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EFFECT OF 3′-DEOXYTHYMIDIN-2′-ENE (d4T) ON NUCLEOSIDE METABOLISM IN H9 CELLS

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Abstract—The effect of 3′-deoxythymidin-2′-ene (d4T) on the metabolism of exogenously supplied radiolabeled nucleosides was investigated. Following a 24-hr exposure to 250 μM d4T, we observed no significant effect on the incorporation of [^{3}H]thymidine or [^{3}H]deoxycytidine into DNA. In contrast, the amounts of [^{3}H]uridine, [^{3}H]deoxyuridine, and [^{3}H]cytidine were significantly lower than those incorporated by control cultures. d4T had no significant effect on the incorporation of [^{3}H]uridine or [^{3}H]cytidine into RNA, or the incorporation of ^{3}H-labeled amino acids into protein. In d4T-treated cells the relative proportions of [^{3}H]dTMP, [^{3}H]dTDP, and [^{3}H]dTTP formed did not change but their absolute concentrations were increased. d4T significantly reduced the level of [^{3}H]dUMP, and a parallel decrease in [^{3}H]dTMP derived from [^{3}H]dUMP was also evident. d4T increased the amounts of labeled deoxycytidine metabolites formed, with increased dCMP levels the most prominent. In a cell-free extract, [^{3}H]d4T was phosphorylated at a rate of 1.6 pmol/30 min. Increasing concentrations of both thymidine and deoxyuridine inhibited the phosphorylation of [^{3}H]d4T with IC_{50} values of 5.7 and 35 μM respectively. d4T was found to be a weak substrate for purified H9 cytosolic thymidine kinase (K_m = 138 μM) and a weak competitive inhibitor of thymidine and deoxyuridine phosphorylation by this enzyme (K_i = 1.37 and 0.33 mM respectively).

Acquired immunodeficiency syndrome (AIDS), first recognized as a new clinical entity in 1981, is caused by infection with the retrovirus termed human immunodeficiency virus (HIV-1). Various nucleoside analogs have been found which inhibit the replication of HIV-1 in cell culture, but to date only 3′-azido-3′-deoxythymidine (AZT‡, Zidovudine) has been approved for therapeutic use in humans. Several examples of nucleoside analogs with anti HIV-1 activity include, in addition to AZT [1], 2′,3′-dideoxycytidine [2, 3], 2′,3′-dideoxyadenosine [2, 3], 2′,3′-dideoxycytidin-2′-ene [4–6], 3′-azido-2′,3′-dideoxy-5-iodouridine [7], and 5′,2-anhydro-3′-azido-3′-deoxythymidine [8].

Recent reports from this laboratory and others have shown 3′-deoxythymidin-2′-ene (d4T) to be a potent and selective inhibitor of HIV-1 replication and of cytopathic effects in a variety of mammalian cells [5, 9–10]. We have shown that d4T is relatively non-toxic to the uninfected human T-cell line H9, is phosphorylated by cellular enzymes to the mono-, di-, and triphosphate level, and ultimately is incorporated into the DNA of growing cells [11]. The 5′-triphosphate of d4T has been shown to be a potent inhibitor of HIV-1 reverse transcriptase [12].

The present report concerns our further study of the cellular pharmacology and mechanism of cytotoxicity of d4T. In particular, we have investigated the effect of d4T treatment of H9 cells on the metabolism of exogenously supplied nucleoside, as well as identifying thymidine kinase as the enzyme most likely responsible for the specific phosphorylation of d4T in H9 cells.

METHODS

Cell growth and cytotoxicity assay. H9 cells were maintained as previously described [11] and had a doubling time of 24 hr. The cytotoxic effect of d4T was assessed by measuring the inhibition of cell proliferation. Cells were suspended in medium at an initial concentration of 5×10^4 cells/mL and incubated at 37° for 72 hr in the presence or absence of 250 μM d4T. Cell number was determined daily on duplicate samples with a model ZM Coulter Counter/model 256 Channelyzer (Coulter Electronics, Hialeah, FL).

