In Vitro and In Vivo Disposition and Metabolism of 3'-Deoxy-2',3'-Didehydrothymidine

ERIKA M. CRETTON,¹ ZHU ZHOU,^{1†} LAUREN B. KIDD,¹ HAROLD M. McCCLURE,² SANJEEV KAUL,³ MICHAEL J. M. HITCHCOCK,^{3‡} AND JEAN-PIERRE SOMMADOSSI^{1*}

Department of Pharmacology, Center for AIDS Research, Comprehensive Cancer Center, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294¹; The Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia 30322²; and the Pharmaceutical Research Institute, Bristol-Myers Squibb Company, Syracuse, New York 13221, and Wallingford, Connecticut 06492³

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The disposition and metabolic fate of 3'-deoxy-2',3'-didehydrothymidine (D4T) were evaluated both in isolated hepatocytes and in nonhuman primates. Rapid formation of thymine and β -aminoisobutyric acid (BAIBA) occurred following incubation of hepatocytes with 10 μ M [5-³H]D4T. Substantial levels of tritiated water were also detected. Exposure of cells to D4T in the presence of either 1 mM thymine or 10 μ M benzyloxybenzyluracil, an inhibitor of dihydropyrimidine dehydrogenase, decreased intracellular BAIBA levels by approximately 89 and 63%, respectively. Concurrently, [³H]thymine levels increased two- to fivefold. These results are consistent with D4T being cleaved to thymine, which is then degraded to BAIBA. A similar metabolic disposition was observed in monkeys following administration of 25 mg of [5-³H]D4T per kg of body weight. BAIBA, thymine, and tritiated water were identified in plasma and urine. Approximately 50% of the administered dose was recovered in urine within 24 h, with the majority of the radioactivity representing unchanged drug. After administration intravenously or orally of 25 mg of [4-¹⁴C]D4T per kg of body weight to monkeys, a novel metabolite, designated X, in addition to unchanged D4T, thymine, and BAIBA, was also detected. The sum of the three metabolites and unchanged drug accounted for virtually all of the radioactivity in plasma and urine. Thymine and X exhibited kinetic profiles similar to that of D4T, with plasma elimination half-life of 2 to 3 h, whereas BAIBA levels remained constant for extended periods and declined slowly; this metabolite could be detected 24 h after intravenous drug administration. Mean oral bioavailability of D4T was high at approximately 70%. As observed in the [5-³H]D4T study performed in monkeys, approximately half of the administered [4-¹⁴C]D4T was recovered unchanged. The remainder was not recovered in urine or feces collected up to 30 days after drug administration. These data suggest that D4T metabolites are further metabolized by salvage pathways and/or converted to biological macromolecules.

3'-Deoxy-2',3'-didehydrothymidine (D4T) is a thymidine analog with potent anti-human immunodeficiency virus activity in vitro comparable to that of 3'-azido-3'-deoxythymidine (AZT) (23, 24). Previous studies on D4T metabolism, by several groups including our own, have demonstrated that D4T is sequentially phosphorylated by cellular enzymes to D4T-TP, the active form which competitively inhibits TTP incorporation by human immunodeficiency virus reverse transcriptase (3, 19, 34, 35). D4T has shown efficacy in patients with AIDS without substantial hematological toxicity (1, 14). The reduced bone marrow toxicity is probably due to the lower steady-state levels of D4T in nuclear DNA of human bone marrow cells compared with those of AZT under similar conditions (34). Peripheral neuropathy, also reported after treatment with 2',3'-dideoxycytidine or 2',3'-dideoxyinosine, was the dose-limiting toxicity and was reversible (6). Inhibition of mitochondrial DNA synthesis has been suggested as a potential mechanism of 2',3'-dideoxynucleoside-induced peripheral neuropathy (8, 21), but other mechanisms may also be involved (31).

Understanding of the catabolic fate of D4T is still limited. Pharmacokinetic studies of D4T in mice, rats, dogs, and monkeys have suggested that this agent is not substantially

degraded. Approximately 50 to 80% of the administered dose of D4T, in animals, is eliminated in urine unchanged (28, 29). In a recent pharmacokinetic study of D4T in humans (13), about half of the administered dose was recovered unchanged. The metabolic fate of the remainder is unknown. The in vivo formation of a glucuronide conjugate is still controversial. Schinazi et al. (29) have suggested the presence of a D4T glucuronide in urine of monkeys, while others have not detected such a conjugate (28). Until recently no D4T metabolites, other than its 5'-phosphorylated derivatives, were reported either in vitro or in vivo (18). D4T was recently demonstrated by this laboratory to be a substrate for *Escherichia coli* thymidine phosphorylase in vitro, leading to the formation of thymine (30). Interestingly, we also detected the in vitro degradation of D4T to thymine, with subsequent formation of thymidine, in human bone marrow cells (34). This suggests that D4T is cleaved to thymine and that subsequent degradation and/or utilization of thymine in vivo accounts for the unrecovered D4T in clinical trials. Understanding the metabolic fate of D4T may be important for clinical trials design, as well as provide information on the mechanism(s) of action and the toxicity of this agent. The present study examines both the in vitro and the in vivo metabolic disposition of D4T in isolated hepatocytes and in nonhuman primates. Isolated rat hepatocytes were chosen, since we had previously demonstrated the usefulness of this in vitro system in examining the metabolic disposition of AZT and 3'-azido-2',3'-dideoxyuridine, which are structur-

* Corresponding author.

† Present address: Department of Pharmacology, School of Medicine, Yale University, New Haven, CT 06510.

