

Anti-Human Immunodeficiency Virus Type 1 Activity, Intracellular Metabolism, and Pharmacokinetic Evaluation of 2'-Deoxy-3'-Oxa-4'-Thiocytidine

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The racemic nucleoside analogue 2'-deoxy-3'-oxa-4'-thiocytidine (dOTC) is in clinical development for the treatment of human immunodeficiency virus (HIV) type 1 (HIV-1) infection. dOTC is structurally related to lamivudine (3TC), but the oxygen and sulfur in the furanosyl ring are transposed. Intracellular metabolism studies showed that dOTC is phosphorylated within cells via the deoxycytidine kinase pathway and that approximately 2 to 5% of dOTC is converted into the racemic triphosphate derivatives, which had measurable half-lives (2 to 3 hours) within cells. Both 5'-triphosphate (TP) derivatives of dOTC were more potent than 3TC-TP at inhibiting HIV-1 reverse transcriptase (RT) *in vitro*. The K_i values for dOTC-TP obtained against human DNA polymerases α , β , and γ were 5,000-, 78-, and 571-fold greater, respectively, than those for HIV RT (28 nM), indicating a good selectivity for the viral enzyme. In culture experiments, dOTC is a potent inhibitor of primary isolates of HIV-1, which were obtained from antiretroviral drug-naïve patients as well as from nucleoside therapy-experienced (3TC- and/or zidovudine [AZT]-treated) patients. The mean 50% inhibitory concentration of dOTC for drug-naïve isolates was 1.76 μ M, rising to only 2.53 and 2.5 μ M for viruses resistant to 3TC and viruses resistant to 3TC and AZT, respectively. This minimal change in activity is in contrast to the more dramatic changes observed when 3TC or AZT was evaluated against these same viral isolates. In tissue culture studies, the 50% toxicity levels for dOTC, which were determined by using [³H]thymidine uptake as a measure of logarithmic-phase cell proliferation, was greater than 100 μ M for all cell lines tested. In addition, after 14 days of continuous culture, at concentrations up to 10 μ M, no measurable toxic effect on HepG2 cells or mitochondrial DNA replication within these cells was observed. When administered orally to rats, dOTC was well absorbed, with a bioavailability of approximately 77%, with a high proportion (approximately 16.5% of the levels in serum) found in the cerebrospinal fluid.

The 2',3'-dideoxy and the 2',3'-dideoxy-2',3'-didehydro classes of nucleoside analogues have given rise to zidovudine (AZT), the first drug approved for the treatment of human immunodeficiency virus (HIV) type 1 (HIV-1) infections (12). Together with other members of this class of nucleoside analogues, including stavudine (d4T) (24), didanosine (ddI) (21), zalcitabine (ddC) (30), the heterosubstituted nucleoside lamivudine (3TC) (1, 2, 22, 27), and more recently, the carbocyclic analogue 1592U89 (abacavir) (29), these classes of nucleoside analogues continue to represent a major chemotherapeutic approach to the management of HIV-1 infections, the causative agent of AIDS. However, despite the number of HIV-1 reverse transcriptase (RT) inhibitors available for clinical use at the present time and the effectiveness of administration of nucleoside RT inhibitors in combination with nonnucleoside RT inhibitors and protease inhibitors, long-term exposure of the patient to these drugs often results in the development of viral resistance or intolerance to the antiviral chemotherapy regimens. For this reason, efforts to identify new agents that have activity against drug-resistant strains of HIV-1 and that

possess a toxicity profile which allows for individual patient tolerance of the drug are still warranted.

The mechanism of action of the 2',3'-dideoxy class of anti-HIV-1 nucleoside analogues is dependent upon their phosphorylation by cellular enzymes in the cytoplasm to yield the corresponding 5'-triphosphate (TP). The nucleoside TP analogue competes with the natural nucleoside TP for binding to the retroviral RT enzyme, and upon incorporation into the nascent DNA strand, these molecules act as terminators of chain elongation (5, 17).

The 2'-deoxy-3'-oxa-4'-thiocytidine (dOTC) class of molecules comprises novel 4'-thio dideoxynucleoside analogues that contain an oxygen heteroatom at the 3' position of the sugar moiety. We have previously reported on the synthesis and anti-HIV-1_{IIIB} properties of the racemate as well as those of the individual enantiomers of dOTC in cell lines and primary cells (1, 15). This class of 2,4-disubstituted 1,3-oxathiolane nucleosides is a hybrid of the 4'-thio and isonucleoside families of compounds. It is isomeric to the 2,5-disubstituted 1,3-oxathiolanes by transposition of the heteroatoms in the sugar moiety of the racemic form of the clinically approved anti-HIV-1 agent 3TC (Epivir). The individual enantiomers of dOTC were relatively equipotent inhibitors of HIV-1_{IIIB}, with (+)-dOTC being less selective in cell culture assays (15).

In the present study we describe how dOTC maintains some of the more desirable features of the individual enantiomers with respect to potency and toxicity. We report that dOTC

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exhibits low levels of toxicity *in vitro*, is well tolerated *in vivo*, and is metabolized into its triphosphate derivatives within cells; the K_i of dOTC-TP for the HIV-1 RT is lower than that of 3TC-TP, resulting in a good selective index with respect to cellular DNA polymerases. In addition, we summarized the results of expanded *in vitro* toxicity studies, including studies of the effect of dOTC on HepG2 mitochondria and on murine bone marrow progenitor cells and activity studies with drug-resistant isolates of HIV-1. This nucleoside analogue is also shown to have good oral bioavailability in rats and is able to penetrate the central nervous systems (CNSs) of these rodents.

MATERIALS AND METHODS

Materials. The cytosine nucleoside analogue dOTC and its enantiomers as well as 3TC were synthesized at BioChem Pharma as described previously (1, 14, 15). For enzyme inhibition studies and/or as controls for intracellular metabolite analysis (–)-dOTC and (+)-dOTC were chemically converted into their monophosphate (MP), diphosphate (DP), or TP derivatives by the methodology reported by Highcock et al. (7). The ^3H -labeled versions of (+)-dOTC and (–)-dOTC (specific activities, 2.3 Ci/mmol) were obtained from International Isotopes Clearing House, while ^3H]3TC (specific activity, 12 Ci/mmol), 3TC-phosphorylated standards, and ^3H]AZT (specific activity, 18.2 Ci/mmol) were purchased from Moravek Biochemicals (Brea, Calif.). ^3H]dCTP was purchased from Dupont-New England Nuclear. The nucleoside analogues ddC and AZT as well as all natural nucleosides and deoxynucleosides, including their phosphorylated derivatives, were purchased from Sigma Chemical Co. (St. Louis, Mo.); 3TC, however, was obtained from Glaxo-Wellcome. High-pressure liquid chromatography (HPLC)-grade acetonitrile (UltimAR grade), methanol (UltimAR grade), acetic acid, and ammonium hydroxide were from Mallinckrodt (Paris, Ky.). Ficoll-Paque and phytohemagglutinin (PHA-P) were purchased from Pharmacia LKB Biotechnology Inc. Human recombinant interleukin-2 (IL-2) and alkaline phosphatase from calf intestine were obtained from Boehringer Mannheim, Laval, Quebec, Canada. Proteinase K, restriction enzymes, salmon sperm DNA, cell culture medium (RPMI 1640 medium and minimal essential medium), fetal calf serum, and other supplements were purchased from Life Technologies (Burlington, Ontario, Canada). [α - ^{32}P]dCTP (specific activity, 3,000 Ci/mmol) and nitrocellulose Hybond-C were purchased from Amersham. Plasmid pA, which contains the 1.5-kb fragment (*Eco*RT) of the human 28S ribosomal DNA (rDNA) in pBR322, and Bluescript SK plasmid N6, which contains the *Nsi*-generated 6-kb (nucleotides 49 to 6089) fragment of the human mitochondrial DNA (mtDNA) and which was used to generate the molecular probes, were generous gifts from Adolf Ruiz-Carrillo (Centre de Recherche Hôtel-Dieu, Quebec City, Quebec, Canada). Agarose gel extraction kits were purchased from Qiagen (Hilden, Germany), and Ready-to-Go kits were purchased from Pharmacia (Baie D'Urfée, Quebec, Canada). Other fine chemicals were purchased from Sigma Chemical Co., Fisher Scientific (Nepean, Ontario, Canada), or Calbiochem (San Diego, Calif.).