Chemicals. The radiolabeled nucleoside precursors [*methyl*-^{3}H]-thymidine (dThd), [5-^{3}H]-2′-deoxycytidine (dCyd), [5-^{3}H]-cytidine (Cyd), [6-^{3}H]-2′-deoxyuridine (dUrd), and [5,6-^{3}H]-uridine (Urd) were obtained from ICN Biomedicals (Costa Mesa, CA). [*methyl*-^{3}H]d4T (20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Non-radioactive d4T was synthesized as previously described [9]. Nucleoside mono-, di-, and triphosphates used as HPLC standards were from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were as previously described [11]. Thymidine-Sepharose affinity matrix was a gift of Dr. Y-C. Cheng.

Uptake of radiolabeled precursors into DNA and RNA of H9 cells and preparation of samples for HPLC analysis. H9 cells (5×10^4/mL) were exposed to 250 μM d4T (IC_{50}) for 23 hr and then pulse labeled

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‡ Abbreviations: AZT, 3′-azido-3′-deoxythymidine; d4T, 3′-deoxythymidin-2′-ene; dThd, thymidine; Urd, uridine; dUrd, 2′-deoxyuridine; Cyd, cytidine; dCyd, 2′-deoxycytidine; IC_{50}, that concentration of a compound resulting in 50% inhibition of the indicated process or reaction; and TCA, trichloroacetic acid.

for 60 min with the following precursors: [*methyl*-^{3}H]dThd (2.5×10^{-7} M), 5 μCi/mL; [5-^{3}H]dCyd (1.9×10^{-7} M), 5 μCi/mL; [5-^{3}H]Cyd (0.8×10^{-7} M), 2 μCi/mL; [6-^{3}H]dUrd (6.5×10^{-7} M), 10 μCi/mL; and [5,6-^{3}H]Urd (1.3×10^{-7} M), 5 μCi/mL. Following the labeling period, cells were harvested by centrifugation at 225 *g* for 12 min, washed twice with ice-cold phosphate-buffered saline (PBS) and extracted three times with 100 μL of 60% methanol/water at 0° for 30 min. The combined supernatant fractions were diluted with H_2O, lyophilized, and analyzed by HPLC. The methanol-insoluble pellet was dissolved in 100 μL of 0.3 N NaOH and incubated at 37° for 1 hr. The DNA was reprecipitated by the addition of 400 μL of ice-cold 5% trichloroacetic acid (TCA), and the pellet was collected by centrifugation at 15,000 *g* for 1 min. The supernatant fraction and an additional 200 μL 5% TCA wash were combined and designated as the "alkaline labile fraction" (RNA). The 5% TCA-insoluble fraction was dissolved in dimethyl sulfoxide and designated as the "alkaline stable fraction" (DNA). The radioactivity in an aliquot of each fraction was determined in 5 mL Optifluor (Packard Instrument Co., Downers Grove, IL).

HPLC. Methanol-soluble extracts were analyzed on an 8 mm × 10 cm Partial-SAX Radial PAK column (Waters, Milford, MA). A linear gradient of 0–90% 0.4 M $(NH_4)_2HPO_4$ (pH 5.0) into 0.01 M $(NH_4)_2HPO_4$ (pH 4.0) was developed over 50 min at 2.0 mL/min. The phosphorylated nucleoside peaks were identified by comparison of retention times with unlabeled standards or by reanalysis on an 8 mm × 10 cm μBondapak C-18 Radial-PAK column after enzymatic digestion with alkaline phosphatase and venom phosphodiesterase (1 unit each for 1 hr at 37°) to the parent nucleoside. The reversed phase system employed a 0–30% linear ingredient of acetonitrile into 0.1 M ammonium acetate (pH 5.5) developed over 35 min at 2 mL/min.

Enzyme preparations. H9 cell extracts were 10,000 *g* supernatant fractions of frozen–thawed (3 cycles) H9 cell pellets in 4 vol. of extraction buffer (10 mM Tris–HCl, pH 7.5/10 mM KCl/1 mM $MgCl_2$/1 mM dithiothreitol) [13]. Cytosolic thymidine kinase was purified by affinity chromatography over thymidine-3′ (4-amino-phenyl phosphate) coupled to carboxyhexyl-Sepharose as described by Cheng and Ostrander [14]. The enzyme was stored in elution buffer, and dThd was removed by gel filtration over Sephadex G-25 just prior to use.