‡ Present address: Gilead Sciences, Inc., Foster City, CA 94404.

ally close analogs of D4T (10, 11). For the in vivo studies, monkeys were chosen, since 2',3'-dideoxynucleosides exhibit disposition and metabolic profiles in those species that are similar to those observed in humans (5, 9, 29).

MATERIALS AND METHODS

Chemicals. [*carboxyl*- ^{14}C]inulin was purchased from DuPont Research Products (Wilmington, Del.). [$5\text{-}^3\text{H}$]D4T (20 Ci/mmol) and [$4\text{-}^{14}\text{C}$]D4T (56 mCi/mmol) were purchased from Moravsek Biochemicals (Brea, Calif.). Radiolabeled compounds were >98% pure at the time of purchase or were otherwise purified by reversed-phase high-pressure liquid chromatography (HPLC) as described below. D4T was chemically synthesized as previously described (24). Thymine, thymidine, β -aminoisobutyric acid (BAIBA), alkaline phosphatase, venom phosphodiesterase I (type VI), and 0.2% ninhydrin in solution were purchased from Sigma Chemical Co. (St. Louis, Mo.). Benzyloxybenzyluracil (BBU) was kindly provided by Mahmoud el Kouni (University of Alabama at Birmingham, Birmingham). All other reagents were commercially available.

Preparation of hepatocyte suspension and incubation conditions. Hepatocytes were freshly isolated from Sprague-Dawley rats (180 to 200 g; Harlan Laboratories, Indianapolis, Ind.) by a modification of the previously described method of Berry and Friend (4, 32). Cell viability was >90%, as determined by trypan blue exclusion in all experiments. Hepatocytes were suspended to a final cytotrit of 4% and were incubated at 37°C in Krebs-Henseleit buffer, containing 0.25% (wt/vol) gelatin and 10 mM glucose, for up to 2 h. The pH was maintained at 7.4 by passing warmed and humidified 95% air–5% CO_2 over the cell suspension.

Experiments were initiated by addition of 10 μM [$5\text{-}^3\text{H}$]D4T (specific activity, 500 dpm/pmol). Preparation of the intracellular and extracellular media for HPLC analysis has been previously described (10, 11). Portions of cell suspension were preincubated for 30 min with 1 mM thymine or 10 μM BBU prior to incubation for 30 min with 10 μM [^3H]D4T, in order to examine the effects of these agents on D4T metabolism.

Determination of intracellular water. Intracellular volume was the difference between the wet and dry weights of the cell pellet minus the [^{14}C]inulin space. This technique has been described previously in detail (10, 11).

Animal experimental design. Rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys were used for the metabolic and pharmacokinetic studies. The animals were maintained at the Yerkes Regional Primate Research Center of Emory University in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Yerkes Center is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Prior to the start of the study, serum chemistry and hematology tests were performed to verify the health of the animals and to establish baseline values. Each monkey was identified by a tattoo number on the chest and was individually housed in a cage in a humidity- and temperature-controlled room with a 12 h:12-h light-dark cycle. Food was provided at 9:00 a.m. every day, and fresh drinking water was offered ad libitum. The animals were fasted overnight before each dose of D4T. On dosing days, food was provided approximately 8 h after dosing. The animals were anesthetized with ketamine (10 mg/kg), with additional ketamine given as needed to maintain anesthesia.

Two young adult rhesus monkeys (RQz-1 and RSg-1) and one cynomolgus monkey (MF403), each weighing approximately 5 to 6 kg, received one subcutaneous dose of 25 mg of D4T per kg of body weight dissolved in sterile normal saline. A tracer amount of 200 μCi of [$5\text{-}^3\text{H}$]D4T was admixed with the "cold" D4T dose to be administered. Samples (2 ml) of blood were collected from the femoral vein into heparinized tubes at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after injection, and plasma was separated by centrifugation ($300 \times g$). Urine was collected at similar times (except at 0.25 h) via urethral catheter. Plasma and urine samples were frozen and stored at -20°C until analysis.

Three young adult cynomolgus monkeys (MF 401, MF 422, and MF 531), each weighing approximately 5 to 6 kg, received one intravenous dose of 25 mg of D4T per kg of body weight dissolved in sterile normal saline into the saphenous vein. A tracer amount of 150 μCi of [$4\text{-}^{14}\text{C}$]D4T was admixed with the cold D4T dose. Intravenous infusion (0.5 ml/min) of Lactated Ringer's solution was given simultaneously for 8 h. Blood samples (2 ml) were drawn immediately before the start of infusion (predose) and at 0.1, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 48, 72, and 96 h after the start of infusion. Blood samples were collected from the femoral vein into heparinized tubes. Plasma was separated by centrifugation ($300 \times g$) and stored at -20°C until assayed. Urine was collected predose and as pooled collections during the 0 to 0.5, 0.5 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 24, 24 to 36, 36 to 48, 48 to 60, 60 to 72, 72 to 84, and 84 to 96 h intervals after the start of the infusion and then every 12 h over 1 month. For the first eight collection intervals, urine was collected via urethral catheter. For subsequent intervals, urine was collected in the cage tray. After measurement of volume, aliquots (10 ml) of urine were stored at -20°C until analysis. Total feces were also collected at 12-h intervals and pooled every 24 h for 30 days. The feces were collected on screens to avoid cross-contamination with urine. At the end of each collection interval, feces were weighed and mixed with a volume of methanol-water mixture (50:50) sufficient to completely immerse the sample. The container was weighed again. The container was stored at -20°C until analysis.