The human T-lymphoblastoid cell line CEM deoxycytidine kinase containing [dCK⁺]; wild type) and the MOLT-4 (acute lymphoblastic leukemia), DU-145 (prostate carcinoma), HT-1080 (fibrosarcoma), and HepG2 (hepatocellular carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, Md.). One normal cell line, human skin fibroblasts (HSFs), was obtained from M. Chevrette (McGill University, Montreal, Quebec, Canada). The dCK-deficient (dCK[–]) CEM cell line was kindly provided by A. Fridland (St. Jude Children's Research Hospital, Memphis, Tenn.).

Cell cultures. Cells in suspension were cultured with RPMI 1640 medium supplemented with 10% standard heat-inactivated fetal bovine serum (st FBS) and 1% L-glutamine, while adherent cells were cultured with minimal essential medium supplemented with st FBS, 1% nonessential amino acids, 1% sodium pyruvate, and 1% L-glutamine. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of HIV-1-negative donors as described previously by Ojwang et al. (19). PBMCs were stimulated by the addition of 5 μg of PHA-P (Pharmacia) per ml and 10 U of IL-2 per ml and were incubated for 48 h prior to use. Fresh medium was added each 24 h to maintain a constant IL-2 concentration. If quiescent cells were required, PHA-P and IL-2 were omitted and the cells were used on the following day. All cultures were routinely checked for *Mycoplasma* infection and were incubated at 37°C in a humidified 5% CO₂ atmosphere.

DNA polymerase assays. Purified recombinant HIV-1 RT was produced as described by Gu et al. (6). DNA polymerases α and β were purchased from Molecular Biology Resources (Milwaukee, Wis.). DNA polymerase γ was purified from the mitochondria of CEM cells by affinity chromatography (single-stranded DNA cellulose column) by a modification of published procedures (20). The effects of nucleoside TPs on DNA polymerase activity were determined by published procedures (6, 16, 19, 20). For HIV-1 RT assays, we used primed 16S rRNA from *Escherichia coli* (3.33 $\mu\text{g}/\text{ml}$) in a 50- μl reaction volume containing 50 mM Tris (pH 8.0), 50 mM KCl, 10 mM MgCl₂, 4 mM β -mercaptoethanol, 3% glycerol, 1 mg of bovine serum albumin per ml, 30 μM RT, 10 μM (each) dATP,

dGTP, and dTTP, and various concentrations of ^3H]dCTP at the measured K_m . DNA polymerase α activity was measured in a 50- μl reaction volume containing 50 mM Tris (pH 8.0), 1 mg of bovine serum albumin per ml, 10 mM MgCl₂, 1 mM dithiothreitol, 20 mM potassium phosphate (pH 8.0), 100 μg of gapped duplex DNA per ml, 100 μM enzyme, 50 μM (each) dTTP, dGTP, and dATP, and various concentrations of ^3H]dCTP. DNA polymerase β and γ activities were measured as described above for measurement of DNA polymerase α activity, except that 20 mM potassium phosphate was replaced with 100 mM KCl. After incubation at 37°C for 60 min, the DNA in each sample was precipitated onto glass fiber filters by using a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. The filters were washed, and the radioactivity was counted. Six half-log dilutions of each compound were run in duplicate. The data presented are the averages of two or more experiments. The 50% inhibitory concentration (IC₅₀) of each compound was determined by fitting the data (percentage of solvent control versus log concentration) to a straight line. Kinetic constants were determined at 37°C as described by Reardon (23).

Antiviral assays. Low-passage virus isolates were obtained from patients treated for 32 weeks with 3TC, AZT, or both at St. Mary's Hospital, London, Ontario, Canada, or at Jewish General Hospital, Montreal, Quebec, Canada. All clinical isolates were amplified by a single passage in cultured PBMCs, and the amino acids present at various positions in the RT gene were characterized by the HIV-1 RT Line Probe Assay (LIPA HIV-1 RT; Murex Innogenetics, Ghent, Belgium) after amplification of RT proviral DNA by PCR as described by Stuyver et al. (32). The sensitivities of the clinical isolates to dOTC, 3TC, and AZT were determined by measuring the reduction in p24 antigen or RT levels in the cell culture fluid after growth in PBMCs as described previously (19).

Intracellular metabolism and catabolism. dCK⁺ CEM cells, dCK[–] CEM cells, and PBMCs were maintained in culture as described above. To ensure exponential and asynchronous growth, cells were plated in 5 ml of culture medium at a density of 0.5×10^6 CEM cells/ml, 0.75×10^6 stimulated PBMCs/ml, or 1.0×10^6 quiescent PBMCs/ml in a 50-ml flask; and the flasks were incubated for different time periods (0 to 24 h) in the presence of ^3H -labeled nucleosides analogues. In addition, to determine the kinetics of clearance (half-life [$t_{1/2}$]) of the accumulated nucleosides and their metabolites, after the 24 h of incubation in the presence of radiolabeled nucleoside, the cells were centrifuged at $500 \times g$ for 10 min at room temperature, resuspended in 10 ml of fresh, drug-free culture medium, and then incubated for an additional 0 to 24 h. At the end of selected incubation periods, the cells were harvested by centrifugation (2,200 rpm for 10 min at 4°C) and washed once in 10 ml of cold phosphate-buffered saline (PBS). The cell pellets were resuspended in 1 ml of cold PBS, centrifuged for 1 min at $16,000 \times g$, and then resuspended in 300 μl of cold PBS. The nucleosides and nucleotides were extracted by adding 100 μl of a 40% trichloroacetic acid solution to the cells and incubating the mixture for 20 min at 4°C. The cells were then centrifuged for 5 min at $16,000 \times g$ at 4°C, neutralized with 400 μl of triethylamine-trichlorotrifluoroethane (1:4), and spun again for 1 min at 14,000 rpm. The aqueous phase was stored at –20°C until HPLC analysis.

Radioactive samples were analyzed by HPLC with a C₁₈ reversed-phase column (5 μm , 120 Å, 4.6 mm [inner diameter] by 250 mm; YMC-005-A). The injection volume was 200 μl per sample. The isocratic mobile phase consisted of sodium dihydrogen phosphate-dissodium hydrogen phosphate buffer (140 mM; pH 6.7) and tetrabutylammonium dihydrogen phosphate (7.5 mM) and was used at a flow rate of 1 ml/min for 50 min. The absorbance determinations were made at 276 nm with a Waters Associates detector, and for the radioactivity measurements, we used an on-line radioactivity detector with a liquid scintillation cell. Chemically synthesized MP, DP, and TP derivatives of (+)-dOTC, (–)-dOTC, or 3TC were used as analytical controls.

To determine the nature of the major unknown metabolite of dOTC in dCK⁺ CEM cells, 2.5×10^6 cells (0.5×10^6 cells/ml) were cultured in RPMI 1640 medium with L-glutamine, penicillin, and streptomycin, supplemented with 10% st FBS or dialyzed fetal bovine serum (dial FBS) for 24 h at 37°C. The medium also contained unlabeled dOTC (0 to 25 μM) and [*methyl*- ^3H]choline (0.05 to 0.1 μM ; specific activity, 85 Ci/mmol) or [^3H]ethanolamine (0.25 μM ; specific activity, 27 Ci/mmol). At the end of the incubation period, the cells were harvested and washed twice in cold PBS and the intracellular nucleotides were extracted and analyzed as described above.

Enzymatic degradation of drug metabolites. Portions of cell extracts (200 μl) obtained from CEM cells incubated with ^3H]dOTC were treated with alkaline phosphatase (50 U/ml for 30 min at 37°C) in order to identify which peaks correspond to nucleotides derivatives. The reaction products were then directly separated by HPLC as described above.

Toxicity evaluation. Cell proliferation studies were performed as described previously (13–15). In addition, we assessed the effects of dOTC and its enantiomers on ^3H]thymidine uptake into logarithmic-phase tumor (solid and leukemic) and normal cell lines including murine bone marrow progenitor cells. We also analyzed the effect of a longer-term treatment (14 days) of dOTC on the mtDNA contents of HepG2 cells and the effect of dOTC *in vivo* using a 14-day repeat dose study with rats.

(i) **Bone marrow cell methods.** Bone marrow was collected from the femoral bone of one CD-1 male mouse per assay. The cells were plated in 24-well plates at a concentration of 1.5×10^6 cells/ml of Iscove's modified Dulbecco's medium with 2% fetal bovine serum in a semisolid medium (MethoCult M3434) which contained recombinant growth factors including IL-3, IL-6, granulocyte-mac-

rophage colony-stimulating factor (GM-CSF), and erythropoietin. The plates were incubated for 10 to 12 days at 37°C with 5% CO₂ with or without the addition of test compound. The colonies (GM-CSF treated) were counted under an inverted microscope. The data were expressed as percent inhibition compared with that for control (nontreated) cells.