Enzyme assays. Phosphorylation of tritiated d4T, dThd, and dUrd was measured by a DEAE-filter disc method as described [13]. Standard reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 5 mM ATP-Mg^{2+}, 1 μM [*methyl*-^{3}H]d4T (250 cpm/pmol), the indicated concentration of unlabeled dThd, dUrd, Cyd or dCyd, and cell extract in a total volume of 50 μL. Assays were incubated at 37° for 30 min, and terminated by spotting 35 μL onto a DE-81 filter disc. The discs were washed four times for 5 min each in H_2O, then twice in 95% ethanol, and the dried discs were counted after standing overnight in 5 mL Instafluor + 3% Soluene Tissue Solubilizer (Packard Instrument Co.).

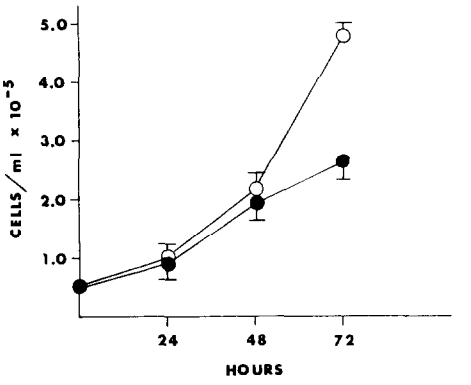

Fig. 1. Effect of 250 μM d4T on H9 cell proliferation. Cells were suspended in growth medium at an initial concentration of 5×10^4 cells/mL and incubated in the absence (○) or the presence (●) of 250 μM d4T. Cell number was determined daily on duplicate samples as described in Methods. Values are means ± SD.

Phosphorylation of [^{3}H]d4T, [^{3}H]dThd, or [^{3}H]dUrd by purified H9 thymidine kinase was measured as described above, except that the standard reaction contained: 50 mM Tris–HCl (pH 7.5), 2 mM ATP-Mg^{2+}, the indicated concentrations of radiolabeled substrate and unlabeled competing nucleoside, and enzyme in a total volume of 50 μL. The enzyme activity was proportional to enzyme concentration and time of reaction for the experiments described.

RESULTS

To elucidate the primary cytotoxic effect of d4T on cellular metabolism, experimental conditions were chosen such that the consequences of the d4T-mediated inhibition of cellular proliferation were still not evident, i.e. 5×10^4 cells/mL and 250 μM d4T for 24 hr. As shown in Fig. 1, under these experimental conditions d4T had no detectable effect on the first round cell multiplication; however, inhibition of cell growth by 20 and 50% after 2 and 3 days of treatment, respectively, was observed.

The effect of d4T on the incorporation of labeled precursors into alkaline stable (DNA) and alkaline labile (RNA) material was evaluated. As shown in Fig. 2A, following a 24-hr exposure, 250 μM d4T had no significant effect on the amount of radioactivity from labeled dThd and dCyd incorporated into DNA. On the contrary, the amounts of labeled Cyd, dUrd, and Urd were lower than those incorporated by control cultures (77, 61, and 54% of control respectively). Figure 2B shows that d4T had no significant effect on the incorporation of labeled Urd and Cyd into RNA. This suggested an interference of the drug with some step of the *de novo* biosynthetic pathways leading to the formation of dTTP and/or dCTP, but prior to the formation of dTMP or of dCMP. Furthermore, d4T did not inhibit the incorporation of ^{3}H-labeled amino acids into protein (data not shown), suggesting that the effects described above are likely a primary and not a secondary effect of d4T.

