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Metabolism and DNA Interaction of 2′,3′-Didehydro-2′,3′-dideoxythymidine in Human Bone Marrow Cells

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SUMMARY

2′,3′-Didehydro-2′,3′-dideoxythymidine (D4T) is a potent inhibitor of human immunodeficiency virus (HIV), with low hematological toxicity. In the present study, the cellular pharmacology of D4T was investigated in human bone marrow cells (BMC), in an attempt to understand the mechanism of the observed low bone marrow toxicity. After exposure of human BMC to 10 μM [^{3}H] D4T for 24 hr, D4T-5′-triphosphate (D4T-TP) was the predominant metabolite, reaching a concentration of 0.3 pmol/10^6 cells. The D4T-5′-monophosphate levels were slightly lower, whereas the D4T-5′-diphosphate levels were about 6-fold lower than those of D4T-TP at 24 hr. Nucleic acids of human BMC exposed to 10 μM [^{3}H]D4T for 24 hr were purified and analyzed by cesium sulfate density gradient centrifugation. No radioactivity was detected in the RNA region, whereas a limited amount was associated with the DNA region. The amount of label incorporated into DNA correlated with the extracellular D4T concentration and the length of incubation time. Enzymatic hydrolysis of radiolabeled DNA and subsequent analysis by high performance liquid chromatography demonstrated incorporation of both D4T and thymidine (dThd) into DNA. Degradation of D4T to thymine and subsequent formation of labeled dThd was also detected in human BMC. Pulse (24 hr)-chase (48 hr) experiments with 10 μM [^{3}H]D4T demonstrated that the amount of radiolabel from D4T in DNA decreased over time during the chase. Under similar conditions, [^{3}H]3′-azido-3′-deoxythymidine (AZT) incorporated into DNA of human BMC did not decrease during the chase. Although D4T-TP standard was demonstrated to be unstable at 37° and neutral pH, D4T was much more stable in solution when incorporated into newly synthesized DNA isolated from human BMC, suggesting that enzymatic excision may be the mechanism for D4T removal from DNA. In summary, although higher concentrations of D4T-TP, compared with AZT-5′-triphosphate, are observed in human BMC, after exposure of cells to similar extracellular concentrations of parent drug, steady state levels of D4T incorporated into DNA are 10–50-fold lower, compared with AZT. Competition with dTTP formed by D4T metabolism and excision of D4T from DNA may be responsible, in part, for these effects. This study further demonstrates that incorporation of 2′,3′-dideoxynucleosides into nuclear DNA of human BMC may be related to the ability of these anti-HIV agents to induce hematological side effects.

Development of anti-HIV agents with a high degree of selectivity represents a major approach to preventing and/or treating AIDS. AZT is currently the only clinically approved drug for the treatment of AIDS, but its use in patients has been hampered by its severe hematological toxicity (1–3). Studies over the past years have demonstrated the importance of the intracellular metabolism of AZT and how this metabolism plays a major role in both the antiviral and the cytotoxic effects of AZT in host cells (4–10).

D4T is a novel 2′,3′-dideoxynucleoside that has been shown in several *in vitro* systems to inhibit HIV replication, at concentrations below 0.01 μM, with limited host cell toxicity (11, 12). In particular, we demonstrated that D4T had IC_{50} values for human CFU-GM and human erythroid burst-forming units 100- and 10-fold, respectively, higher than those of AZT (13); thus, D4T was predicted to show less bone marrow suppression.

That prediction proved accurate in animal studies (14) and phase I clinical trials (15–17). In patients, following administration of D4T at doses up to 4 mg/kg/day, preliminary indications of efficacy with reduced p24 antigenemia were observed, without substantial bone marrow toxicity (15–17). However, an

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ABBREVIATIONS: HIV, human immunodeficiency virus; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; D4T-MP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-monophosphate; D4T-DP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-diphosphate; D4T-TP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine 5′-monophosphate; AZT-TP, 3′-azido-3′-deoxythymidine 5′-triphosphate; dThd, thymidine; BMC, bone marrow cells; AIDS, acquired immune deficiency syndrome; HPLC, high performance liquid chromatography; CFU-GM, colony-forming unit granulocyte-macrophage; TCA, tricholoracetic acid.

unpredicted peripheral neuropathy, already reported with 2′,3′-dideoxycytidine and 2′,3′-dideoxyinosine, was the major limiting toxicity (15–17). Research on the mechanism(s) by which these dideoxynucleosides, including D4T, induce this neurotoxicity suggests that their effects on mitochondrial DNA synthesis may play a role in the observed neuropathies (18, 19). The absence of D4T-induced myelosuppression is particularly interesting, because other dThd analogs, including 3′-fluoro-3′-deoxythymidine, AZT, and its recently identified metabolite 3′-amino-3′-deoxythymidine, have been demonstrated to be highly toxic for human BMC (20, 21). Previous studies have suggested that differences in metabolism of D4T and AZT may be responsible for the decreased host toxicity (8, 22). AZT, unlike D4T, accumulates as its 5′-monophosphate derivative within cells, and these high intracellular AZT-MP levels may lead to inhibition of dTMP kinase. This, in turn, results in reduction of dTTP pools (8, 23), a mechanism that has not been observed with D4T (8, 24). However, our recent studies using human BMC and a pharmacologically relevant concentration of 10 μM AZT demonstrated that imbalance of deoxyribonucleotide pools was not a critical factor in AZT inhibition of DNA synthesis (4). These data were also confirmed by several other groups using different cell lines and experimental procedures (5, 7, 10, 25). In contrast, although AZT is a poor substrate for human DNA polymerase α and δ (25–27), substantial amounts of that drug are incorporated into nuclear DNA (4, 25, 28), and this biochemical event has been demonstrated by us to correlate, to some degree, with inhibition by AZT of CFU-GM colony formation (4). The decreased toxicity of D4T in that system may, thus, result from a low steady state level of D4T incorporation into host cellular DNA, possibly subsequent to poor phosphorylation of D4T in human BMC and/or a different interaction of D4T with DNA, compared with AZT.