Following a 1-month washout period, the three cynomolgus monkeys who had received intravenous doses received an oral dose of 25 mg of D4T per kg of body weight, admixed with a tracer amount of 150 μCi of [$4\text{-}^{14}\text{C}$]D4T, via a nasogastric tube. The tube was rinsed with 2 ml of distilled water prior to removal. An intravenous infusion of Lactated Ringer's was again administered for 8 h to stimulate urine formation in monkeys. Except for the 0.1-h blood sample, blood, urine, and fecal samples were collected in the same manner as for the intravenous dose.

Measurement of radioactivity in feces. Aliquots of homogenized fecal samples (about 0.3 g in duplicate) were placed in combustion boats, allowed to dry at ambient conditions, and combusted in a model OX-300 biological oxidizer (R. J. Harvey Instrument Co., Hillsdale, N.J.). $^{14}\text{CO}_2$ was trapped in 15 ml of ^{14}C liquid scintillation cocktail, and radioactivity was determined by liquid scintillation spectrophotometry (model LS 5801; Beckman Instruments, Fullerton, Calif.).

HPLC analysis of D4T and metabolites. Fifty- to 100- μl aliquots of the intracellular and extracellular media were analyzed by HPLC with a Hewlett-Packard (Avondale, Pa.) model 1050 liquid chromatograph equipped with a manual injector and a C_{18} Hypersil column (5- μm particle size, 4.6 by 250 mm; Jones Chromatography, Lakewood, Colo.). Elution was performed isocratically by using a mobile phase

of 5 mM tetrabutylammonium hydrogen sulfate and 1.5 mM potassium phosphate adjusted to pH 8, with a flow rate of 0.5 ml/min. The column effluent was monitored at 254 nm with a model 440 UV detector (Waters Associates, Milford, Mass.). An integrator model 3396A (Hewlett-Packard) was part of the data collection system. Eluent from the column was directed via a low-dead-volume connection line into a model 2112 Redirac fraction collector (LKB Instruments, Rockville, Md.), and timed fractions of 0.5 ml were collected into miniscintillation vials over 50 min. After 5 ml of Econo-Safe scintillation fluor (Beckman Instruments, Palo Alto, Calif.) was added, radioactivity was measured by using a model 5000 TA LS counter equipped with an automatic quench correction program (Beckman Instruments). The retention times of the referenced compounds, BAIBA, thymine, thymidine, and D4T, were determined with the UV detector and were 6 to 7, 11 to 12, 31 to 32, and 37 to 38 min, respectively. The unknown metabolite, X, eluted as a single peak at 18 to 19 min.

The urinary and plasma profiles of unchanged drug and metabolites were determined by identical HPLC analysis of 10- to 200- μ l samples of urine and 200- μ l samples of plasma. These biological samples were filtered through a 0.45- μ m-pore-size Acro LC 13 filter (Gelman Sciences, Ann Arbor, Mich.) prior to analysis. The total radioactivity applied to the column was recovered for all samples in 45 min (95% $\pm$

was calculated as the product of CL_T and mean residence time. Renal clearance (CL_R) of D4T was determined by dividing the amount of drug excreted in urine during an 8-h period by the AUC value corresponding to the same period (15). The peak concentration in plasma (C_{max}) values and time at which C_{max} occurred (T_{max}) were observed experimental values.

The absolute bioavailability (F) of D4T was estimated from the concentration in plasma and/or the urinary excretion data by using the Kwan-Till method (22) which was modified by Gibaldi and Perrier (15). The correction method assumes that nonrenal clearance remains constant following intravenous, subcutaneous, or oral dosing but that CL_R estimates can be variable. The plasma concentration data are as follows:

$$F(AUC) = \frac{AUC(INF)_{po(s.c.)} \cdot D_{i.v.} (1 - UR_{i.v.})}{AUC(INF)_{i.v.} \cdot D_{po(s.c.)} + UR_{po(s.c.)}} \quad (1)$$

where D is the dose, the subscripts indicate routes (subcutaneous [s.c.], oral [po], or intravenous [i.v.]) of administration, and UR is urinary recovery.

Alternatively, urinary excretion data were corrected for differences in estimated CL_R to yield estimates of F :

$$F(UR) = \frac{UR_{po(s.c.)} \cdot D_{i.v.} [(CL_R/UR)_{i.v.} - (CL_R)_{i.v.} + (CL_R)_{po(s.c.)}]}{UR_{i.v.} \cdot D_{po(s.c.)} [(CL_R)_{po(s.c.)}/UR_{i.v.}]} \quad (2)$$

2% recovery). The concentrations of D4T, BAIBA, thymine, and X were based on recovery of radioactivity. Levels of X represent equivalent micromole concentrations. The intra- and interday coefficients of variation were less than 10% for all analyses. The detection limit was 0.2 μ mol/liter for D4T, thymine, and BAIBA and 1.0 μ mol/liter for X. Standard curves for D4T and metabolites (with the exception of X) were linear, with $r^2 > 0.99$.

Pharmacokinetic analyses. Plasma concentration versus time data for each individual data set from each monkey studied were evaluated by using a SIPHAR/Base (SIMED, Creteil Cedex, France) (16). Initial parameter estimates were determined by using a curve-stripping algorithm with a two-compartment open model which assumed zero-order input and first-order elimination for the intravenous administration. All fits were forced through the origin for the oral and subcutaneous administration. Final parameter estimates were determined by using the terminal disposition slope (K) generated by a weighted least-squares algorithm (25) with weighing $1/y^2$. Compartment model selection was based upon visual inspection of the fit, comparison of the Akaike Information Criterion value (33), evaluation of standard residual data, comparison of the correlation coefficients, and comparison of the coefficients of variation for the determined parameters. The area under the curve (AUC) was calculated by the trapezoidal rule with extrapolation to time infinity. Elimination half-life ($t_{1/2}$) values of unchanged drug and metabolites were calculated as $0.693/K$. The total plasma clearance (CL_T) of D4T was calculated by dividing the intravenous dose by the resulting AUC. The apparent volume of distribution in the terminal elimination phase (V) was calculated as the quotient of CL_T and K . The steady-state V