An insight into the mechanism whereby d4T

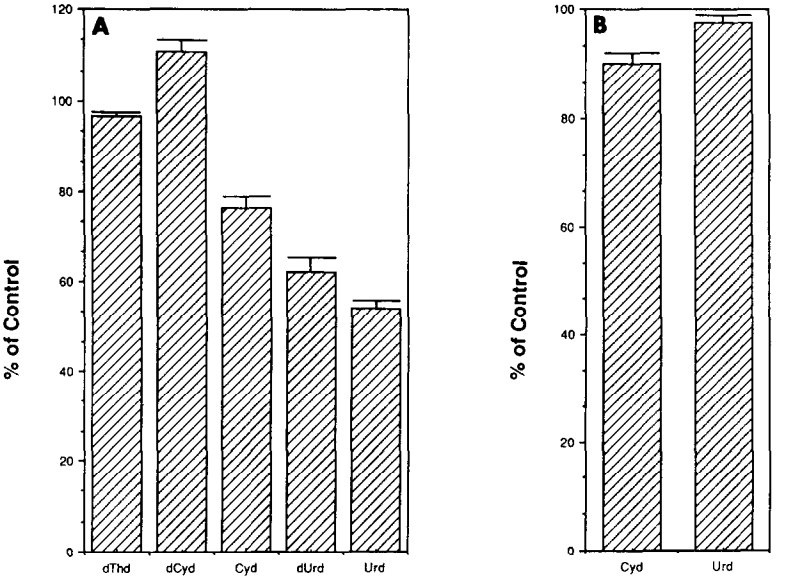

Fig. 2. Effect of d4T (250 μM) on the incorporation of ^{3}H-labeled nucleoside precursors into DNA (A) and RNA (B) of H9 cells. Cells were exposed to 250 μM d4T for 24 hr, and the radiolabeled nucleoside was added during the final hour. Alkaline stable (A) and labile (B) fractions were prepared as described in Methods. At the end of the incubation, the cells were at a density of 1.2×10^5 cells/mL in both control and drug-treated cultures. All determinations were performed on duplicate cultures, with SD as shown. Absolute values of incorporation in non-drug treated control cultures were (pmol/10^6 cells): dThd 78.5; dCyd, 29.0; Cyd (A), 5.7; dUrd, 43.3; Urd (A), 2.8; Cyd (B), 63.9; and Urd (B) 38.5.

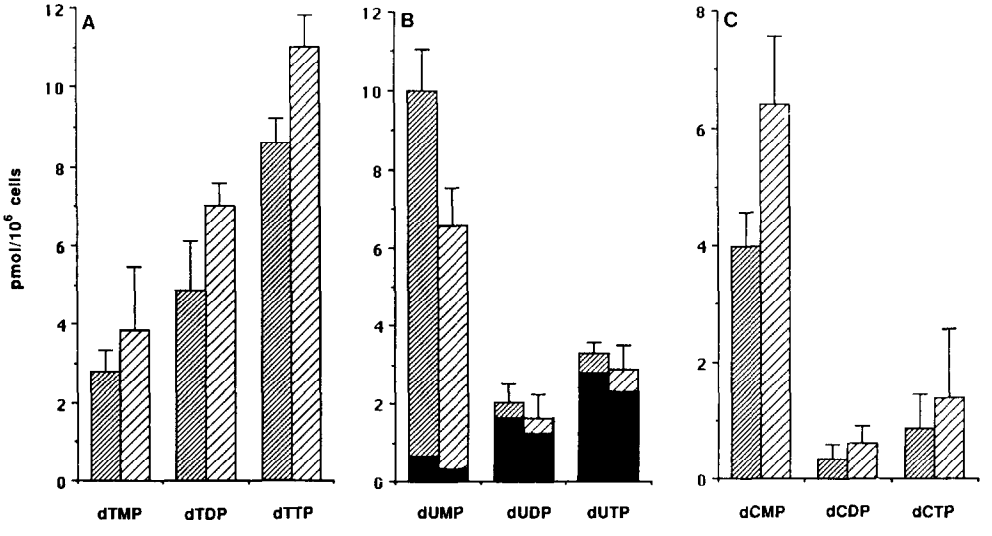

Fig. 3. Effect of d4T (250 μM) on the metabolism of [^{3}H]dThd (A), [^{3}H]dUrd (B), and [^{3}H]dCyd (C) in H9 cells. Cells were treated with 250 μM d4T as described for Fig. 2, and a 60% methanol-soluble fraction was prepared and analyzed on anion exchange HPLC as described in Methods. Data are shown as picomoles of [^{3}H]metabolite/10^6 cells in control (■) and d4T-treated cultures (▨). In Fig. 3B, the dUrd metabolites were enzymatically digested to the parent nucleoside and reanalyzed on reverse phase HPLC as described in Methods, and each bar is shaded to reflect amounts of dThd-derived radioactivity. All experiments were performed in duplicate, with SD as shown.

affects pyrimidine nucleoside metabolism was obtained by HPLC analysis of the methanol-soluble fraction of H9 cells treated with 250 μM d4T for 24 hr. As can be seen in Fig. 3A, the labeled nucleotide pools of control cultures treated with [^{3}H]dThd contained 2.8, 4.9 and 8.6 pmol of dTMP, dTDP and dTTP/10^6 cells respectively. In d4T-treated cells the relative proportions of these three labeled nucleotides (dTMP, dTDP and dTTP) did not change but their absolute concentrations were increased to 3.9, 7.0, and 11.0 pmol/10^6 cells.