(ii) **Analysis of mtDNA.** HepG2 cells were plated into 25-cm² tissue culture flasks and were treated or were not treated with various nucleoside compounds at concentrations of 0.1 to 10 μM. Fresh medium containing drug was added twice per week, and the cells were split once per week at a dilution of 1:5. After 14 days of incubation, the cells were washed once in 12 ml of cold PBS, briefly pelleted (500 × g for 7 min), resuspended in 3 ml of lysis buffer (100 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulfate) containing proteinase K to a final concentration of 200 μg/ml, and incubated overnight at 37°C. An equal volume of phenol was then added, and the mixture was rocked for 1 h at room temperature before centrifugation (2,000 × g for 5 min). The aqueous layer was then extracted with an equal volume of chloroform, and then the nucleic acids were precipitated by standard laboratory methods (25).

Plasmid pA (which contains the 1.5-kb fragment of 28S rDNA) was digested with *SacI* and plasmid N6 (which contains the 6-kb mtDNA fragment) was digested with *HindIII* and *BamHI* for 2 h at 37°C. The restriction fragments obtained were separated by electrophoresis on an agarose gel. The corresponding molecular probe bands were excised from the gels and radiolabeled to a specific activity of 2 × 10⁹ cpm/μg of DNA as described previously (18). The hybridization probes were used within 24 h of labeling.

Hybridization reactions were performed on nitrocellulose (Hybon-C) according to the manufacturer's instructions (Amersham), with few modifications. Approximately 6 μg of purified genomic DNA was digested with 80 U of *SacI* for 4 h at 37°C. The DNA fragments (1.7 μg of total DNA) were separated on a 0.8% agarose gel before transfer to the nitrocellulose membranes (25). The filter membranes were then prehybridized, hybridized with the molecular probes as described previously (18), and then washed before exposure to X-ray film. The resultant autoradiograms were scanned with a CS9000U dual-wavelength flying-spot densitometer (by using alpha imager 2000 software [Packard]). The amount of mtDNA present in each sample was determined as a ratio of the 6-kb fragment of human mtDNA probe signal to the 1.5-kb fragment of human 28S rDNA probe signal, which was independent of the amount of DNA loaded.

(iii) **[³H]thymidine uptake studies.** MOLT-4, DU-145, HSF, HT1080, and HepG2 cells were cultured as described above and were treated at the mid-logarithmic phase with various concentrations of test compounds for 4 days. [³H]thymidine (0.5 μCi/well) was added to each culture 18 h before it was harvested. The cells were then washed once in PBS, adherent cultures were trypsinized, and cells were collected with a cell harvester (Wallac, Turku, Finland) and then filtered onto glass fibers. The degree of intracellular radioactivity was determined with a liquid scintillation counter (Microbeta 1451; Wallac).

(iv) **Repeat-dose oral toxicity study with rats.** A total of 48 Sprague-Dawley rats (24 males and 24 females) were used in the repeat-dose oral toxicity study. The rats were divided into four groups with six rats of each sex per group. The rats in the control group received 0.5% (wt/vol) carboxymethyl cellulose, while the rats in the treatment groups received dOTC at dosages of 50, 250, or 500 mg/kg of body weight per day in 0.5% carboxymethyl cellulose. The parameters monitored during the course of the study included clinical signs, body weight, food consumption, and ophthalmological condition. Plasma samples for toxicokinetic determinations were obtained from three rats of each sex per group per time point at 0.5, 1, and 4 h after the 1st and 14th drug administrations. Hematology, coagulation, clinical chemistry, and urine analyses were performed for all study animals at the end of the treatment period (day 15). The animals were killed and necropsied on day 15. Organ weights were recorded, and histopathological examinations of tissues from all control animals and all animals in the high-dose group, all gross lesions, and the adrenals from males in the low- and intermediate-dose groups were performed. In addition, evaluation of bone marrow smears was performed for control animals and animals in the high-dose group.

Pharmacokinetics in rats. The pharmacokinetic profiles of dOTC and its two enantiomers were assessed in male and female Sprague-Dawley rats (15 rats of each sex per group). The rats received a dosage of 10 mg/kg once a day orally by gavage or intravenously (i.v.) by bolus injection. For oral administration, the test compounds were suspended in carboxymethyl cellulose (0.5% [wt/vol]), while for i.v. administration, the test compounds (2 mg/ml) were suspended in 0.9% sodium chloride. Blood samples were collected from five animals of each sex per time point at 15 or 30 min and again at 1, 2, 4, 8, 12, or 24 h following i.v. or oral administration of the test compound. Following i.v. administration, one additional sample was collected at 5 min postdosing. Blood samples were collected in heparinized tubes. Following collection, the samples were centrifuged and the plasma was aspirated and stored frozen (-20°C). The animals were deprived of food but not water (overnight) prior to the day of dosing.

Plasma samples (200 μl) were diluted 1:1 with reverse osmosis-treated water, and the dOTC enantiomers were extracted by the addition of acetonitrile. To do this, 560 μl of acetonitrile was added to the diluted plasma samples and the mixtures were then mixed vigorously. The mixture was centrifuged at 2,000 × g at 4°C for 30 min, after which 800 μl of the supernatant was transferred to a new tube and was dried under vacuum at 50°C. The dried sample was reconstituted in 50 μl of the mobile-phase HPLC buffer, which consisted of 1% (vol/vol)

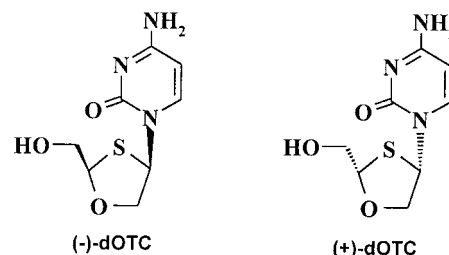


FIG. 1. Chemical structures of the enantiomers of dOTC.

acetonitrile-1% triethylamine (pH 6.8). Chiral HPLC analysis was performed with a Cyclobond 1 RSP 2000 column under isocratic conditions (1% [vol/vol] acetonitrile, 1% [vol/vol] triethylamine [pH 6.8]) run at approximately 0°C for 40 to 60 min.

Pharmacokinetic analysis was performed with the standard computer software program WinNonlin (version 1.5A). All results were verified by running the same analyses on the standard computer software program NonLin 84. Plasma concentration-versus-time curves were fitted to pharmacokinetic models, and the appropriate parameters were derived.

CNS penetration studies. Male Sprague-Dawley rats were fitted with cisterna magna catheters and tail vein catheters as described by Sarna et al. (26). The test compounds were administered orally at a dose of 5 mg of the unlabeled nucleoside per kg with different amounts of labeled compound on the basis of their respective specific activities: (i) AZT, 10 μCi (specific activity, 18.2 Ci/mmol); (ii) 3TC, 15 μCi (specific activity, 12.0 Ci/mmol); (iii) dOTC as 50 μCi of (-)-dOTC and 50 μCi of (+)-dOTC (specific activity of each, 2.3 Ci/mmol); and (iv) (-)-dOTC, 80 μCi (specific activity, 2.3 Ci/mmol). Blood and cerebrospinal fluid (CSF) samples (25 μl) were simultaneously collected over a 5-h period, and radioactivity was determined by liquid scintillation counting. The blood samples were collected at 0, 5, 15, 30, 60, 90, 120, 180, 240, and 300 min postadministration, while the CSF samples were collected at 0, 30, 60, 120, 180, 240, and 300 min postadministration of test compound. Each drug experiment was replicated with six animals.

The analysis proceeded in two stages: the first stage dealt with a general statistical analysis of data, and the second dealt with calculations of the area-under-the-curve (AUC) integrals, i.e., integrals of the concentrations over time, and evaluations of the quantities of the drugs that passed through the individual compartments. These evaluations were fitted to a simple two-compartment model of transport. Since the time interval covered by the experiment did not reach the end of drug presence (i.e., until the time when the concentration drops below a measurable threshold), the AUC was evaluated by using corresponding compartment models.

RESULTS

Inhibition of cellular polymerases. For enzyme inhibition studies, (-)-dOTC and (+)-dOTC (Fig. 1) as well as 3TC were chemically converted into their TP derivatives which, along with the parent nucleosides, were assessed for their ability to interfere with HIV-1 RT as well as DNA polymerases α, β, and γ. The *K_m*s of dCTP for the four polymerases were determined first (Table 1), and these values were used, along with the measured nucleotide inhibition constant (*K_i*), to assess the relative biochemical selectivity of dOTCs for HIV-1 RT.