Therefore, in the present study we evaluated the intracellular metabolism and incorporation into nucleic acids of D4T in human BMC. The pattern of D4T phosphorylation to its 5′-phosphate derivatives was similar to that observed in other human primary cells (29) or established cell lines (8, 22, 30). Of note, degradation of D4T to thymine and subsequent formation of dThd was demonstrated within cells, consistent with the detection of labeled dThd in DNA of cells that had been exposed to labeled D4T. Steady state levels of D4T incorporated into DNA were 10–50-fold lower, compared with AZT, after exposure of cells to similar concentrations. Removal of D4T from DNA was detected under conditions in which no excision of AZT was detected.

Materials and Methods

Chemicals. [*methyl*-^{3}H]D4T (20 Ci/mmol), [*methyl*-^{3}H]AZT (11 Ci/mmol), and [*methyl*-^{3}H]dThd (65 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). The purity of all radiolabeled compounds used was >99%, as ascertained by HPLC techniques described below. Nonlabeled standard AZT was a generous gift of Dr. Raymond F. Schinazi (Veterans Administration Medical Center and Emory University, Atlanta, GA). D4T and its nucleotides were chemically synthesized as previously described (12). Nucleosides, RNase A and T_1, proteinase K, alkaline phosphatase, and snake venom and spleen phosphodiesterase were obtained from Sigma Chemical Co. (St. Louis, MO). Micrococcal nuclease was purchased from Worthington Biochemicals (Fairfield, NJ). All other chemicals and reagents were of the highest analytical grade available.

Cells and extraction of intracellular nucleotides. Human BMC were obtained by procedures described in detail by us previously (4). Cell viability, as determined by trypan blue dye exclusion, was 95% or greater during experiments. Cells (2×10^6 cells/ml) were suspended in McCoy's 5A nutrient medium supplemented with 15% dialyzed heat-inactivated fetal bovine serum. After addition of various concentrations of D4T, cells were maintained at 37° under an atmosphere of 5% CO_2, for specified time periods. Cells were then pelleted at 1200 rpm for 10 min, in a Beckman GPR centrifuge, and were washed three times with 5 ml of cold phosphate-buffered saline. Nucleotides present in the cell pellet were extracted by incubation overnight at −20° with 1 ml of 60% methanol and were then extracted with 200 μl of 60% methanol for 30 min in an ice bath. Combined extracts were dried under a gentle nitrogen stream at room temperature, and the samples were stored at −20° until analysis.

HPLC methods. Separation of nucleotides was performed on a Hewlett-Packard 1090 HPLC system equipped with automatic injector, filter spectrophotometric detector, and chromatographic terminal (Hewlett Packard 3393A). Analysis of D4T and its 5′-phosphorylated derivatives, and cell extracts containing them, was performed using a Partisil 10 SAX column (Whatman, Inc., Clifton, NJ) as stationary phase. Elution was carried out at 1 ml/min with 15 mM KH_2PO_4 (pH 3.5) and a 45-min linear gradient of 1 M KH_2PO_4 (pH 3.5) from 0 to 100%, starting 10 min after the time of injection. Under the conditions defined above, the retention times of the unlabeled markers D4T, D4T-MP, and D4T-TP were 7, 14, and 54 min, respectively. The assignment of D4T-DP at 33 min was assumed from its position between the mono- and triphosphate derivatives and its dephosphorylation to D4T by enzymatic hydrolysis with alkaline phosphatase, as previously described (22). For sample analysis, dried residues were dissolved in 250 μl of distilled water, and an aliquot (180 μl) was injected. After fractionation (1 ml) of the eluate and addition of scintillation fluor (5 ml), radioactivity was measured by using a Beckman LS 5000TA scintillation counter equipped with an automatic quench correction program. Reverse phase chromatography was used to separate D4T or AZT from thymine and dThd. Extracts were examined using a Hypersil 5-μm column (Jones Chromatography, Littleton, CO) as stationary phase. Elution was carried out at 1 ml/min with 25 mM ammonium phosphate (pH 7.2) and a 35-min linear gradient of 60% methanol from 0 to 30%, starting at the time of injection. The retention times of authentic standards of thymine, dThd, D4T, and AZT were 11, 21, 27, and 35 min, respectively.

Incorporation of D4T into nucleic acids of human BMC. To examine D4T incorporation into DNA, cells (2×10^6 cells/ml) were incubated at 37° for 24 hr after addition of various concentrations of [^{3}H]D4T, from 1 to 25 μM, with a final specific activity of approximately 800 mCi/mmol. DNA was extracted by phenol/chloroform and proteinase K and RNase A and T_1 digestion procedures, as previously described (4). The amount of DNA in each sample was determined by a fluorometry technique that includes binding of bisbenzimidazole to DNA (31). Samples (100 μl) were then spotted on Whatman 3MM filter paper, and filters were washed twice with ice-cold 5% TCA, followed by 100% methanol. Filters were dried at room temperature, and radioactivity was determined in a Beckman LS 5000 TA scintillation counter.

Cesium sulfate density gradient centrifugation. Cellular nucleic acids were extracted and analyzed by cesium sulfate gradient centrifugation, as described previously (4).