The total radioactivity from [6-^{3}H]dUrd in mono-, di-, and triphosphate peaks showed a decrease in the extracts of d4T-treated cells: [^{3}H]dUMP decreased

Table 1. Phosphorylation of [^{3}H]d4T by cell-free extracts of H9 cells*

	([^{3}H]d4T phosphorylated 30 min)	% Control
d4T, 1 μM	1.6	100
d4T, 1 μM + dThd, 1 μM	1.6	100
d4T, 1 μM + dThd, 10 μM	0.06	3.8
d4T, 1 μM + dThd, 50 μM	0.04	2.5
d4T, 1 μM + dThd, 100 μM	0.03	1.9
d4T, 1 μM + dUrd, 1 μM	1.6	100
d4T, 1 μM + dUrd, 10 μM	1.5	93.8
d4T, 1 μM + dUrd, 50 μM	0.4	26.3
d4T, 1 μM + dUrd, 100 μM	0.2	13.8
d4T, 1 μM + dCyd, 100 μM	1.7	101
d4T, 1 μM + Cyd, 1 μM	1.7	101
d4T, 1 μM + Cyd, 10 μM	1.8	101
d4T, 1 μM + Cyd, 50 μM	2.0	102
d4T, 1 μM + Cyd, 100 μM	1.9	102

* H9 cell extracts were prepared as described in Methods. Assays were carried out for 30 min at 37° and terminated by spotting on DE 81 filter disks.

from 10 to 6.6 pmol/10^6 cells (Fig. 3B), and a small decrease in [^{3}H]dUDP and [^{3}H]dUTP was observed. Since the corresponding phosphorylated derivatives of dUrd and dThd were not resolved satisfactorily on the anion exchange HPLC system, each peak was then, in turn, digested to the parent nucleoside as described in Methods, and reanalyzed on reverse phase HPLC. As shown in Fig. 3B, the amount of [^{3}H]dTMP derived from [^{3}H]dUMP decreased by approximately 50% in the d4T-treated cells, paralleling the sharp decrease in total [^{3}H]dUMP. In contrast, the amounts of labeled dTDP and dTTP derived from [^{3}H]dUrd were affected only slightly by d4T; however, the proportion of dThd/dUrd in each peak remained stable relative to control. The decreased formation of [^{3}H]dTMP may be due to the decrease in the amount of [^{3}H]dUMP present, which may, in turn, be a consequence of decreased uptake or phosphorylation of [^{3}H]dUrd.

As shown in Fig. 3C, d4T increased the amounts of labeled dCyd metabolites formed from [^{3}H]dCyd. This effect was especially prominent in the level of the monophosphate, which increased 61% relative to control. A slight increase in di- and triphosphate levels was noted.

The data (Figs. 2 and 3) are compatible with a competition of d4T with dUrd either for a membrane carrier or for the enzyme responsible for dUrd phosphorylation (dThd kinase). However, we have determined recently that d4T enters the cell independent of a membrane carrier.* Thus, we investigated the ability of a cell-free extract to phosphorylate [^{3}H]d4T and the effect of dUrd (and dThd) on this activity.

In a cell-free extract of H9 cells, 1 μM [^{3}H]d4T was phosphorylated at a rate of 1.6 pmol/30 min (Table 1). Increasing concentrations of both dThd and dUrd inhibited the phosphorylation of d4T, with IC_{50} values of <10 μM and approximately 35 μM

* August EM and Prusoff WH, manuscript submitted for publication.

Table 2. Kinetic values for the interaction of d4T with purified H9 cell thymidine kinase

Substrate	K_m (μM)	V_{max} (pmol/min)	K_i (mM d4T)
dThd	1.91	1.13	1.37
dUrd	7.67	0.84	0.33
d4T	138	0.13	

respectively. Neither Cyd nor dCyd had a significant effect on d4T phosphorylation. These data suggest that the conversion of d4T to d4TMP is carried out by the cellular thymidine kinase.