As expected, the IC₅₀s of the parent nucleosides were >1 mM for all four polymerases tested (data not shown). All of the nucleosides were inactive at 1 mM, the highest test concentration, with the possible exception of (-)-dOTC with DNA polymerase α. At 1 mM this compound inhibited the polymerase by 25%, which was not significant compared to the inhibition seen when the nucleoside TP derivatives were tested (Table 1).

The *K_i* of dOTC-TP, the TP derivatives of its enantiomers, and 3TC-TP were then determined for HIV-1 RT and the three human polymerases (Table 1). For HIV-1 RT reactions, dOTC-TP, (-)-dOTC-TP, and (+)-dOTC-TP were all determined to be more potent inhibitors of RT than 3TC-TP. The two most active compounds, dOTC-TP and (+)-dOTC-TP, were 5- to 10-fold more potent than 3TC-TP (Table 1). The *K_i/K_m* ratio in the RT assay for dOTC was 0.049 (Table 1),

TABLE 1. K_i s of dOTC-TP for HIV-1 RT and human DNA polymerases^a

Enzyme	dCTP K_m (μ M)	K_i (μ M) of TPs of the following nucleosides:				K_i/K_m ratio			
		(+)-dOTC	(-)-dOTC	dOTC	3TC	(+)-dOTC	(-)-dOTC	dOTC	3TC
HIV-1 RT	0.57 ± 0.15	0.012	0.08	0.028	0.16	0.02	0.14	0.049	0.28
DNA polymerase α	1.45 ± 0.49	100	300	140	56	68.9	206.9	96.5	38.6
DNA polymerase β	1.69 ± 0.23	2.2	12	2.2	14.5	1.3	7.1	1.3	8.6
DNA polymerase γ	0.53 ± 0.18	19.25	111.25	16	55	36.3	209.9	30.1	103.8

^a The K_m determinations are presented as the means \pm standard deviations. All other values are the averages of two or more independent experiments.

which compares favorably with those reported for other nucleoside analogue TPs such as carbovir-TP (CBV-TP) and ddA-TP (4). In the human DNA polymerase studies, all test compounds were more active against DNA polymerase β than DNA polymerase α or γ . Even though different assay conditions were used to evaluate the nucleoside analogues against the viral and human polymerases, the K_i/K_m ratios were sufficiently high for all test compounds to allow for good selectivity comparisons. The K_i values of dOTC for DNA polymerases α , β , and γ were 5,000-, 78-, and 571-fold higher, respectively, than those for HIV-1 RT (Table 1).

Intracellular metabolism of dOTC. The intracellular metabolism of dOTC was studied with CEM cells and PBMCs. In these experiments dCK⁺ and dCK⁻ CEM cells (9) or PBMCs were incubated for 24 h with [³H]dOTC or [³H]3TC, at which time the intracellular metabolites were isolated and analyzed by HPLC. The results revealed that in dCK⁺ CEM cells and PBMCs the parent nucleoside of dOTC is metabolized into readily identifiable MP, DP, and TP derivatives (Fig. 2; Tables 2 and 3). Several unknown metabolites (UMs) were also identified in the HPLC chromatogram. In dCK⁺ CEM cells and PBMCs, two major UMs for dOTC were observed; these UMs had retention times of approximately 6.8 (UM1) and 8.3 (UM2) min, respectively (Fig. 2). It is interesting that a peak that migrated to a point analogous to that of UM2 was observed in cells incubated in the presence of [³H]3TC (Tables 2 and 3). After a 24-h incubation of dCK⁺ CEM cells with the individual enantiomers of dOTC, the intracellular levels of UM1 and UM2 were determined to be approximately 1.5- and

3.6-fold higher in the (+)-dOTC-treated cells than in the (-)-dOTC treated cells (Table 2). The differences between UM1 and UM2 were more pronounced in the studies with activated PBMCs (Table 3). In addition, we observed a higher level of the MP derivative but lower levels of the DP and TP derivatives of (+)-dOTC relative to the levels of these derivatives of (-)-dOTC in the two cell systems studied (Tables 2 and 3). In the assays with dCK⁻ CEM cells (Table 2) there was no accumulation of dOTC-MP, -DP, or -TP derivatives, suggesting that dOTC is converted to its MP derivatives in dCK⁺ cells by the normal cellular enzymatic pathway (9). In dCK⁻ CEM cells a third UM (UM3, with an HPLC retention time of approximately 14 min) which accounted for 12% of the recovered radioactivity was observed. This peak was not observed in dCK⁺ CEM cells or PBMCs. dOTC extracts obtained from the treated cells were also subjected to alkaline phosphatase digestion, and as expected, the phosphorylated nucleoside metabolites were converted back into the parent nucleoside, while the levels of UM1 and UM2 remained relatively unchanged (data not shown). It is interesting that UM3, which was observed in dOTC-treated dCK⁻ cell extracts, was sensitive to alkaline phosphatase treatment.

A time course experiment was used to monitor the accumulation of [³H]dOTC and its metabolites in PHA-P- and IL-2-stimulated PBMCs. The results were compared to those obtained with the control compound [³H]3TC (Fig. 3). In both PBMCs (Fig. 3) and CEM cells (data not shown), the accumulation of the MP, DP, and TP derivatives of dOTC was fairly rapid over the first 8 h of incubation and leveled off by the 24-h

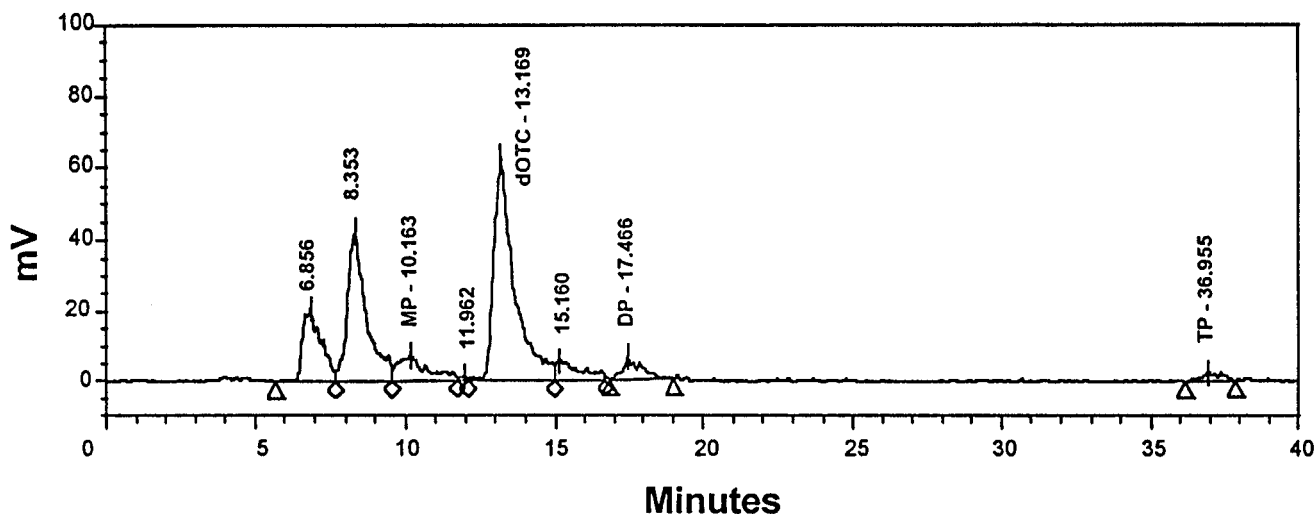


FIG. 2. Reverse-phase (C_{18}) HPLC elution profile of a 10% trichloroacetic acid extract of activated PBMCs incubated for 24 h with 2.5μ M [³H]dOTC (specific activity, 2.3 Ci/mmol). The column was equilibrated in the isocratic running buffer as described in Materials and Methods before application of the test sample. For this particular experiment, the retention times of the parent nucleoside (13.169 min) and its MP (10.163 min), DP (17.466 min), and TP (36.955 min) derivatives were determined by using chemically synthesized standards.