The extent of absorption (EA) was calculated from the ratio of urinary recovery of total radioactivity after oral or subcutaneous and intravenous doses as follows:

$$EA = \frac{(\% \text{ dose as total radioactivity in urine})_{po(s.c.)}}{(\% \text{ dose as total radioactivity in urine})_{i.v.}} \quad (3)$$

The extent of first pass metabolism (FPM), calculated as the percent of absorbed dose after oral or subcutaneous administration, was determined by using the equation:

$$FPM = \frac{EA_{po(s.c.)} - F(AUC)}{EA_{po(s.c.)}} \times 100 \quad (4)$$

Preliminary identification of unknown metabolites. (i) **Ninhydrin test.** Preliminary identification was performed by using a ninhydrin dye which produces a colored product when it reacts with a free amino group. The unknown radiolabeled chromatographic peak, with a retention time of 7 min, was isolated from intracellular medium by HPLC and concentrated by lyophilization. The dry residue was redissolved in 0.5 ml of ethanol and spotted on the center of a 3MM disk filter (Whatman, Maidstone, England). A drop of 0.2% ninhydrin solution in ethanol was added, and the filter was air dried at room temperature. Aspartic acid was used as a positive control. After 30 min, color development was determined.

(ii) **Amino acid analysis.** Intracellular samples were passed through Econo-Pac 10 DG desalting columns (Bio-Rad, Richmond, Calif.) to eliminate cellular associated proteins prior to amino acid analysis. The columns were equilibrated with distilled water. Fractions (0.5 ml) were collected, and

those eluting radioactivity after the column void volume were pooled and concentrated by lyophilization. Following this process, samples were hydrolyzed at 110°C in the presence of 6 N HCl for 20 h by using a Pico Tag Work Station (Waters Associates, Milford, Mass.). Samples were then redried with methanol-water-triethylamine (2:2:1), derivatized by addition of methanol-water-triethylamine-phenylisothiocyanate (7:1:1:1), and finally dissolved in a sample buffer to be applied to a Pico Tag column (150 by 3.0 mm) (Waters Associates). Analysis was performed with a Perkin-Elmer series 4 liquid chromatograph, equipped with a Waters 715 Ultra Wisp autoinjector, an LDC/Milton Roy detector with UV at 254 nm, and a Hewlett-Packard 3396A integrator. Authentic amino acids were included with the unknown samples and applied to the column simultaneously. β -Alanine and BAIBA eluted at 5.6 and 7.7 min, respectively, and the compounds did not overlap with any of 20 endogenous amino acids. Samples (0.5 ml) were fractionated, and radioactivity was measured as described above.

Measurement of tritiated water. Formation of tritiated water after incubation of [^3H]D4T with hepatocytes or following administration of [^3H]D4T to monkeys was determined. Briefly, aliquots were applied to the inside surface of a petri dish (35 by 10 mm). The dish was covered, sealed with tape, and placed on a hot plate (50°C). A beaker containing ice was placed on top of the dish. After the sample had evaporated and condensed on the lid, the dish and beaker were removed from the hot plate and allowed to cool. A 0.5-ml portion of distilled water was then added to the condensed water, mixed, and collected into a miniscintillation vial. Radioactivity in the condensed water was measured. Exposure of samples to air was kept to a minimum. This technique has been previously described in detail (28).

RESULTS

In vitro studies with isolated hepatocytes. (i) **HPLC analysis of D4T and metabolites after incubation of hepatocytes with [^3H]D4T.** Figure 1 represents the HPLC radiochromatogram of intracellular ^3H following the incubation of hepatocytes with 10 μM [^3H]D4T. In addition to D4T and thymine, which were initially identified by retention time and coelution with an authentic standard and then confirmed by comparing spectrophotometric scans of compounds with those of standards, a peak, labeled B, with a retention time of 6.5 min, was detected. This unknown derivative did not correspond to any known analate of D4T, since it was resistant to both alkaline phosphatase and 5'-phosphodiesterase hydrolysis (data not shown).

(ii) **Identification of B.** The ninhydrin test, as previously described, was positive for the unknown peak B, indicating that this metabolite contains a free amino group. The chromatographic profiles of several authentic standards were compared with that of B. The amino acid BAIBA, a catabolic product of thymine (7), coeluted with this unknown D4T metabolite.

Further identification was carried out through amino acid analysis. Following hydrolysis and derivatization of intracellular extracts, samples were analyzed by HPLC. The labeled metabolite, B, coeluted with authentic BAIBA.

Interestingly, in the process of concentrating samples by lyophilization, a partial loss of radioactivity was observed. Previous studies have reported the formation of tritiated water in humans following the administration of [^3H]thymidine (27). If tritiated water was formed in hepatocytes

FIG. 1. HPLC profile of intracellular D4T and metabolites in freshly isolated rat hepatocytes 10 min after exposure to 10 μM [^3H]D4T. D4T and its catabolites were resolved on a Hypersil octyldodecyl silane column as described in Materials and Methods.

following incubation with [^3H]D4T, it would be lost during lyophilization. This could explain the partial loss of radioactivity observed during this procedure. Using an evaporation technique, tritiated water was detected in both intracellular and extracellular samples. Substantial amounts of tritiated water were also detected in plasma and urine samples from nonhuman primates following the administration of [^3H]D4T.