Using cytosolic thymidine kinase purified from H9 cells, we observed that d4T was indeed a weak substrate for this enzyme (K_m = 138 μM, V_{max} = 0.13 pmol/min) (Table 2); and also that d4T was a weak competitive inhibitor of both dThd (Fig. 4A) and dUrd (Fig. 4B) phosphorylation by thymidine kinase (K_i = 1.37 and 0.3 mM respectively).

DISCUSSION

To elucidate the primary cytotoxic effect of d4T on cellular metabolism, H9 cells were exposed to 250 μM d4T for 24 hr. Under these conditions, d4T had no effect on the first-round of cell replication (Fig. 1), although we have shown previously that 240 μM d4T is the IC_{50} for H9 proliferation in a 72-hr growth assay.

Using a pulse label of various radiolabeled nucleoside precursors, we traced the effects of a 24-hr treatment with 250 μM d4T on the uptake, metabolism, and distribution of these exogenous nucleosides. As shown in Fig 2A, d4T had no significant effect on the uptake of [^{3}H]dThd into DNA within 24 hr, and a modest (10%) increase in the amount of dCyd incorporation into DNA was also observed.

In contrast, the amounts of labeled Cyd, Urd, and dUrd incorporation into DNA were inhibited

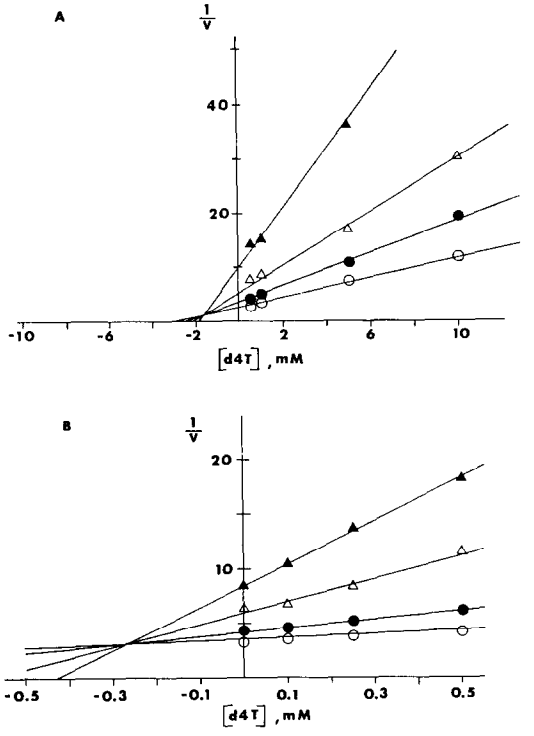

Fig. 4. Dixon plots of the effect of d4T on the phosphorylation of [^{3}H]dThd (A) or [^{3}H]dUrd (B) by purified H9 cell cytosolic thymidine kinase. The enzyme was purified by affinity chromatography and assayed as described in Methods. Velocity (v) is expressed as picomoles phosphorylated/min. Substrate concentrations were: (A) 0.25 (▲), 0.5 (△), 1.0 (●), and 2.0 μM dThd (○); (B) 1 (▲), 2.5 (△), 5 (●), and 10 μM dUrd (○). Data points represent the average of at least triplicate experiments, with triplicate assays in each experiment.

strongly by d4T to 77, 54, and 61% of control respectively. The incorporation of labeled Urd and Cyd into RNA were not affected significantly by d4T (Fig. 2B), suggesting that d4T does not interfere with the direct phosphorylation of these compounds to the corresponding 5′-ribonucleotides but rather may inhibit the conversion of Cyd and Urd (CDP and UDP) to the corresponding 2′-deoxyribonucleotides (dCDP and dUDP) by ribonucleotide reductase. The inhibition of [^{3}H]dUrd incorporated into DNA suggests an effect either on thymidylate synthetase, the transport of dUrd, or its phosphorylation by thymidine kinase.