TABLE 2. Intracellular metabolites of dOTC and 3TC in dCK⁺ and dCK⁻ CEM cells

Peak ^a	% of the indicated total intracellular [³ H]nucleoside metabolites in the following cells ^b :				
	dOTC-treated dCK ⁻ CEM cells	dCK ⁺ CEM cells			
		(+)-dOTC	(-)-dOTC	dOTC	3TC
UM1	0	16	9	15 ± 3	0
UM2	1	58.5	17.5	53.0 ± 2.0	12
UM3	12	0	0	0	0
MP	0	18	8	13 ± 4	10
Parent	81	4	21.5	8 ± 2	3
DP	0	2	29	6.0 ± 0.9	53
TP	0	0.3	13.5	3.6 ± 2.3	18

^a The peak names correspond to the UMs (UM1, UM2, or UM3) and MP, DP, or TP derivative of the parent nucleoside determined by reversed-phase (C₁₈) HPLC as described in Materials and Methods.

^b Percentage of total intracellular metabolites following 24 h of incubation with 1 μM [³H]3TC (specific activity, 12 Ci/mmol) or 2.5 μM ³H-labeled dOTC, (-)-dOTC, or (+)-dOTC (specific activity, 2.3 Ci/mmol). The data are presented as the averages of two independent experiments or as the averages of three or more experiments with the standard deviations.

time point (Fig. 3). The maximum accumulation of the TP derivative of 3TC in stimulated PBMCs reached 1.2 pmol/10⁶ cells (17% of total intracellular 3TC) at 24 h; however, in the same cell system dOTC-TP levels were only 0.05 pmol/10⁶ cells (2.3% of total intracellular dOTC) at 24 h (Table 3; Fig. 3). The values obtained for the intracellular metabolism of 3TC after a 24-h incubation with 1 μM drug are consistent with those reported in the literature (3, 10).

The *t*_{1/2} of dOTC and its intracellular metabolites was determined with PHA-P- and IL-2-stimulated PBMCs and dCK⁺ CEM cells. In this experiment the cells were incubated in the presence of 2.5 μM [³H]dOTC for 24 h, at which time drug was removed from the culture medium and the cells were further cultured for defined periods of time. The results from this experiment determined that the intracellular *t*_{1/2} of the parent nucleoside, dOTC, was approximately 1 h, while the *t*_{1/2}s of the MP, DP, and TP derivatives were found to be 3.1, 2.2, and 3.0 h, respectively. The *t*_{1/2}s of UM1 and UM2 were also measurable and were determined to be 2.0 and 6.3 h, respectively.

When Tornevik and Eriksson (34) reported on the toxicity of ddC for cultured CEM cells, they identified several metabolites of ddC as liponucleotides, presumably, ddCDP-choline and ddCDP-ethanolamine. For this reason, we incubated dCK⁺

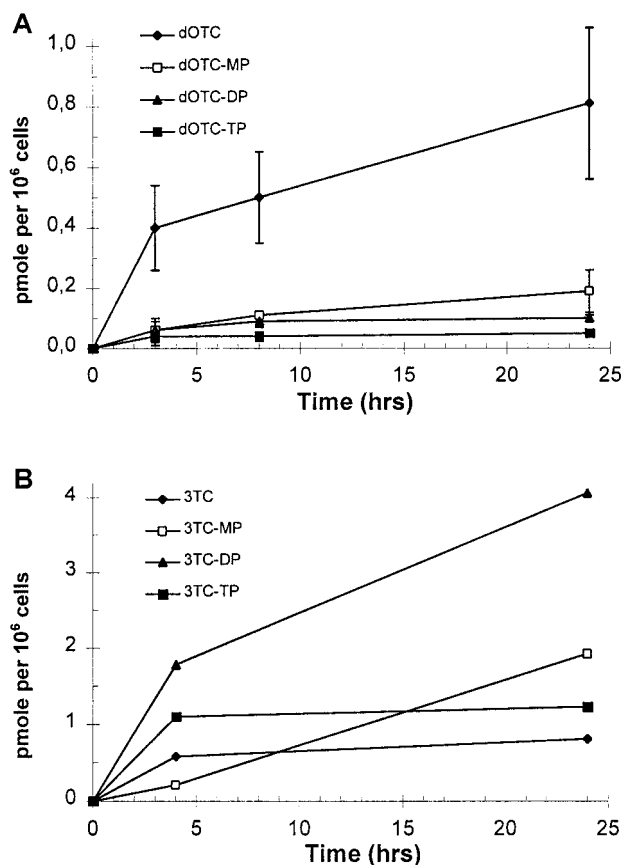


FIG. 3. Intracellular levels of dOTC or 3TC and their metabolites in PHA-P- and IL-2-stimulated PBMCs following continuous exposure of the cells to ³H-labeled nucleoside for up to 24 h. In this experiment the cells were incubated in the presence of 2.5 μM [³H]dOTC (specific activity, 2.3 Ci/mmol) (A) or 1 μM [³H]3TC (specific activity, 12 Ci/mmol) (B) for the indicated lengths of time. The data presented for dOTC are the averages of two or more independent experiments, while the data presented for 3TC represent data from one experiment.

CEM cells in the presence of [³H]ethanolamine or [³H]choline and increasing concentrations of dOTC. When the cells were incubated in the presence of [³H]ethanolamine, upon HPLC analysis one peak with a retention time of approximately 8.1 to 8.4 min (in two different experiments) was observed. This peak, which has a retention time similar to that of UM2 found in the

TABLE 3. Intracellular metabolites of dOTC and 3TC in quiescent and activated PBMCs

Peak ^a	% of the indicated total intracellular [³ H]nucleoside metabolites in the following PBMCs ^b :							
	Quiescent				PHA-P IL-2 activated			
	(+)-dOTC	(-)-dOTC	dOTC	3TC	(+)-dOTC	(-)-dOTC	dOTC	3TC
UM1	46	32	45	0	16	5	14.8 ± 2.4	0
UM2	33	5	28	18	50	7	34.4 ± 4.7	6
MP	12.5	5	11	2	15	6	7.7 ± 2.6	17
Parent	6	12	6	4	17	64	34.4 ± 5.6	10
DP	2	31	7	41	2	13	3.7 ± 1.1	50
TP	<1	12	3	34	<1	9	2.3 ± 0.6	17

^a The peak names correspond to the UMs (UM1 and UM2) and the MP, DP, or TP derivative of the parent nucleoside determined by reversed-phase (C₁₈) HPLC as described in Materials and Methods.

^b Percentage of total intracellular metabolites following 24 h of incubation with 1 μM [³H]3TC (specific activity, 12 Ci/mmol) or 2.5 μM ³H-labeled dOTC, (-)-dOTC, or (+)-dOTC (specific activity, 2.3 Ci/mmol). The data are presented as the averages of two independent experiments or as the averages of three or more experiments with standard deviations.

TABLE 4. Effect of dOTC on choline and ethanolamine metabolism in dCK⁺ CEM cells

Medium ^a	Compound concn (μM) ^b			Radioactivity (cpm [10 ⁶]) at the following peak retention times (min) ^c :			
	[³ H]choline	[³ H]ethanolamine	dOTC	3.8	5.3	6.2	8.1
dial FBS	0.05	0	0	8.9	0	0	0
dial FBS	0.05	0	2.5	10.0	1.4	0	0
dial FBS	0.05	0	10	11.8	0	0	0
dial FBS	0.05	0	25	12.6	0	0	0
dial FBS	0.1	0	0	19.5	0	0	0
dial FBS	0.1	0	2.5	21.3	0	0	0
dial FBS	0.1	0	10	18.4	0	0	0
dial FBS	0	0.25	0	41.8	9.8	3.6	0
dial FBS	0	0.25	2.5	24.1	4.8	2.1	0.6
dial FBS	0	0.25	10	30.8	6.5	2.6	2.2
dial FBS	0	0.25	25	27.7	8.2	0	3.8
st FBS	0	0.25	0	51.1	8.3	5.8	0.1
st FBS	0	0.25	2.5	52.1	9.4	5.5	1.4
st FBS	0	0.25	10	53.8	12.6	2.4	2.4
st FBS	0	0.25	25	56.4	14.0	2.4	6.6

^a The medium used to culture the CEM cells was supplemented with either dial FBS or st FBS.

^b The test compounds, dOTC, [³H]choline, or [³H]ethanolamine, were added to the culture medium at the indicated concentrations.

^c The data presented are the radioactivity in each peak after chromatographing 10 μl of cell extract on a reversed-phase HPLC column under the same conditions used for chromatography of the [³H]dOTC cell extracts. The data represent the averages of two or more experiments.

chromatogram obtained by HPLC of dOTC (Fig. 2), increased in size upon addition of increasing amounts of unlabeled dOTC to the culture medium (Table 4). In similar experiments with [³H]choline, no peaks with retention times similar to those seen for UM1 and UM2 were observed by HPLC. These data suggest that UM2 is the ethanolamine adduct of the two cytosine nucleoside analogues found in dOTC.

