZT of 7.6 μM. Essentially similar results have also been found by using thymidine kinase purified from Vero cells (data not shown).

In light of the interest in using anti-HIV agents in combinations, an experiment was performed to measure the effect

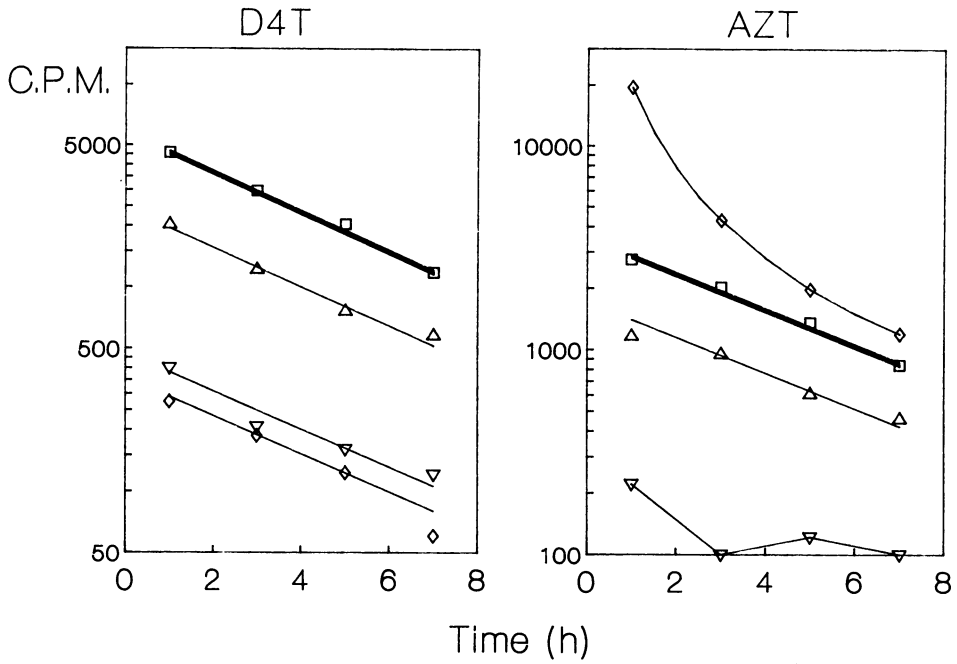

FIG. 2. Decay of phosphorylated forms of D4T and AZT after removal of extracellular drug. Cells were incubated for 24 h with 2 μM labeled drug. At zero time, the cells were pelleted by centrifugation and washed once with medium at room temperature. They were then suspended in fresh medium. At the times indicated, cells were removed and processed to quantitate intracellular metabolites: free drug (▽), monophosphate (◇), diphosphate (△), and triphosphate (□).

of AZT on phosphorylation of D4T and vice versa. Both compounds were added to CEM cells simultaneously at a 2 μM concentration with one compound labeled and one unlabeled. The results are shown in Fig. 3. It is apparent that AZT inhibited production of phosphorylated forms of D4T. The monophosphate level was only about 50% of the control value, whereas di- and triphosphate were below 5% of their control levels. By contrast, D4T had no observable effect on phosphorylated forms of AZT.

These findings are consistent with AZT having a higher affinity for the first enzyme of the activation sequence, thymidine kinase. They also support the view that activation of D4T is catalyzed by the same enzyme.

DISCUSSION

The metabolism of D4T by a human lymphocytic cell line, CEM, is considerably different from that of AZT under similar conditions. Each drug is phosphorylated to mono-, di-, and triphosphate. However, the ratios of these phosphates are considerably different. This is mostly the result of the rate-limiting enzyme being different for the two drugs (thymidine kinase for D4T, thymidylate kinase for AZT). The difference in ratios may also result from a difference in the mechanism of limitation; with D4T, it is a decreased affinity for the enzyme, whereas for AZT, the affinity of thymidylate kinase for AZT monophosphate is similar to that of thymidine monophosphate but there is a decreased V_{max} (11).

TABLE 3. Effect of nucleosides on phosphorylation of D4T[a]

Nucleoside added	% of control value			
	Free drug	Mono-phosphate	Diphos-phate	Triphos-phate
Thymidine	115	11.7	<0.4	<0.4
2'-Deoxycytidine	122	104	94.4	88.2
2',3'-Dideoxyadenosine	107	106	108	97
2',3'-Dideoxyinosine	107	120	132	94.1

[a] Cells were incubated with unlabeled nucleosides at a 20 μM final concentration for 3 h prior to the addition of 2 μM labeled D4T. After 24 h with label, the cells were processed to quantitate the intracellular forms of D4T.

The results shown in Table 2 indicate that the mechanism of action of AZT may be more complex than has been proposed. The production of the triphosphate species, which has been shown to be the active inhibitory species against HIV reverse transcriptase, is barely changed over a wide range of input drug. Thus, it appears that any enhanced antiviral effect of higher concentrations is most likely the result of the accumulation of monophosphate suppressing TTP formation and thus reducing the competition for AZT triphosphate at the reverse transcriptase. This 18-fold drop in the level of TTP initially described (11) is now claimed to be transient (10), the levels returning to near normal in at least some cell lines by 24 h. However, up to fivefold decreases in TTP are seen at 4 h after exposure to the drug (10). Thus, this may still be the event responsible for toxicity in vivo since the drug is given very frequently (every 4 h). Under these conditions, levels of AZT monophosphate will change dramatically because AZT is phosphorylated efficiently, but when extracellular drug is removed, the concentration of AZT monophosphate decays rapidly (seen here and in reference 10).