The nucleotides present in the soluble fraction were analyzed by HPLC (Fig. 3). The absolute amounts of [^{3}H]dThd metabolites increased in response to d4T. Similarly, an increase in the intracellular level of [^{3}H]dTTP as well as the ratio of labeled dTMP/(dTDP + dTTP) upon exposure of MT-4 cells to 500 μM d4T has been reported [15]. This may be an early effect of the slowing of DNA synthesis. An increase in cellular dTTP upon inhibition of DNA synthesis was also found by Chen *et al* [16], in a study of the mechanism of action of 3′-amino-3′-deoxythymidine. The amino nucleoside analog was phosphorylated to the triphosphate but not incorporated into DNA, and the consequence was a decrease in the uptake of [^{14}C]dThd into the DNA of L1210 cells and an 88% increase in the pool of dTTP. Also, Furman *et al.* [17] showed that dGTP and dATP pool levels in HSV-1 infected cells treated with acyclovir increased dramatically compared to uninfected cells. These increases were due to either an inhibition of viral DNA polymerase activity or a terminal addition of acyclovir to the growing DNA chain, or both, which resulted in reduced utilization of the dNTPs.

The overall level of [^{3}H]dUMP, but not that of di- and triphosphates showed a sharp decrease in the d4T-treated cells (Fig. 3B). Upon enzymatic digestion and rechromatography to identify the parent nucleosides, we found that, although the absolute amounts of [^{3}H]dThd metabolites derived from [^{3}H]dUrd decreased in d4T-treated cells, the proportion of each peak corresponding to dThd was unchanged by d4T (i.e. the percentage of dThd was 6.6 and 5.5% of the total monophosphate peak in control and d4T-treated cells, 79.8 and 77.5% of the diphosphate, and 83.3 and 80.1% of the triphosphate respectively). This suggests that d4T does not affect the conversion of dUMP to dTMP catalyzed by thymidylate synthetase.

The metabolism of [^{3}H]dCyd was increased in d4T-treated cells (Fig. 3C), which agrees with the increase in the amount of labeled dCyd incorporated into DNA (Fig. 2). If, as suggested above, d4T (or one of its phosphorylated forms) inhibits the activity of ribonucleotide reductase, then the observed increase in phosphorylated labeled dCyd may be a result of lowering of "competing" (nonradiolabeled) dCDP derived from CDP through this enzyme. Frick *et al.* [18] reported an increase in the endogenous dCTP pools in H9 cells treated with 200 μM AZT. They attributed this rise to a transient decrease in dTTP pools which allosterically enhances the reduction of CDP to dCDP by ribonucleotide reductase. Effector studies of d4T and d4TTP with purified ribonucleotide reductase are currently in progress.

In a cell-free extract of H9 cells, 1 μM [^{3}H]d4T was phosphorylated at a rate of 1.6 pmol/30 min (Table 1). To elucidate the enzyme responsible, the ability of exogenous natural nucleosides to inhibit d4T phosphorylation was investigated. Only dThd and dUrd inhibited d4T phosphorylation (IC_{50} values of 5.7 and 35 μM respectively), indicating that thymidine kinase is the probable primary enzyme responsible for this phosphorylation. This is in agreement with similar observations made in MT-4 cells [15] and CEM cells [19], where dThd suppressed phosphorylation of d4T.

Using purified H9 cytosolic thymidine kinase, we observed that d4T is a weak substrate for this enzyme (K_m = 138 μM) with a V_{max} one-tenth of dThd. d4T was found also to be a weak competitive inhibitor of both dThd and dUrd phosphorylation by thymidine kinase (K_i = 1.37 and 0.33 mM respectively). Similar values have been reported by Balzarini *et al.* [15] in MT-4 cell extracts and Ho and Hitchcock [19] using CEM cell-thymidine kinase. Due to this high K_m, and in light of a recent report of phosphorylation of d4T in Raji/TK$^-$ cells [14], the possibility that other cellular enzymes may also contribute to the overall phosphorylation of d4T is currently under investigation.

We previously reported that d4T accumulates in H9 cells in a concentration-dependent manner [11], and that there is a considerably large intracellular pool of unphosphorylated nucleoside present. In light of this observation, the decrease in dUrd incorporation into DNA, as well as the decrease in formation of dUrd metabolites, are most likely a result of inhibition at the level of dUrd phosphorylation by thymidine kinase, rather than an inhibition of dUrd transport.

We have shown that the major target of d4T disruption of nucleoside metabolism appears to be an effect on ribonucleotide reductase. In contrast to previous reports that AZT depresses TTP pools [13, 15], we and Balzarini *et al.* [15] find no evidence for such disruption of cellular metabolism by d4T. This is especially significant, in that the concentration of d4T evaluated (250 μM) is some 200-fold greater than that required for antiviral activity.

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