Tissue culture studies. The RT genotypes of the laboratory strains and low-passage clinical isolates of HIV-1 were assessed by the line probe assay as described in Materials and Methods. The viruses were grouped into three categories: those with wild-type RT genomes, those which were predominantly 3TC resistant (i.e., those in which only the M184V mutation was detected), and those which harbored multiple mutations in their RT genes and which were, at a minimum, resistant to both 3TC and AZT. We also compared these results to the efficacies of the nucleoside analogues against HIV-1_{RF}. The sensitivities of clinical isolates to dOTC, 3TC, and AZT were determined by measuring the reduction in viral p24 antigen levels in the cell culture fluid after growth in PBMCs.

In all, five viral isolates with no mutations at the indicated positions (wild type), 12 isolates with the M184V mutation, and 8 isolates with multiple mutations were assessed, in addition to HIV-1_{RF} (Table 5). In these experiments dOTC or its

enantiomers were less effective than 3TC and AZT against HIV-1_{RF} and wild-type (wild-type RT gene) isolates of HIV-1. However, there was only a small change in the activity of dOTC or (–)-dOTC (approximately twofold) when they were tested against strains resistant to 3TC or to both 3TC and AZT. In the control experiment, resistance to 3TC increased more than 100-fold (Table 5) when the same resistant clinical isolates were used.

Development of viral resistance. To study the kinetics of the emergence of HIV-1 resistance to dOTC, C1866 cells were infected with HIV-1_{RF} and were then cultured in the presence of increasing concentrations of dOTC, its individual enantiomers, or 3TC. After 12 passages no phenotypic resistance was observed for virus cultured in the presence of dOTC or (–)-dOTC; however, by passage 6, virus resistant to 3TC and (+)-dOTC had emerged (data not shown). Sequence analysis identified the presence of an M184I mutation in the RT gene of virus grown in the presence of (+)-dOTC. It is interesting that (+)-dOTC closely resembles L-nucleoside analogues 3TC and fluorothiacytidine (FTC) in its modified furanosyl ring (15), and as shown here, like 3TC and FTC, (+)-dOTC can quickly elicit the M184 mutation (28). The delay in the development of resistance to dOTC and (–)-dOTC, given the quick emergence of M184I changes in the RT gene for viruses treated only with

TABLE 5. In vitro efficacy of dOTC against clinical isolates of HIV-1 in cultured PBMCs

Virus phenotype	No. of isolates ^a	LiPA results ^b	Mean ± SE IC ₅₀ (μM)				
			dOTC	(–)-dOTC	(+)-dOTC	3TC	AZT
HIV-1 _{RF}	1		0.5	0.2	0.28	0.045	0.0035
Wild type	5	M41, T69K70, L74V75, M184, F214T215	1.76 ± 0.77	1.45 ± 0.74	1.54 ± 0.71	0.50 ± 0.21	<0.01
3TC resistant	12	V184 or M184/V184	2.53 ± 0.67	2.2 ± 0.86	7.0 ± 2.5	>100	<0.01
3TC and AZT resistant	8	V184, L41, Y215*	2.5 ± 0.41	2.4 ± 0.45	14.8 ± 2.9	>100	0.08 ± 0.04

^a Number of isolates of a given phenotype tested.

^b The RT genotype was assessed by a line probe assay (LiPA) as described in Materials and Methods.

TABLE 6. Analysis of cellular toxicities of dOTC, its enantiomers, AZT, and 3TC

Cell line ^a	CC ₅₀ (μM) ^b				
	(+)-dOTC	(-)-dOTC	dOTC	3TC	AZT
MOLT-4	>100	72	>100	ND ^c	3
DU-145	>100	>100	500	>500	>10
HSF	>100	>100	200	400	>10
HT-1080	>100	>100	>500	>500	5
HepG2	>100	>100	500	350	3
Bone marrow ^d	ND	ND	>500	>100	7.8

^a The data presented for MOLT-4, DU-145, HSF, HT-1080, and HepG2 cells are the results of [³H]thymidine uptake experiments. The bone marrow toxicity evaluation was performed by visual inspection, as described in Materials and Methods.

^b CC₅₀, 50% cytotoxic concentration.

^c ND, not determined.

^d Murine bone marrow cells were stimulated with GM-CSF, and the results presented are the averages of four experiments for 3TC and AZT and two experiments for dOTC.

(+)-dOTC, suggests that the desirable features of (-)-dOTC are maintained in the racemate.

Toxicity assays. We have previously reported on the cytotoxicities of the enantiomers of dOTC, which were monitored by a cell viability assay (15). In the current study, we have expanded on these observations using several different assay systems. The ability of dOTC to inhibit cell growth was determined by using [³H]thymidine uptake into logarithmic-phase tumor (solid and leukemic) and normal cell lines and by monitoring the effects of these compounds on colony formation of murine bone marrow progenitor cells. The results from these experiments show that dOTC was nontoxic in all cell systems tested (Table 6).

The results of a quantitative analysis of the mtDNA content in relation to the content of a cellular gene and a visual evaluation of cell confluence and morphology are presented in Table 7. In this experiment there was little effect on the ratio of mtDNA to cellular DNA when either 3TC or dOTC was used at concentrations up to 10 μM. This is in contrast to the

TABLE 7. Effects of dOTC, ddC, and 3TC on cell growth and mtDNA content^a

Compound	Concn (μM)	mtDNA signal (cpm)	28S rDNA signal (cpm)	mtDNA signal/28S rDNA signal	mtDNA signal/control mtDNA signal	% CF cells ^b
Control	0	93,342	25,872	3.6	100	60–75
ddC	0.1	87,318	36,036	2.4	67	60–75
	1.0	83,622	32,340	2.6	72	50
	10.0	0	0	0	5	5
3TC	0.1	82,236	26,796	3.1	85	50–60
	1.0	95,172	28,644	3.3	92	50–60
	10.0	91,476	24,024	3.8	105	60–75
dOTC	0.1	97,482	23,562	4.1	115	60–75
	1.0	98,868	23,562	4.2	116	60–75
	10.0	99,330	26,796	3.7	103	50–60

^a Nucleic acids were extracted from drug-treated HepG2 cells after 14 days in culture and were analyzed by hybridization to specific molecular probes as described in Materials and Methods. The data (averages of two experiments) are presented as the quantitation of the resultant hybridization analysis.

^b Percent confluent (CF) cells (visual inspection) at day 14.

effect seen with the control nucleoside analogue ddC, even when 0.1 μM drug was tested (Table 7).

To determine the toxic effects of dOTC in vivo we first attempted to obtain a maximum tolerated dose by performing a single dose escalation study. In this experiment no toxicity was observed when dOTC was administered orally to rats at concentrations of 50, 100, 500, 1,000, or 2,000 mg/kg (data not shown). Plasma drug concentrations increased in a dose-dependent manner following administration of 50, 100, and 500 mg of dOTC per kg. At doses of 1,000 and 2,000 mg/kg, concentrations in plasma were comparable to those obtained following administration of 500 mg/kg, indicating saturation of exposure at doses of between 500 and 1,000 mg/kg (data not shown). For this reason, 500 mg/kg was the highest dose administered in the repeat-dose toxicokinetic studies. In these studies similar plasma drug concentrations were observed on days 1 and 14 at 0.5, 1, 2, and 4 h postdosing. In addition, there was no suggestion of drug accumulation at these doses. For example, after administration of the first 500-mg/kg dose, the maximum concentrations of drug in serum (C_{max} s) were 18.2 μg/ml for males and 26.6 μg/ml for females. After administration of the last dose (day 14) the C_{max} s were 22.2 μg/ml for males and 29.4 μg/ml for females. Similarly, the AUCs from 0 to 4 h (AUC_{0-4} s) obtained after administration of the first and last doses were 3,496.2 and 3,640.2 μg · min/ml, respectively, for males and 4,542 and 4,878 μg · min/ml, respectively, for females. There were no deaths or compound-related changes in clinical signs, body weight, or food consumption levels associated with the administration of dOTC. All values were comparable between control and dOTC-treated groups (data not shown). There were no compound-related effects in clinical pathology. dOTC did not produce any significant effects on organ weights or significant findings upon macroscopic or microscopic examination of tissues. In addition, examination of bone marrow after administration of the highest dosage (500 mg/kg/day) did not reveal any changes (data not shown).