(iii) **Analysis of intracellular and extracellular D4T and metabolites following the exposure of cells to [^3H]D4T.** Figure 2 illustrates the presence of unchanged D4T, thymine, and BAIBA in the intracellular medium when hepatocytes were incubated for various times with 10 μM [^3H]D4T. Unmetabolized D4T was detected within the hepatocytes at all sampling times over the 2-h exposure period. The intracellular levels of D4T equaled those in the extracellular compartment at any given time point, consistent with the described nonfacilitated diffusion of this drug (2). The predominant intracellular D4T metabolite was BAIBA. Substantial levels of BAIBA were rapidly detected within 10 min and reached a mean $\pm$ standard deviation (SD) maximal concentration of $6.95 \pm 2.84 \mu\text{M}$ in 90 min. Thymine reached a steady-state level of $0.43 \pm 0.13 \mu\text{M}$ within 45 min. Extracellular concentrations of unmetabolized D4T decreased at a rate of 0.018 $\mu\text{M}/\text{min}$, and the metabolites formed within the cell accumulated to concentrations approximating those in the intracellular compartment (data not shown).

(iv) **Effect of thymine and BBU on D4T metabolism in hepatocytes.** To further delineate the formation of thymine and BAIBA from [^3H]D4T in hepatocytes, the effects of

FIG. 2. Intracellular profile of D4T (■), BAIBA (▨), and thymine (□) after exposure of cells to 10 μ M [3 H]D4T. At timed intervals, portions of the cell suspension were separately centrifuged and extracted. Total intracellular 3 H was then analyzed by HPLC. Data represent the mean $\pm$ SD of four experiments.

adding exogenous thymine or BBU, a potent inhibitor of dihydropyrimidine dehydrogenase (12), were studied in this *in vitro* system. Addition of 1 mM thymine or 10 μ M BBU to cell suspensions containing 10 μ M [3 H]D4T decreased the intracellular concentrations of radiolabeled BAIBA by $89.3\% \pm 3.4\%$ ($n = 3$) and $63.5\% \pm 3.2\%$ ($n = 3$), respectively, while the radiolabeled intracellular concentration of thymine increased two- to fivefold. In these same experiments, the extracellular concentrations of [3 H]BAIBA decreased approximately four- to fivefold compared with the control. [3 H]thymine levels increased as much as 10-fold by 30 min. These data support the contention that D4T is cleaved, releasing thymine, which then undergoes normal endogenous catabolism.

In vivo metabolic and pharmacokinetic studies. (i) **Plasma kinetics of unchanged drug and metabolites following administration of [4 - 14 C]D4T.** A representative plasma concentration-time profile of D4T and metabolites after intravenous administration of 25 mg of D4T per kg of body weight to a cynomolgus monkey is illustrated in Fig. 3A. In all animals, thymine, BAIBA, and X (an unidentified metabolite) were detected as early as 6 to 15 min, suggesting rapid metabolism. Unchanged D4T, thymine, and X exhibited similar kinetic profiles, with apparent elimination $t_{1/2}$ values in the range between 2.5 and 3 h (Table 1). In contrast, BAIBA plasma levels quickly plateaued and slowly declined with an apparent elimination $t_{1/2}$ of 13.8 ± 2.7 h. This metabolite was still detected 24 h after drug administration. Pharmacokinetic parameter values for D4T and metabolites after intravenous administration were consistent among all three monkeys (Table 1).

A representative plasma concentration-time profile of D4T and metabolites after oral administration of 25 mg of D4T per kg to a cynomolgus monkey is illustrated in Fig. 3B. After oral administration, plasma concentration of D4T peaked at 3.3 h. Peak concentrations of metabolites were delayed, with

FIG. 3. Representative plasma concentration-time profiles of D4T (■), BAIBA (▲), X (□), and thymine (●) in two monkeys after administration of 25 mg of D4T per kg intravenously (monkey MF401) (A), orally (monkey MF401) (B), or subcutaneously (monkey MF403) (C).

TABLE 1. Pharmacokinetic parameters after intravenous administration of 25 mg of D4T per kg in nonhuman primates

Monkey and compound	$C_{\max}$ ($\mu\text{mol/liter}$)	$T_{\max}$ (h)	AUC ($\mu\text{mol/liter/h}$)	CL (ml/h/kg)	V (liters)	V_{ss}^a (liters)	Apparent $t_{1/2}$ (h)
MF 401							
D4T	318.8	0.10	381.2	292.5	4.8	3.7	2.18
Thymine	20.5	0.10	89.9				2.96
BAIBA	7.9	2.00	126.8				11.8
X	5.2	2.00	31.9				1.71
MF 422							
D4T	398.0	0.10	405.7	274.8	6.6	4.4	3.13
Thymine	38.1	0.10	58.5				1.50
BAIBA	8.4	1.00	157.8				12.80
X	4.8	1.00	38.0				4.30
MF 531							
D4T	455.2	0.10	513.6	217.0	3.8	3.1	2.11
Thymine	24.6	0.10	75.4				4.30
BAIBA	8.0	1.00	145.2				16.90
X	7.3	2.00	46.3				3.40
Mean (SD)							
D4T	390.7 (68.5)	0.10 (0.0)	433.5 (70.4)	261.4 (39.5)	5.1 (1.4)	3.7 (0.7)	2.5 (0.6)
Thymine	26.2 (11.1)	0.10 (0.0)	74.6 (15.7)				2.9 (1.4)
BAIBA	8.1 (0.3)	1.30 (0.60)	143.3 (15.6)				13.8 (2.7)
X	5.8 (1.3)	1.70 (0.60)	38.7 (7.2)				3.1 (1.3)

^a V_{ss} , volume of distribution at steady state.