In contrast to this, the concentration of D4T triphosphate is increased 100-fold over a 400-fold increase in extracellular drug concentration. Even at 0.05 μM external D4T, 0.06 μM D4T triphosphate is made, a concentration which is above the 0.03 μM K_i for HIV reverse transcriptase (17). Thus, the increased antiviral effect can be explained on the basis of the

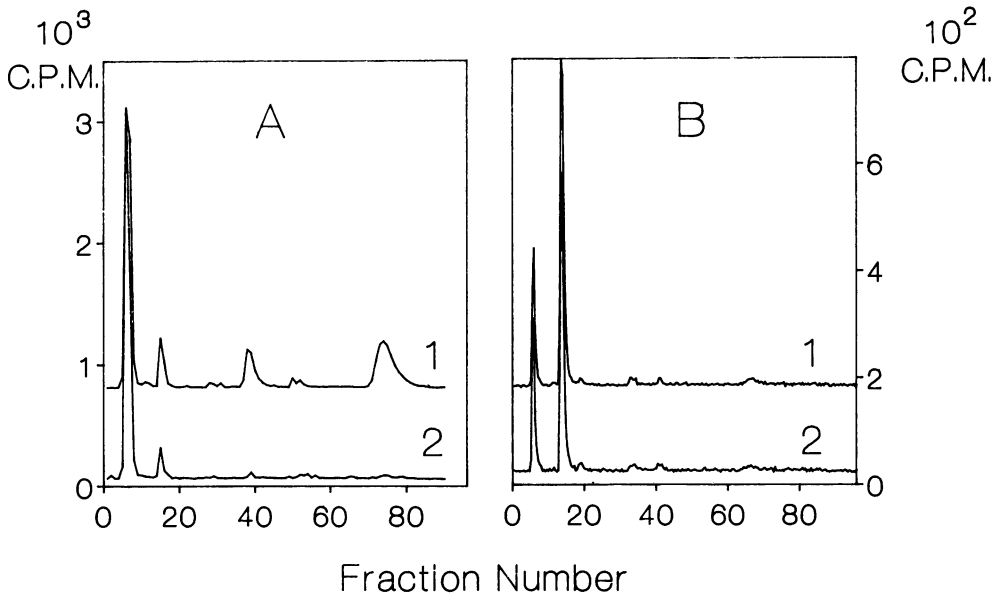

FIG. 3. Effect of AZT on phosphorylation of D4T and vice versa. Cells were incubated with 2 μM labeled drug in the presence or absence of the other unlabeled drug, also at 2 μM, for 24 h. (A) Phosphorylation of D4T in the absence (1) or presence (2) of AZT. (B) Phosphorylation of AZT in the absence (1) or presence (2) of D4T.

increase in concentration of triphosphate alone, and in fact D4T does not inhibit phosphorylation of thymidine to TTP (17). This ability to provide antiviral concentrations of D4T triphosphate concomitantly with normal levels of TTP needed to support cellular DNA synthesis may explain the lower toxicity to cells which is seen with D4T (17).

An overall rationale for the antiviral efficacy of D4T is still not clear. At concentrations of D4T (0.15 μM) and AZT (0.1 μM) which inhibit HIV replication in CEM cells (17) there is less D4T triphosphate than AZT triphosphate (Table 2). D4T triphosphate seems to have a slightly lower affinity for HIV reverse transcriptase, and D4T, unlike AZT, does not suppress the synthesis of the competing ligand, TTP (17). If only these three parameters were responsible for activity, one would not expect D4T to have antiviral activity similar to that of AZT. One possible explanation is that D4T triphosphate may be a better substrate for reverse transcriptase than is AZT triphosphate, thus causing more efficient termination of chain elongation. Its termination activity has been described, although not with HIV reverse transcriptase and not in comparison to AZT triphosphate (8).

The narrow therapeutic window of AZT in patients with acquired immunodeficiency syndrome has led many investigators to explore the potential utility of combinations of AZT with other agents. These combinations have the potential to improve the efficacy, reduce the toxicity, or both, thus leading to a wider therapeutic margin. The results of combining AZT with D4T, shown in Fig. 3, show that AZT inhibits formation of D4T phosphates whereas D4T has no effect on phosphorylation of AZT. This suggests that the two compounds together would have no better activity or toxicity profile than AZT alone, and there would be no utility from such a combination.

The biochemical differences in the metabolism of D4T compared with that of AZT described here suggest that D4T may have a different toxicity potential in humans and possibly a wider therapeutic margin.

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ADDENDUM

Intracellular production of D4T triphosphate without accumulation of monophosphate has recently been described by August et al. (1) using H-9 cells and by Perno et al. (21) using human monocyte-macrophage cells.

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