Pharmacokinetics of dOTC in rats. The pharmacokinetic profiles of dOTC and its two enantiomers were assessed in male and female Sprague-Dawley rats (15 rats of each sex per group). The rats received dOTC at 10 mg/kg once a day orally by gavage or i.v. by bolus injection (Table 8). The plasma concentration-versus-time curves for dOTC in rats following oral administration were fitted to one-compartment models with first-order elimination. There was a rapid absorption of dOTC following oral administration (data not shown), giving an apparent C_{max} of between 2 and 2.5 μg/ml, depending upon the gender of the rat. The time to C_{max} (T_{max}) for oral dosing, the plasma elimination $t_{1/2}$, and the mean AUCs extrapolated to infinity ($AUC_{0-\infty}$) are presented in Table 8. The oral bioavailability of the racemate was 77.7% for males and 76.4% for females. Pharmacokinetic parameter ratios of one enantiomer over the other did not deviate substantially from unity, indicating similar body dispositions of each enantiomer following the administration of the racemate.

CNS penetration. The radiolabeled nucleosides dOTC, (-)-dOTC, AZT, and 3TC were administered once to six male rats per group at 5 mg/kg orally by gavage, and blood and CSF samples were collected over a 5-h period. Using a two-compartment model, the measured uptake into blood was highest for dOTC and 3TC (Table 9). These two compounds also exhibited the largest AUC profiles in blood. In CSF the measured uptake and AUC values were again highest for dOTC and 3TC, respectively (Table 9). In all cases, most of the drug was cleared from the blood during the first 300 min. These results suggest that although all four compounds exhibit relatively short circulation times in blood and CSF, when admin-

TABLE 8. Pharmacokinetic parameters of dOTC in rats following administration of a single i.v. or oral dose^a

Gender	Compound	i.v. dosing				Oral dosing		
		AUC _{0-∞} (μg · min/ml)	t _{1/2} (min)	C _{max} (μg/ml)	T _{max} (min)	AUC _{0-∞} (μg · min/ml)	T _{1/2} (min)	% oral bioavailability
Male	(-)-dOTC	199.3	12.1	0.97	51.6	135.4	NA ^b	68.0
	(+)-dOTC	197.3	12.3	1.07	56.8	165.5	NA	83.9
	dOTC ^c	396.7	12.2	2.02	55.2	308.3	46.1	77.7
Female	(-)-dOTC	226.9	12.7	1.16	37.3	157.4	62.2	69.4
	(+)-dOTC	225.2	14.6	1.36	41.5	185.1	57.3	82.2
	dOTC ^c	450.2	13.6	2.51	39.4	343.9	60.4	76.4

^a The i.v. and oral dose was 10 mg/kg, as described in Materials and Methods.

^b NA, data not available.

^c Calculated as the sum of the values for (-)-dOTC and (+)-dOTC, as measured by a chiral HPLC assay.

istered at this low dose (5 mg/kg), dOTC is the compound that is the most efficiently retained.

DISCUSSION

The efficacy data presented here focused on the activity of dOTC against clinical isolates that harbor mutations against the structurally related nucleoside 3TC. When evaluated in primary cell cultures (PBMCs), dOTC and its enantiomeric constituents exhibited potent activities against HIV-1 clinical isolates which had wild-type (according to genotypic and phenotypic analyses) RT genes. In general, only a slight degree of cross-resistance to dOTC (two- to threefold) was observed when virus isolates with phenotypic resistance (as confirmed by genotypic analysis) to 3TC or 3TC and AZT were tested. However, when the enantiomers were tested individually, viruses that harbored the M184V mutation were moderately cross-resistant to (+)-dOTC. This result is consistent with a stereochemical rationale in that the TP and cytosine moieties of (+)-dOTC are oriented similarly to those in the L-nucleoside analogues 3TC and FTC (15), and as described above, virus cultured in the presence of (+)-dOTC alone quickly selects the M184I mutation. It is interesting that dOTC, the racemate, behaved in these assays in a fashion similar to that in which (-)-dOTC behaved. In addition, the mean IC₅₀s of dOTC and (-)-dOTC obtained for wild-type and 3TC- and/or AZT-resistant viruses were not statistically significant. These data suggest that the positive effect of (-)-dOTC on drug-resistant virus is maintained in the racemate, even though the overall concentration of (-)-dOTC in the racemate is only one-half that of the overall dOTC concentration.

No virus resistance has been observed up through 12 passages of HIV-1_{RF} in the presence of dOTC and (-)-dOTC. This feature of dOTC may provide a significant advantage when the drug is administered to humans. At the same time the

emergence of the M184I mutation was observed after only six passages in virus cultured in the presence (+)-dOTC. This is consistent with the observation that (+)-dOTC is less effective than (-)-dOTC against clinical isolates that harbor the M184V mutation. However, from the studies conducted with clinical isolates resistant to 3TC and/or AZT the level of cross-resistance of viruses with these particular mutations to (+)-dOTC is moderately low (4.6- to 9.8-fold) compared to that to 3TC in the same experiments (>100-fold; Table 5). The (-) enantiomer has a sugar configuration more like that seen in the natural deoxynucleoside triphosphate substrates and nucleoside analogues such as d4T, ddI, 1592U89, and ddC (4, 11, 33), and in a fashion similar to that seen with these other nucleoside analogues, the emergence of viral resistance takes longer. In addition, as observed for the activity of dOTC against drug-resistant clinical isolates of HIV-1, the desirable feature of (-)-dOTC is maintained in the racemic mixture in that the emergence of viral resistance is much slower for virus grown in the presence of dOTC than for virus grown in the presence of (+)-dOTC.

dCK (NTP:deoxycytidine 5'-phosphotransferase) is responsible for the formation of dCMP, and this enzyme is controlled by feedback regulation by the natural end product dCTP. The results of intracellular metabolism studies obtained with dCK⁻ and dCK⁺ CEM cells determined that dOTC is most likely converted to its MP derivative via the dCK pathway, since no dOTC-MP was observed within dCK⁻ CEM cells. In dCK⁺ CEM cells and activated PBMCs, all three phosphorylated derivatives (MP, DP, and TP) of both dOTC enantiomers were observed. The intracellular levels of dOTC-TP reached 2.3 to 3.6% (approximately 0.04 to 0.15 pmol/10⁶ cells, depending upon the cell type used) of the total intracellular dOTC levels; these levels are lower than those observed for AZT, 3TC, d4T, and carbocvir when tested at similar concentrations and most likely account for much of the difference in *in vitro* antiviral potency (4, 8, 10, 33, 35). The intracellular t_{1/2} of dOTC-TP was on the order of 2 to 3 h, which is similar to those observed for other nucleoside analogues such as AZT, d4T, and ddC but which is much shorter than those observed for 3TC and ddI (31). An examination of the conversion of (-)-dOTC and (+)-dOTC from their MP derivatives into their DP and TP derivatives (Tables 2 and 3) indicates that a greater accumulation of the DP and TP derivatives of (-)-dOTC was occurring. These data suggest that (+)-dOTC-MP is not as viable a substrate for dCMP kinase as (-)-dOTC-MP is. If this is true, then the accumulated (+)-dOTC-MP within the cell would be available for conversion into the ethanolanime or other cytosine adduct or would become a substrate for dCMP deaminase. This observation might explain the differential accumulations

TABLE 9. Pharmacokinetic parameters of dOTC in blood and CSF of rats^a

Compound	Blood		CSF	
	C _{max} (μg/ml)	AUC ₀₋₅ (μg · min/ml)	C _{max} (μg/ml)	AUC ₀₋₅ (μg · min/ml)
AZT	0.095 ± 0.01	14 ± 2.8	0.031 ± 0.016	5.8 ± 2.4
3TC	0.44 ± 0.14	72 ± 28	0.17 ± 0.12	22.0 ± 11.1
(-)-dOTC	0.317 ± 0.014	61 ± 22	0.067 ± 0.021	15.6 ± 3.6
dOTC	1.27 ± 0.3	235 ± 35	0.18 ± 0.023	39.2 ± 3.8

^a Six male rats per group were orally administered 5 mg of ³H-labeled test compound per kg.

of UM1 and UM2 between the two enantiomers of dOTC. In dCK⁻ cells we observed the accumulation of an alkaline phosphatase-sensitive metabolite (UM3) which at this time remains unidentified. It is possible that in dCK⁻ cells dOTC is deaminated, possibly by cytidine deaminase, and then is shunted into uracil metabolic pathways.