Enzymatic digestion of ^{3}H-labeled DNA. After cesium sulfate density gradient centrifugation, fractions with a density of 1.41–1.43 g/ml were pooled and dialyzed overnight against 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, after which DNA was precipitated in 0.3 M sodium acetate and ice-cold absolute ethanol. DNA was then dissolved in 20 μl of Tris·HCl (pH 7.4), 1 mM EDTA, and heat denatured by boiling at 100° for 5 min. The DNA was hydrolyzed at 37° for 30 min with 50 μl of micrococcal nuclease (12 units/ml), in 10 mM Tris·HCl (pH 8.8), 2 mM $CaCl_2$. After addition of a mixture containing 1 μl of 50 mM EDTA, 3 μl of 1 M sodium acetate buffer (pH 6.5), and 3 μl of 0.1 M $MgCl_2$,

degradation to nucleosides was carried out at 37° for 24 hr, with snake venom 5′-phosphodiesterase (2 units), spleen 3′-phosphodiesterase (2 units), and alkaline phosphatase (0.3 units). The digested material was then analyzed by the reverse phase HPLC method described above.

Hydrolysis of DNA to nucleoside monophosphates was carried out at 37° by sequential action of micrococcal nuclease (12 units/ml) and spleen 3′-phosphodiesterase (2 units). The fraction containing the radioactive nucleoside monophosphates was analyzed by anion exchange HPLC technique, except that a different elution mode was used. Elution was performed isocratically at 0.5 ml/min with 15 mM K_2HPO_4 (pH 3.5) for 20 min, followed by a 45-min linear gradient of 1 M K_2HPO_4 (pH 3.5) from 0 to 100%. The retention times for dThd 5′-monophosphate, dThd 3′-monophosphate, and D4T-MP were 15, 18, and 21 min, respectively.

Pulse-chase labeling of DNA with ^{3}H-nucleosides. Human BMC were exposed to either 10 μM [^{3}H]D4T (360 mCi/mmol), 10 μM [^{3}H]AZT (360 mCi/mmol), or 10 μM [^{3}H]dThd (360 mCi/mmol), for 24 hr at 37°. After the 24-hr pulse, the radioactive medium was removed and the cells were washed with a fresh phosphate-buffered saline solution at 0°. The cells were then chased for 0, 6, 24, and 48 hr at 37°, in McCoy's 5A nutrient medium containing 10 μM (nonlabeled) levels of the corresponding nucleoside or nucleoside analog being investigated. The chase was terminated by pelleting of cells and placement in an ice-cold water bath. Radioactivity was then analyzed in total DNA, following procedures described above.

Degradation of D4T by cell extracts. Catabolic conversion of D4T to thymine was investigated in human BMC using a modified radioisotopic assay (32). Human BMC (80 × 10^6 cells/ml) were lysed by two cycles of 30-sec sonic oscillation at 0°, in 0.5 ml of 2 mM potassium phosphate (pH 7.4). The reaction consisted of either 2 μM [^{3}H]D4T (450 mCi/mmol), 2 μM [^{3}H]AZT (450 mCi/mmol), or 2 μM [^{3}H]dThd (450 mCi/mmol), in 100 mM Tris·HCl (pH 6.0), 12.5 mM potassium phosphate (pH 6.0), with cell extract (60 μl), in a final volume of 150 μl. Boiled cell extracts were used as negative control. The reaction was performed at 37° for specified time periods. Aliquots (25 μl) were extracted overnight at −20° with cold 60° methanol. Samples were dried and injected into HPLC using the reverse phase method described above, which resolves thymine, dThd, D4T, and AZT.

Stability of labeled D4T in cellular DNA. Cells (2 × 10^6 cells/ml) were incubated for 24 hr with 10 μM [^{3}H]D4T, at a final specific activity of 450 mCi/mmol, and DNA was purified as described above. The DNA extracted from 20 × 10^6 cells was dissolved in 0.5 ml of a buffer containing Tris·HCl (pH 7.4) and 1 mM EDTA, to which was added 1.5 ml of McCoy's 5A nutrient medium. Mixtures were incubated at 37° for times between 0 and 48 hr. Aliquots (0.5 ml) were spotted on Whatman 3MM discs, and radioactivity was determined after treatment with TCA and methanol as described above. Stability of D4T-TP was also investigated in McCoy's 5A nutrient medium, at similar time intervals between 0 and 48 hr. D4T-TP concentrations were determined using the anion exchange HPLC technique described above.

Results

Metabolism of D4T in human BMC. After exposure of human BMC to 10 μM [^{3}H]D4T, D4T was rapidly phosphorylated within cells to its 5′-mono-, 5′-di-, and 5′-triphosphate derivatives, as revealed by anion exchange chromatography (Fig. 1A). The intracellular concentrations of D4T and its 5′-phosphate metabolites after 2-, 6-, 24-, and 48-hr incubation periods are illustrated in Table 1. A continuous increase in D4T-MP, D4T-DP, and D4T-TP levels was observed between 2 and 24 hr, and an equilibrium was maintained for the remaining 24 hr of the experiment. We previously demonstrated that the percentage of BMC in S phase did not change over the first 24 hr (4), thus demonstrating that the increase in D4T phosphorylated metabolites is not the result of an increased activity of dThd kinase, the enzyme proposed to be responsible in D4T activation (22, 24). By 24 hr, D4T-TP was the predominant intracellular metabolite and reached a mean concentration of 0.3 pmol/10^6 cells. The earlier eluting fraction (Fig. 1A)