$T_{\max}$ for thymine, BAIBA, and X occurring at 4.0, 6.0, and 4.7 h after dosing, respectively. The pharmacokinetic parameter values for D4T and metabolites following oral administration are presented in Table 2. Mean $C_{\max} \pm \text{SD}$ were $39.4 \pm 17.4 \mu\text{M}$ for D4T and 12.6 ± 3.3 , 5.1 ± 2.4 , and $6.2 \pm 1.7 \mu\text{M}$ for the metabolites thymine, BAIBA, and X, respectively. The apparent elimination $t_{1/2}$ of D4T was not markedly different from that following intravenous administration,

TABLE 2. Pharmacokinetic parameters after oral administration of 25 mg of D4T per kg in nonhuman primates

Monkey and compound	$C_{\max}$ ($\mu\text{mol/liter}$)	$T_{\max}$ (h)	AUC ($\mu\text{mol/liter/h}$)	Apparent $t_{1/2}$ (h)
MF 401				
D4T	40.4	2.0	218.2	2.10
Thymine	13.3	4.0	— ^a	—
BAIBA	5.3	6.0	—	—
X	5.3	4.0	32.8	3.80
MF 422				
D4T	56.3	4.0	337.5	1.96
Thymine	15.4	6.0	—	—
BAIBA	7.3	8.0	—	—
X	7.6	4.0	59.2	2.50
MF 531				
D4T	21.6	4.0	151.1	2.85
Thymine	9.0	2.0	61.9	1.70
BAIBA	2.6	4.0	35.8	7.90
X	5.6	6.0	—	—
Mean (SD)				
D4T	39.4 (17.4)	3.3 (1.2)	235.6 (94.4)	2.3 (0.5)
Thymine	12.6 (3.3)	4.0 (2.0)	—	—
BAIBA	5.1 (2.4)	6.0 (2.0)	—	—
X	6.2 (1.7)	4.7 (1.2)	46.0 (18.7)	3.2 (0.9)

^a —, values could not be estimated because of insufficient data.

tion, suggesting route-independent disposition. The $t_{1/2}$ of X was also similar following oral or intravenous dosing, while the mean $t_{1/2}$ s of thymine and BAIBA were substantially shorter after oral dosing than after intravenous administration. The $t_{1/2}$ estimations of thymine, BAIBA, and X in two of three, two of three, and one of three monkeys, respectively, were not possible because of insufficient data. Mean values for absolute bioavailability (F) of D4T after a 25-mg/kg oral solution dose were $71.3\% \pm 25.5\%$ and $70.7\% \pm 38.5\%$ on the basis of AUC or urinary excretion data. The mean extent of absorption of the oral solution dose of D4T was $87.0\% \pm 26.1\%$. The mean extent of first-pass metabolism of D4T was $19.1\% \pm 5.9\%$.

(ii) **Plasma kinetics of unchanged drug and metabolites following subcutaneous administration of [5-³H]D4T.** A representative plasma concentration-time profile of D4T and metabolites after subcutaneous administration of 25 mg of [5-³H]D4T per kg to a cynomolgus monkey is illustrated in Fig. 3C. Pharmacokinetic parameter values for this cynomolgus monkey and for two rhesus monkeys similarly dosed are shown in Table 3. Note that no quantitative or qualitative differences in D4T disposition and metabolism were observed between rhesus monkeys (RQZ-1 and RSG-1) and cynomolgus monkeys (MF403). When [5-³H]D4T was used, the unidentified metabolite, X, was not detected in the plasma of any monkey, while a substantial amount of tritiated water was detected. The plasma concentrations of D4T and thymine peaked at 1 h, while the $T_{\max}$ of BAIBA ranged from 0.5 to 2.0 h. The mean $C_{\max} \pm \text{SD}$ of D4T was $76.9 \pm 24.1 \mu\text{M}$, and those of the metabolites thymine and BAIBA were 4.9 ± 2.1 and $4.8 \pm 3.1 \mu\text{M}$, respectively. The apparent elimination $t_{1/2}$ s of D4T and thymine were similar to those observed after intravenous or oral administration, while the mean $t_{1/2}$ of BAIBA following subcutaneous dosing (3.2 ± 0.4 h) was far shorter than that calculated after intravenous or oral administration of D4T. The mean bioavailability (F) of D4T after a 25-mg/kg subcutaneous dose was 78% on the

TABLE 3. Pharmacokinetic parameters after subcutaneous administration of 25 mg of D4T per kg in nonhuman primates

Monkey and compound	$C_{\max}$ ($\mu\text{mol/liter}$)	$T_{\max}$ (h)	AUC ($\mu\text{mol/liter/h}$)	Apparent $t_{1/2}$ (h)
RQZ-1				
D4T	55.2	1.0	161.4	1.49
Thymine	3.7	1.0	12.9	1.25
BAIBA	1.5	0.5	39.5	3.50
RSG-1				
D4T	72.1	1.0	196.7	1.52
Thymine	3.6	1.0	13.5	1.80
BAIBA	7.6	2.0	41.9	2.30
MF 403				
D4T	103.3	1.0	274.3	1.92
Thymine	7.3	1.0	49.6	3.92
BAIBA	5.5	1.0	21.9	3.25
Mean (SD)				
D4T	76.9 (24.1)	1 (0)	210.8 (57.8)	1.6 (0.2)
Thymine	4.9 (2.1)	1 (0)	25.3 (21.0)	2.3 (1.4)
BAIBA	4.8 (3.1)	1.2 (0.8)	34.4 (10.9)	3.20 (1.4)

basis of AUC data. The mean extent of absorption of D4T after subcutaneous administration was 110%.