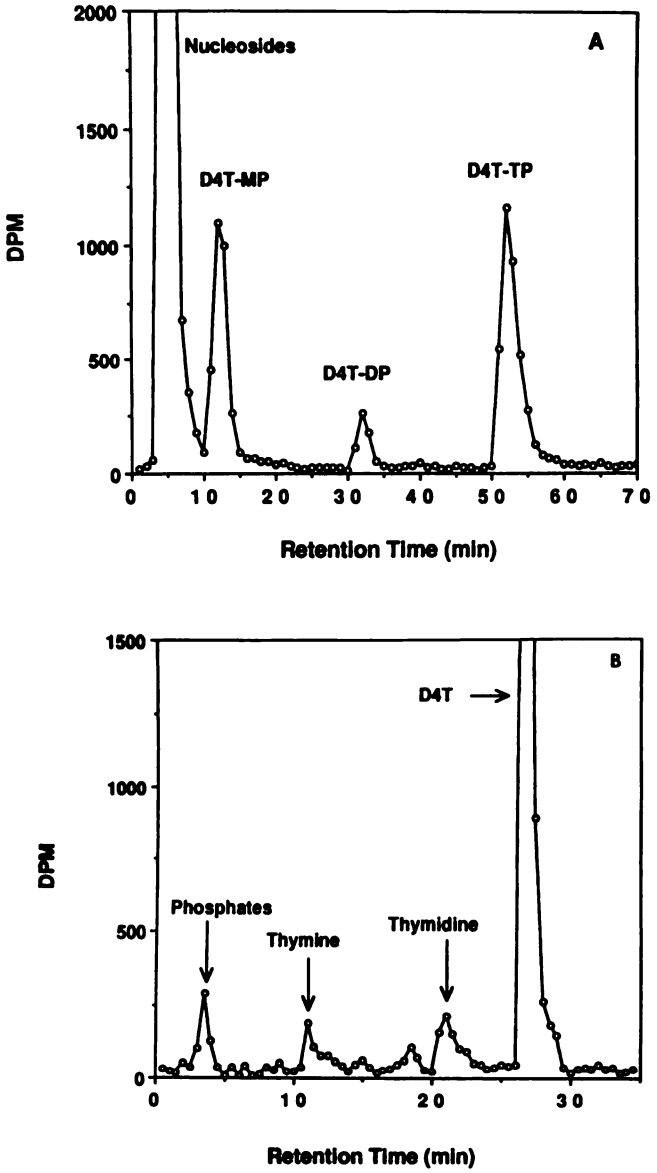

Fig. 1. Radiochromatogram of intracellular ^{3}H after exposure of human BMC for 24 hr to 10 μM [^{3}H]D4T and analysis by anion exchange HPLC (A) or analysis by reverse phase HPLC (B). Elution positions of authentic standards were monitored at 254 nm.

TABLE 1

Metabolism of 10 μM [^{3}H]D4T in human BMC

Values are mean ± standard deviation of at least three experiments, with cells from different donors.

Time of exposure	Metabolite		
	D4T-MP	D4T-DP	D4T-TP
hr	*pmol/10^6 cells*		
2	0.129 ± 0.092	0.011 ± 0.007	0.043 ± 0.033
6	0.128 ± 0.047	0.019 ± 0.011	0.084 ± 0.062
24	0.267 ± 0.055	0.049 ± 0.042	0.300 ± 0.163
48	0.189 ± 0.087	0.052 ± 0.035	0.260 ± 0.095

coeluted with an authentic D4T standard and represented an average of 80–85% of the total intracellular radioactivity at every time point of sampling. Further analysis by reverse phase chromatography revealed that approximately 14% of that fraction actually represented thymine and dThd and only 80% was D4T (Fig. 1B). Because D4T used in these experiments was >98% pure, these data suggest that D4T may be cleaved within cells to release labeled thymine, with subsequent conversion to labeled dThd.

The extent of degradation of D4T was assessed by incubating 2 μM [^{3}H]D4T with human BMC extracts, as described in Materials and Methods. Parallel studies were performed with similar concentrations of [^{3}H]AZT and [^{3}H]dThd. After 48 hr of incubation and analysis by reverse phase HPLC, D4T represented only 69% of the total radioactivity, whereas thymine and dThd accounted for 25.5 and 3.5%, respectively. No substantial degradation of D4T was detected in control boiled cell extracts. Under similar conditions, dThd was completely converted to thymine, whereas AZT was very stable, with >95% of radioactivity being still associated with the parent drug. These data confirm that dThd phosphorylase activity is present in human BMC (33, 34) and indicate that D4T is probably a substrate for that enzyme. When purified dThd phosphorylase derived from *Escherichia coli* was used, D4T was also found to be a substrate for that enzyme.[1] In contrast, AZT was not degraded by bacterial dThd phosphorylase,[1] as suggested by cellular studies described above.

Analysis of [^{3}H]D4T incorporation into nucleic acids. In order to evaluate the interaction of D4T with host cell nucleic acids, human BMC were exposed for 24 hr to various concentrations of [^{3}H]D4T, from 1 to 25 μM, and extracted nucleic acids were partitioned by cesium sulfate density gradient centrifugation, as described in Materials and Methods. No radioactivity was detected in the RNA region, whereas a substantial amount of tritium label was associated with the DNA region (density, 1.42 g/ml) (Fig. 2). To determine quantitatively the amount of ^{3}H incorporated into DNA, as a function of extracellular D4T concentration and time of exposure, radioactivity was measured in purified DNA using a 'disk filter' assay, as previously described (4). As illustrated in Fig. 3, the amount of tritium incorporated into DNA correlated with the extracellular concentrations of [^{3}H]D4T used in the experiment (from 1 to 25 μM), and the amount of tritium associated with cellular DNA also increased from 0 to 24 hr, when a concentration of 10 μM [^{3}H]D4T was used.

Identification of ^{3}H detected in cellular DNA. Identification of the radioactivity detected in DNA was performed by enzymatically hydrolyzing the DNA to nucleosides or nucleoside monophosphates, with subsequent HPLC analysis. When the DNA was digested with micrococcal nuclease, snake venom and spleen phosphodiesterase, and alkaline phosphatase, two radioactive peaks, which coeluted with dThd and D4T standards, were detected by reverse phase HPLC analysis (Fig. 4). The ratio of D4T to dThd was approximately 1 to 3. To confirm the identification of D4T and dThd as the sole radioactive entities detected in DNA, another digestion strategy was used.

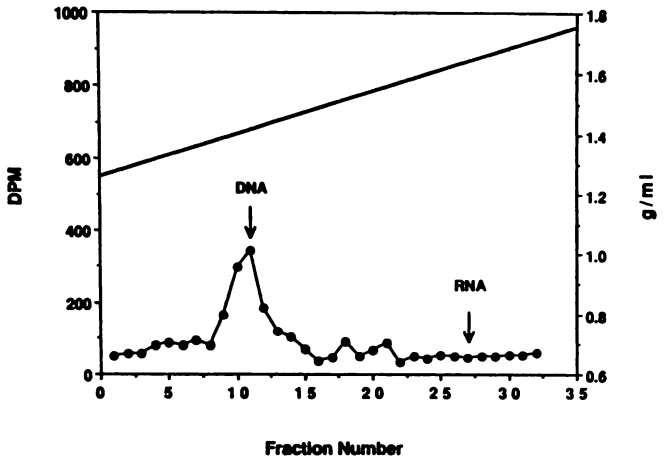

Fig. 2. Cesium sulfate density gradient centrifugation of [^{3}H]D4T-radiolabeled nucleic acids. After centrifugation of purified nucleic acids, fractions of 200 μl were collected from top to bottom and 100-μl samples were spotted on Whatman 3-mm filter paper. Filters were washed and dried, and radioactivity was determined.

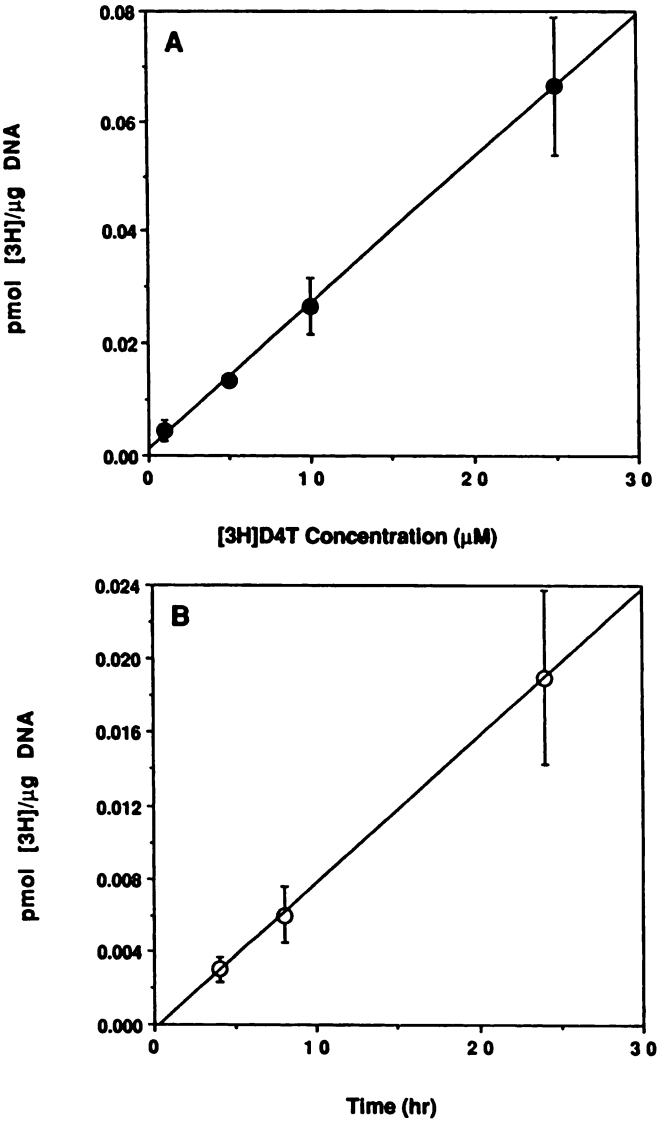

Fig. 3. Incorporation of radioactivity into DNA after incubation of cells for 24 hr with increasing concentrations of [^{3}H]D4T (A) and after incubation of cells with 10 μM [^{3}H]D4T for varying time periods (B) Values are the mean ± standard error of three experiments, with cells from different donors.

[1] R. F. Schinazi and J. P. Sommadossi, unpublished data.

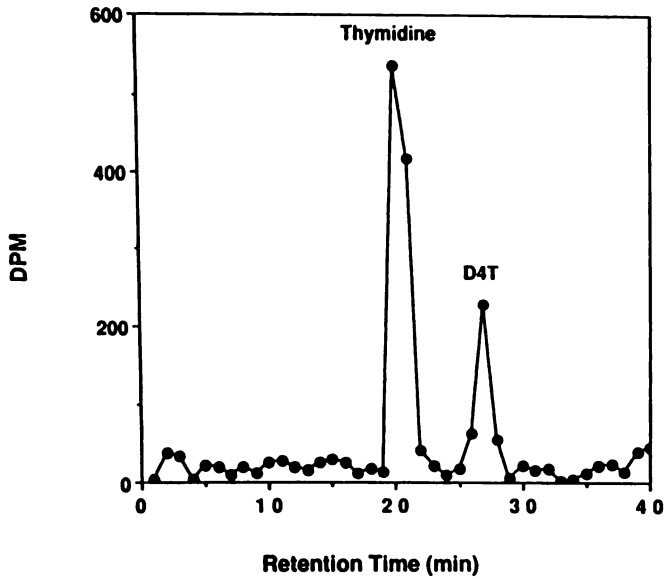

Fig. 4. Reverse phase HPLC analysis of ^{3}H-labeled digested DNA isolated from cells exposed to 10 μM [^{3}H]D4T for 24 hr. DNA digestion was performed by the sequential addition of micrococcal nuclease, snake venom and spleen phosphodiesterase, and alkaline phosphatase.