(iii) **Urinary excretion of D4T and its metabolites in urine and feces.** Urinary excretion of unchanged D4T and metabolites was similar among the three monkeys, with the most occurring between 0.25 and 1 h after D4T administration (Table 4). BAIBA and D4T were detected, although at low concentrations, up to 96 h after drug administration. Total urinary recovery averaged 47.8% (total radioactivity) within 96 h. D4T, BAIBA, thymine, and X represented $44.4\% \pm 6.6\%$, $1.8\% \pm 0.2\%$, $1.1\% \pm 0.6\%$ and $1.0\% \pm 0.5\%$, respectively. No radioactivity was detected beyond 96 h and during a 30-day sample collection period. Over the same period, approximately 0.1% of the dose was excreted in feces. Therefore, approximately 50% of the dose as radioactivity was unaccounted for. The mean CL_R value of D4T was 128 ± 20.2 ml/h/kg. The mean relative ratio of CL_R/CL_T

TABLE 4. CL_R and urinary excretion of D4T and its metabolites after administration of 25 mg of D4T per kg in nonhuman primates^a

Route and compound	Amt (% of total dose)	CL_R (ml/h/kg)
Intravenous		
D4T	44.4 (6.6)	128.0 (20.2)
Thymine	1.1 (0.6)	
BAIBA	1.8 (0.2)	
X	1.0 (0.5)	
Oral		
D4T	37.4 (5.7)	177.7 (41.5)
Thymine	0.9 (0.1)	
BAIBA	1.1 (0.3)	
X	1.1 (0.1)	
Subcutaneous		
D4T	50.4 (7.2)	250.4 (28.3)
Thymine	2.00 (1.60)	
BAIBA	1.00 (0.60)	

^a Data are given as mean $\pm$ SD values.

FIG. 4. Proposed metabolic pathways of D4T.

indicates that approximately 50% of the administered dose undergoes nonrenal elimination in monkeys, consistent with observations in humans (13).

Urinary excretion of D4T and metabolites was delayed compared with that following intravenous administration, with most occurring during the period up to 24 h. In contrast to excretion after intravenous dosing of D4T, unchanged drug and metabolites were detectable in urine only up to 48 h, with a cumulative mean recovery of 37.4%. No additional radioactivity was detected up to 30 days after dosing. Most of the dose recovered in urine was identified as D4T. BAIBA, X, and thymine represented $1.1\% \pm 0.3\%$, $1.1\% \pm 0.1\%$, and $0.9\% \pm 0.1\%$, respectively, of the total recovery. The cumulative recovery of radioactivity in feces was 0.26% over 30 days after dosing. The mean CL_R value of D4T was 177.7 ± 41.5 ml/h/kg, about 40% greater than that after intravenous administration.

Urinary excretion of D4T and its metabolites occurred mostly between 2 and 4 h after administration. Total urinary recovery approximated 50.4% within 24 h. Unchanged D4T represented the bulk of the total radioactivity, with thymine and BAIBA accounting for $2.0\% \pm 1.6\%$ and $1.0\% \pm 0.6\%$, respectively. As observed after oral administration, the mean CL_R value for D4T was higher than that measured after intravenous administration.

DISCUSSION

The present study provides a detailed analysis of D4T disposition and metabolism in vitro and in vivo. The identified metabolic pathways of D4T (Fig. 4) indicate that the glycosidic bond of D4T is cleaved to liberate thymine, which is subsequently degraded to an amino acid, BAIBA. An unidentified metabolite, which is probably a direct product of D4T, was detected in monkeys after administration of [$4\text{-}^{14}\text{C}$]D4T.

Initial studies in monkeys indicated that this species

excretes only approximately 50% of the administered dose within 24 h (28). In contrast, a higher recovery, between 70 and 80% of a dose, was observed in rats and dogs (28). In a recent pharmacokinetic study of D4T in humans (13), approximately 50% of the administered dose was recovered, suggesting, as our group has shown with AZT (9), that nonhuman primates may represent an appropriate animal model for the *in vivo* disposition of D4T. The present study, with either [5-³H]D4T or [4-¹⁴C]D4T in nonhuman primates, confirms this hypothesis. Several possibilities have been suggested to explain the limited urinary recovery of D4T, including incomplete absorption, biliary excretion, insufficient sampling time (28), or unknown routes of metabolism or clearance (13). Incomplete absorption was ruled out by pharmacokinetic studies in humans, since mean oral bioavailability is approximately 90% in patients (13). The mean absolute bioavailability of D4T was approximately 70% after a 25-mg/kg oral solution dose to monkeys. Oral absorption was delayed in monkeys, confirming a previous report (29), for unknown reasons. This is in contrast to the rapid oral absorption observed in humans.

In previous studies, a low and variable oral bioavailability of D4T was reported in monkeys, implying poor absorption from the gastrointestinal tract (29). Some variability was observed in our study, but the extent of absorption was high. The estimated renal clearance of D4T increased after oral or subcutaneous administration compared with that after intravenous dosing. Plasma levels achieved after oral or subcutaneous administration of D4T were approximately 10-fold lower than those observed after intravenous administration, suggesting that the tubular reabsorption of D4T and/or other processes including metabolism of D4T to unknown pathways are saturated after intravenous administration. Our present study also excluded a delayed elimination of D4T or a significant fecal excretion. Most of the radioactivity (>98% of total urinary recovery) was recovered in urine within 48 h after administration of D4T regardless of routes of administration. No additional radioactivity was detected up to 30 days after dosing. Biliary excretion of D4T is probably negligible, since radioactivity in feces accounted for less than 1% of an administered dose after either oral or intravenous administration, although potential enterohepatic circulation of D4T and metabolites has not been evaluated.

Potential metabolic pathways were investigated by using an *in vitro* system which has been shown to be useful in evaluating hepatic catabolism of 2',3'-dideoxynucleosides (10, 11). D4T was demonstrated to be cleaved in cells to thymine with subsequent formation of BAIBA, the predominant intracellular metabolite, which accumulated at concentrations approximating those of unchanged D4T. A loss of tritium from [5-³H]D4T was observed in these *in vitro* studies and also in monkeys. By using an evaporation technique, formation of tritiated water was demonstrated in both *in vitro* and *in vivo* studies. Rubini et al. (27) have previously reported that as much as 50% of [5-³H]thymidine administered to humans was recovered as tritiated water. These data suggested that thymidine and D4T undergo a metabolism leading to loss of the label and resulting in formation of tritiated water. In the study by Rubini et al., [³H]BAIBA was the only metabolite found in urine (27). Although both [³H]thymine and [³H]BAIBA were also detected in urine of monkeys receiving [5-³H]D4T, these metabolites accounted for 3% of the total D4T dose. Unchanged D4T represented 50% of the dose; therefore, approximately 47% of administered D4T remained unaccounted for. Loss of radioactivity from [5-³H]thymidine

administered to humans was also explained as possible incorporation into newly synthesized DNA in proliferating cells (27). Recent studies by our laboratory showed that 70% of the radioactivity in nucleic acids of human bone marrow cells exposed to [5-³H]D4T is present as thymidine (34). This metabolism of D4T may account, in part, for the lack of significant bone marrow toxicity of D4T in humans (6). The conversion of D4T to thymine could theoretically be mediated by thymidine phosphorylase, since D4T was recently demonstrated to be a substrate for *E. coli* thymidine phosphorylase (30). Thymine would then be salvaged with deoxyribose-1-phosphate, as shown for other nucleosides (20). However, with purified human liver thymidine phosphorylase, D4T was not a substrate (25a), suggesting that it is metabolized to thymine by another enzymatic pathway. Consistent with that hypothesis, thymine and BAIBA were detected when D4T was incubated in either rat or human liver homogenates (36).

In order to further clarify its metabolism, [¹⁴C]D4T (with the label at the 4 position of the thymine base) was administered to monkeys. In contrast to the study with 5-³H-labeled D4T, in addition to BAIBA, thymine, and unchanged D4T, a novel metabolite was detected in both plasma and urine. This metabolite represented 1% of the total dose. As in the [5-³H]D4T study, BAIBA and thymine accounted for approximately 3% of the dose, with unchanged D4T representing approximately 40 to 50%. No substantial levels of radioactivity (¹⁴C) were detected in fecal samples. In a previous study, radioactivity associated with [2,6-¹⁴C]thymidine was recovered in both expired CO₂ and in cellular macromolecules, but not in DNA, with the latter representing about 50% of total radioactivity (26). Therefore, it would appear that in addition to salvage pathways which lead to reincorporation of purine and pyrimidine bases into nucleic acids, the breakdown products of these bases may be reutilized for the synthesis of proteins, carbohydrates, and lipids. Indeed, labeled macromolecules such as glycogen, glucose, ribose, alanine, aspartate, glutamate, and lipids have been isolated following exposure of cells to radiolabeled thymidine (26). Since D4T is cleaved *in vivo* to thymine and subsequently metabolized to BAIBA, degradation and/or conversion to macromolecules and products which are shuttled into salvage pathways may account for the unrecovered portion of the D4T. It is very likely that expired ¹⁴CO₂ may also account for a substantial amount of the loss of radioactivity. Indeed, within the hepatic cell, BAIBA is transported into mitochondria, where it is further metabolized to propionyl-coenzyme A and CO₂ derived from the carbonyl group at the 4 position of the thymine base (17). These possibilities need to be explored further.

The pharmacokinetic data for D4T generated in this study were comparable to those values reported previously for nonhuman primates (29) and humans (13), although the apparent elimination *t*_{1/2} was twofold longer by all routes of administration. Also, consistent with the pharmacokinetic studies in humans (13), the renal clearance was greater than the creatinine clearance, confirming that in addition to glomerular filtration, D4T undergoes active renal tubular secretion. The concentrations of metabolites thymine and X decayed similarly to those of the unchanged drug, whereas BAIBA elimination *t*_{1/2} was substantially prolonged. In contrast to previous suggestions made by Schinazi et al. (29), no D4T glucuronide conjugate was detected. The sum of the three metabolites and unchanged drug accounted for virtually all of the radioactivity in plasma and urine, confirming previous studies with monkeys (28) and humans (13).

In summary, the results of the present study provide the first detailed analysis of D4T metabolism in an appropriate animal model which mimics the disposition of this anti-HIV agent in humans. These studies demonstrate that the metabolism of D4T is route independent and that D4T is converted to thymine, which subsequently is metabolized to BAIBA. Formation of a novel metabolite is also observed. Identification of this metabolite is in progress and will be reported at a later time. The mean relative ratio of CL_R/CL_T indicates that approximately 50% of the administered dose undergoes nonrenal elimination. This percentage corresponds to the radioactivity which is not accounted for in cumulative radioactivity recovered in urine and feces up to 30 days. A complete mass-balance study, with evaluation of radioactivity distribution in tissues and radioactive expired air, will be necessary to precisely identify the distribution of the remaining radioactivity. The detection of thymine and BAIBA as D4T metabolites suggests that the base is salvaged to thymidine, which may be utilized for nucleic acid synthesis, and BAIBA can be degraded to metabolites including CO_2 involved in the synthesis of proteins, carbohydrates, and lipids.

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