Briefly, after micrococcal nuclease digestion, only spleen 3′-phosphodiesterase was added to the reaction solution. This enzyme cleaves 5′-ester bonds and leaves the 3′-nucleoside monophosphates intact. Thus, under these conditions, nucleotides that are incorporated at the terminus of the DNA chain and have no 3′-hydroxyl group will be converted to their corresponding nucleosides rather than their 3′-monophosphates. Fig. 5 shows the radiochromatogram of the DNA digest after exposure of cells, for 24 hr, to either 10 μM [^{3}H]dThd or 10 μM [^{3}H]D4T. The dThd 3′-monophosphate represented the major radioactive component of the newly synthesized DNA after exposure to [^{3}H]dThd, consistent with the natural pyrimidine being localized in the intranucleotide linkage. In contrast, digestion of DNA isolated from cells treated with [^{3}H]D4T led to the detection of a portion of radioactivity eluting as a deoxyribonucleoside derivative and a major fraction corresponding to the dThd 3′-monophosphate region. These data confirm that labeled D4T and dThd are both present in newly synthesized DNA after exposure of cells to [^{3}H]D4T and demonstrate, as expected, that D4T accumulates at the chain termini of host cell DNA. Of note, the ratio of D4T to dThd was similar to that described above when different DNA hydrolysis and HPLC analysis were performed.

Removal of D4T from newly synthesized DNA in human BMC. In order to gain insight into potential mechanisms responsible for the rather small amount of D4T detected in cellular DNA of human BMC, compared with AZT (4), we investigated whether D4T could be removed from DNA of human BMC. Cells were incubated for 24 hr with either 10 μM [^{3}H]D4T or [^{3}H]AZT, and the label was then chased as described in Materials and Methods. Control experiments were also performed by incubating cells with [^{3}H]dThd. Fig. 6 illustrates the tritium remaining in DNA over the 48-hr chase in the presence of the corresponding nonlabeled compound. In control as well as in AZT experiments, no decrease of radioactivity in DNA was observed over the 48-hr chase period. In contrast, over this same interval, approximately 30–40% of the tritium in total DNA from D4T-treated cells was removed.

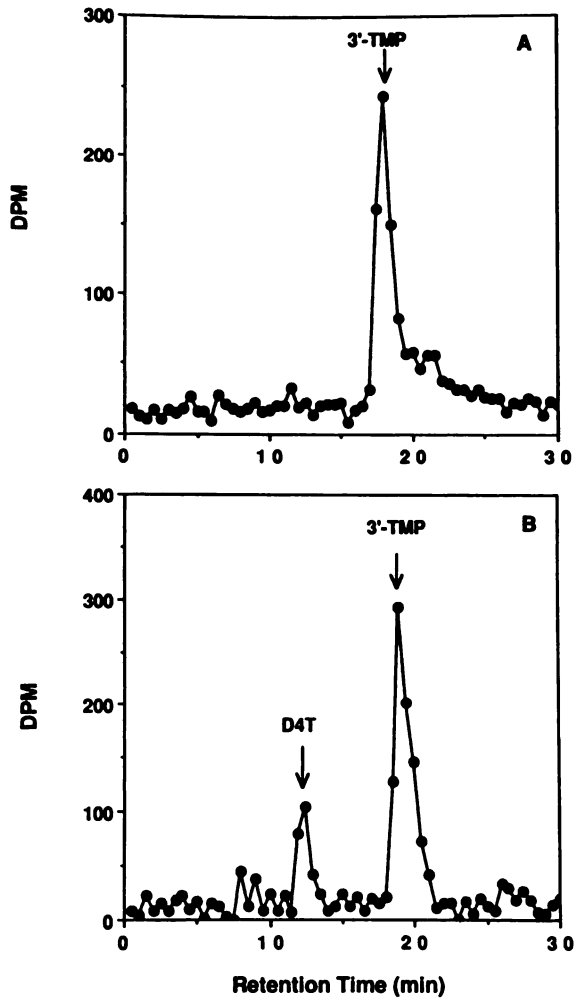

Fig. 5. Anion-exchange HPLC analysis of ^{3}H-labeled digested DNA isolated from cells exposed to either 10 μM [^{3}H]dThd (A) or 10 μM [^{3}H]D4T (B) for 24 hr. Purified DNA was digested by micrococcal nuclease and spleen 3′-phosphodiesterase.

Chemical stability of D4T-TP and ^{3}H-labeled D4T in cellular DNA. When D4T-TP, at a final concentration of 500 μM, was incubated at 37° and neutral pH in either McCoy's 5A nutrient medium or phosphate-buffered saline solution, a time-dependent degradation was observed, with approximately 60% of the initial concentration remaining after 48 hr of incubation (Fig. 7). Because this chemical degradation of D4T-TP was in a similar range, compared with the extent of D4T removal from DNA, as described above, it was important to determine the stability of D4T in newly synthesized DNA. After incubation of cells with 10 μM [^{3}H]D4T for 24 hr, DNA was purified and incubated at 37°, in McCoy's 5A nutrient medium, for time periods between 6 and 48 hr. At the specified times, aliquots were removed and spotted on disk filters, and radioactivity was determined after treatment with TCA and methanol. Of importance, the amount of radioactivity in DNA was not substantially altered over the 48-hr time of incubation, demonstrating that, once incorporated into cellular DNA, D4T as the D4T-MP form is chemically stable. Therefore, enzymatic excision, rather than chemical instability, of D4T from DNA of human BMC is a more likely mechanism for the observed removal of D4T from newly synthesized DNA.

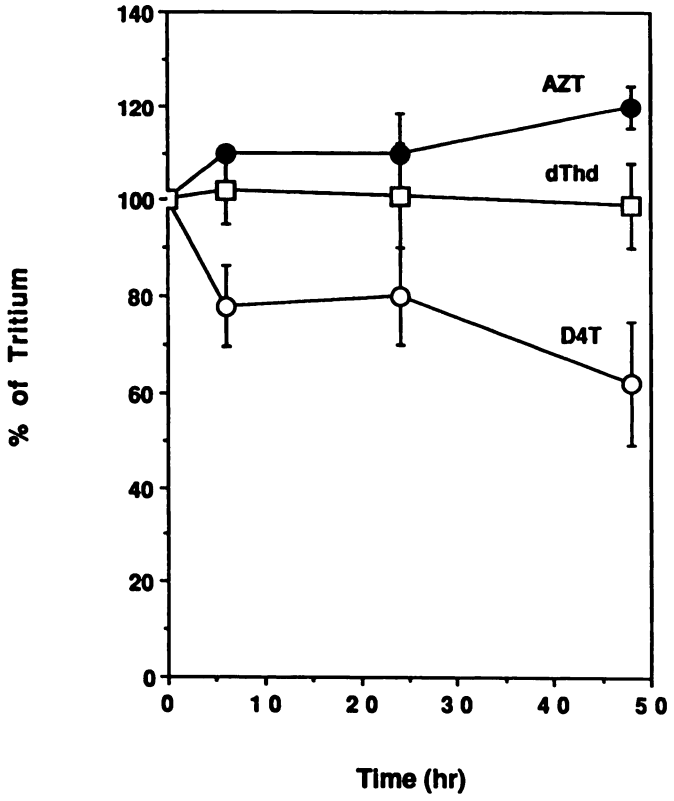

Fig. 6. ^{3}H removal from DNA over a 48-hr chase after a 24-hr exposure of cells to either 10 μM [^{3}H]D4T (○), 10 μM [^{3}H]dThd (□), or 10 μM [^{3}H] AZT (●). DNA was purified as described in Materials and Methods. Values are the mean ± standard error of three experiments, with cells from different donors.

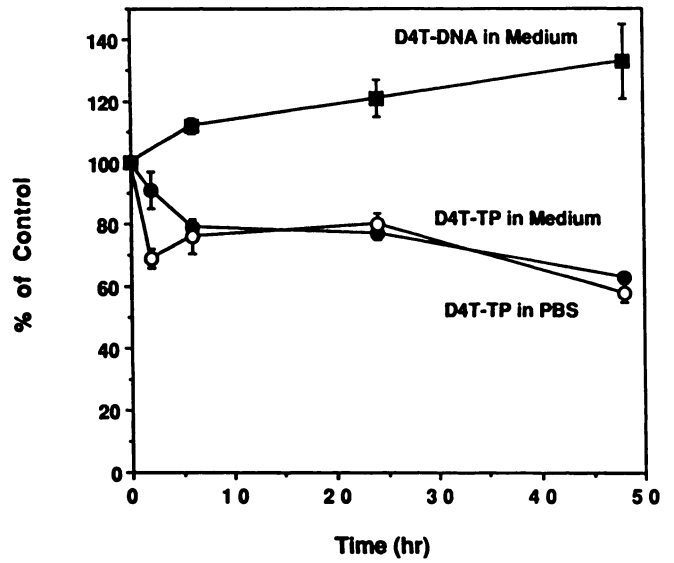

Fig. 7. Chemical stability in McCoy's 5A nutrient medium or PBS, at 37° and pH 7.4, of D4T-TP and [^{3}H]D4T in newly synthesized DNA isolated from human BMC exposed to 10 μM [^{3}H]D4T for 24 hr. D4T-TP concentration was determined by anion exchange HPLC, whereas the tritium remaining in DNA was determined with a filter disk assay. Values are the mean ± standard error of three experiments.

Discussion

D4T is a potent inhibitor of HIV replication, with activity comparable to that of AZT in a large variety of cell lines (11), and yet is 20–100-fold less toxic than AZT in a human CFU-GM clonogenic assay (13, 20). This limited *in vitro* cytotoxicity against human BMC is consistent with the absence of major hematological toxicity in phase I clinical trials (15–17). In an attempt to elucidate the mechanism(s) of toxicity of 2′,3′-dideoxynucleosides for human BMC, it was of particular interest to study the cellular mechanism(s) that may be responsible for the decreased sensitivity of these human host cells to D4T. Previous studies have demonstrated that D4T has a different pattern of phosphorylation, compared with AZT, with dThd kinase being the rate-limiting step (8, 22, 24). In contrast, AZT-MP being poorly converted to the diphosphate is the rate-limiting step in AZT metabolism (23). Perturbation of intracellular deoxyribonucleoside 5′-triphosphate pools by AZT, through inhibition of thymidylate kinase by AZT-MP, had been suggested in early studies (8, 23), whereas D4T has been shown to exhibit little effect on the metabolism of exogenous labeled dThd and other nucleosides (8, 22, 24). Although this difference may have played a role in the decreased host toxicity of D4T, compared with AZT, more recent studies, including those from our group using human BMC, have ruled out depletion of dTTP by AZT as one mechanism responsible for AZT-induced bone marrow toxicity (4, 5, 7, 10, 25). Although the mechanism of anti-HIV activity of 2′,3′-dideoxynucleosides can be explained by their intracellular sequential phosphorylation by cellular kinases to their active 5′-triphosphate derivative (5), inconsistent data have been reported on the relationship between intracellular concentration of 5′-triphosphate metabolites of 2′,3′-dideoxynucleosides in host cells and their cellular toxicity (13, 35, 36). Table 1 demonstrates that D4T is also phosphorylated to its 5′-mono-, 5′-di-, and 5′-triphosphates, with a pattern similar to that reported in other cells (8, 22, 29, 30); of interest, intracellular D4T-TP values were about 2–3-fold higher, compared with AZT-TP levels, after incubation with similar extracellular concentrations of the parent drug (4). These data would, thus, further suggest that cellular toxicity cannot be correlated with the relative intracellular triphosphate levels. We, and others, have suggested that incorporation of AZT into nuclear DNA of host cells may be an important toxicity target (4, 25, 28), with DNA polymerase β being possibly responsible for that incorporation (26). In contrast, DNA polymerase α is probably not able to incorporate AZT-TP into elongating nuclear DNA, and the potential involvement of DNA polymerase δ is still debatable (25–27). In the present study, using cesium sulfate gradient centrifugation, D4T was detected in DNA of human BMC. But, of note, steady state levels of D4T incorporated into DNA were at least 10–50-fold lower than values measured after exposure of cells to AZT, under similar conditions. These data are, thus, consistent with the hypothesis that the extent of incorporation of 2′,3′-dideoxynucleosides into nuclear DNA correlates with bone marrow toxicity (4).

Because D4T-TP and AZT-TP do not exhibit major differences in affinity for human nuclear DNA polymerases (11), other mechanisms accounting for the substantial difference in incorporation into nuclear DNA between the two drugs were explored. Further analysis of radiolabeled DNA extracted from cells exposed to [^{3}H]D4T revealed that approximately 70% of the radioactivity was associated with dThd (Fig. 4). Formation of dThd was demonstrated to result from intracellular conversion of D4T to dThd, possibly by dThd phosphorylase. Subse-

quently, dTTP metabolically formed from D4T via dThd is rapidly utilized for DNA synthesis, competing with D4T-TP for its incorporation into DNA. Consistent with these data, Huang *et al.* (37) recently reported, by using *in vitro* polymerization assays, that high molecular weight DNA was still synthesized by DNA polymerase α and ϵ in the presence of D4T-TP and dTTP at a ratio as high as 30 to 1. Although D4T is probably converted to dThd through dThd phosphorylase, it is important to note that dThd phosphorylase activity is minimal in highly purified human lymphocytes (38) and even absent or undetectable in human T lymphocytes (39), a cell population that is a major target for HIV *in vivo* (40).

Consequently, the toxicity and probably the anti-HIV activity of D4T are dependent on cellular dThd phosphorylase levels, and modulation of D4T degradation by that cellular enzyme may contribute to the observed increased selectivity, compared with AZT.

Recent studies by Cheng and co-workers (25) have identified an exonucleolytic enzyme in human K-562 erythroleukemia cells that is suggested not to be associated with DNA polymerase activity and can remove 3′-terminal AZT or dideoxycytidine residues from DNA. In our study, no excision of AZT from DNA was detected, whereas label from D4T was substantially removed (Fig. 6). Although the D4T-TP was chemically unstable (Fig. 7), D4T, as the polynucleotide monophosphate form, was protected against chemical hydrolytic degradation after its incorporation into DNA (Fig. 7). This observation has been reported with other nucleosides (41) and suggests that an enzymatic repair mechanism is responsible for D4T excision from DNA. The lack of AZT excision from DNA in our study is inconsistent with the recently published data (25). However, a difference in our study is that nonradiolabeled compounds were added in the chase portion of the experiment. The rationale was to dilute the ^{3}H-labeled triphosphate pool (which declines slowly during that phase), thereby preventing further incorporation into DNA. A possible explanation for the absence of excision of AZT from DNA during the chase is that the exonuclease activity may be inhibited by AZT nucleotides. The inhibitory effects of high concentrations of nucleoside 5′-monophosphates on the 3′ to 5′ exonuclease activity are well recognized (42) and have been suggested to play a role in the mutagenic effects of 6-mercaptopurine, which, similar to AZT, mostly accumulates within cells as its 5′-monophosphate derivative. Preliminary studies[2] from our group have demonstrated the ability of AZT-MP, at concentrations of >100 μM, to inhibit the proofreading exonuclease activity associated with mammalian DNA polymerase δ, which may also provide a molecular mechanism for the carcinogenic effects observed with AZT. Although the effect of D4T-MP on 3′ to 5′ exonuclease activity has not yet been evaluated, cellular concentrations of D4T-MP are never high and are thus unlikely to have an influence on the excision process. Another, more speculative, possibility that could account for the different data between our study and that of Cheng's group (25) and the observed different patterns of excision from DNA of human BMC between D4T and AZT might be the presence of a specific repair enzyme. Differential expression in human BMC, compared with K-562 cells, and differential recognition of 2′,3′-dideoxynucleosides would also explain the observations.

[2] E. G. Bridges and J. P. Sommadossi, unpublished data.

In summary, decreased D4T incorporation into DNA of human BMC is probably a major mechanism for its low hematological toxicity, compared with AZT. The role of degradative host enzymes, including dThd phosphorylase, and repair enzymes in the minimal steady state incorporation of D4T into DNA further emphasizes the importance of cellular function, in addition to kinases, in modulating the anti-HIV activity of 2′,3′-dideoxynucleosides.

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