dOTC-TP was a selective inhibitor of HIV-1 RT over cellular DNA polymerases α , β , and γ . The assay methods used for the study described in this report were not strictly equivalent between the viral and cellular polymerases, such that an accurate selectivity index is not obtainable; however, with respect to the control drug, 3TC-TP, several conclusions can be drawn. The TP derivatives of dOTC were all more potent inhibitors of HIV-1 RT than 3TC-TP was (Table 1). In these experiments (+)-dOTC-TP was the preferred enantiomer among the dOTC enantiomers, with a K_i of 0.012 μM compared to a K_i of 0.08 μM for (-)-dOTC-TP, while dOTC (K_i , 0.028 μM) was 5.7-fold more potent than 3TC-TP (K_i , 0.16 μM). However, (+)-dOTC was also the most potent inhibitor of the cellular polymerases so that when the K_i/K_m ratios were plotted (-)-dOTC had the most favorable characteristics (Table 1). Against cellular polymerases, dOTC-TP was a less potent inhibitor of DNA polymerase α but was a slightly more potent inhibitor of DNA polymerase β and γ than 3TC-TP. When comparing the K_i/K_m ratios between RT and cellular polymerases, given 3TC as a control, dOTC is a highly selective inhibitor of RT with respect to DNA polymerases α and γ , and while it is less selective for RT over DNA polymerase β , it is in the same range as that observed for 3TC-TP. In the studies describing efficacy against clinical isolates and the emergence of viral resistance to dOTC, the racemate tended to have activity aligned closely to that of (-)-dOTC. In studies with these polymerases, however, the opposite was true, in that the potency of dOTC was much closer to that of (+)-dOTC against both the viral and cellular polymerases (Table 1).

In pharmacokinetic studies, there was a rapid absorption of dOTC following administration of a 10-mg/kg oral dose to rats, with C_{max} reaching 8 to 10 μM , which is, even with this low dose of drug, approximately three- to fourfold higher than the mean IC_{50} of dOTC defined for drug-resistant clinical isolates (Table 5). dOTC had good oral bioavailability (approximately 77%), and in the comparative analysis of nucleoside uptake into blood and CSF, both blood and CSF took up high levels of dOTC, such that the concentrations of dOTC in plasma were well above the IC_{50} s of the compound in tissue culture activity studies for extended periods of time.

The cytotoxicities of the enantiomers of dOTC against logarithmic-phase tumor and normal cell lines were minimal, with no toxicity observed in most cell lines with the highest concentration tested. The safety of dOTC was further confirmed with murine bone marrow progenitor cells, in which no toxicity was observed when dOTC was used at 500 μM (Table 6), indicating a low potential for inhibition of hematopoiesis. In further studies, after a 14-day incubation of HepG2 cells with 10 μM dOTC, no gross changes in mtDNA content were observed. Taken together, the in vitro studies demonstrate that dOTC is a selective inhibitor of HIV-1 and has little cellular toxicity and therefore has a favorable selectivity index. In further support of these findings, after a 14-day oral toxicity evaluation of dOTC, the highest concentration tested (500 mg/kg/day) had no observable toxic effects on rats. In conclusion, the data presented in this report support the advancement of dOTC into clinical trials. Furthermore, the effectiveness of dOTC against drug-resistant clinical isolates of HIV-1 suggests that the full potential of antiviral nucleoside analogues has not yet

been realized and that further investigation into novel nucleoside analogues is warranted.

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REFERENCES

- Belleau, B., L. Brasili, L. Chan, M. DiMarco, B. Zacharie and N. Nguyen-Ba. 1993. A novel class of 7,3-oxathiolanucleotide analogues having potent anti-HIV activity. *Bioorg. Med. Chem. Lett.* **3**:1723-1728.
- Cameron, J. M., P. Collis, M. Daniel, R. Storer, and P. Wilcox. 1993. Lamivudine. *Drugs Future* **18**:319-323.
- Chang, C. N., V. Skalski, J. H. Zhou, and Y.-C. Cheng. 1992. Biochemical pharmacology of (+) and (-) 2',3'-dideoxy-3'-thiacytidine as anti-hepatitis B virus agents. *J. Biol. Chem.* **267**:22414-22420.
- Daluge, S. M., S. S. Good, M. B. Faletto, W. H. Miller, M. H. St. Clair, L. R. Boone, M. Tisdale, N. R. Parry, J. E. Reardon, R. E. Dornsife, D. R. Averett, and T. A. Krenitsky. 1997. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. *Antimicrob. Agents Chemother.* **41**:1082-1093.
- Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. Nussinoff Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333-8337.
- Gu, Z., E. J. Arts, M. A. Parniak, and M. A. Wainberg. 1995. Mutated K65R reverse transcriptase of human immunodeficiency virus type 1 shows diminished chain termination in the presence of 2',3'-dideoxycytidine 5'-triphosphate and other drugs. *Proc. Natl. Acad. Sci. USA* **92**:2760-2764.
- Highcock, R. M., H. Hilpert, P. L. Myers, S. M. Roberts, and R. Storer. 1991. The potential for using carbocyclic nucleosides for the treatment of AIDS. Part 1. Preparation of some analogues of azidothymidine (AZT). *Chem. Soc. Perkin Trans. I* **5**:1127-1134.
- Hoggard, P., S. Kewn, M. G. Barry, S. H. Khoo and D. J. Back. 1997. Effects of drugs on 2',3'-dideoxy-2',3'-didehydrothymidine phosphorylation in vitro. *Antimicrob. Agents Chemother.* **41**:1231-1236.
- Johnson, M. A., and A. Fridland. 1989. Phosphorylation of 2',2'-dideoxyinosine by cytosolic 5' nucleotidase of human lymphoid cells. *Mol. Pharmacol.* **36**:291-295.
- Kewn, S., G. J. Veal, P. G. Hoggard, M. G. Barry, and D. J. Back. 1997. Lamivudine (3TC) phosphorylation and drug interactions in vitro. *Biochem. Pharmacol.* **54**:589-595.
- Larder, B. A. 1995. Viral resistance and the selection of antiretroviral combinations. *J. Acquired Immune Defic. Syndr. Hum. Retroviro.* **10**(Suppl. 1): S28-S33.
- Levantis, P., C. Stein, and J. Oxford. 1993. Molecular and biological aspects of AZT resistant viruses derived from HIV-1 infected individuals. *J. Antivir. Chem. Chemother.* **4**:131-138.
- Lewis, A. F., J. C. Drach, S. M. Fennewald, J. H. Huffman, R. G. Ptak, J.-P. Sommadossi, G. R. Revankar, and R. F. Rando. 1994. Inhibition of human cytomegalovirus in culture by alkenyl guanine analogs of the thiazolo[4,5-*d*]pyrimidine ring system. *Antimicrob. Agents Chemother.* **38**:2889-2895.
- Mansour, T. S., H. Jin, W. Wang, D. M. Dixit, C. A. Evans, H. L. A. Tse, B. Belleau, J. W. Gillard, E. Hooker, C. Ashman, N. Cammack, H. Salomon, A. R. Belmonte, and M. A. Wainberg. 1995. Structure activity relationships among a new class of antiviral heterosubstituted 2',3'-dideoxynucleoside analogues. *Nucleosides Nucleotides* **14**:627-635.
- Mansour, T. S., H. Jin, W. Wang, E. U. Hooker, C. Ashman, N. Cammack, H. Salomon, A. R. Belmonte, and M. A. Wainberg. 1995. Anti-human immunodeficiency virus and anti-hepatitis B virus activities and toxicities of the enantiomers of 2'-deoxy-3'-oxa-4'-thiocytidine and their 5'-fluoro analogues in vitro. *J. Med. Chem.* **38**:1-4.
- Martin, J. O., C. E. Brown, N. Matthews-Davis, and J. E. Reardon. 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. *Antimicrob. Agents Chemother.* **38**:1230-1238.
- Mitsuya, H., and S. Broder. 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**:1911-1915.
- Ojwang, J. O., B. K. Bhattacharya, H. B. Marshall, B. E. Korba, G. R. Revankar, and R. F. Rando. 1995. Inhibition of episomal hepatitis B virus

- DNA in vitro by 2,4-diamino-7-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-pyrrolo[2,3-*d*]pyrimidine. *Antimicrob. Agents Chemother.* **39**:2570-2573.
19. **Ojwang, J. O., R. W. Buckheit, Y. Pommier, A. Mazumder, K. DeVreese, J. Esté, D. Reyman, L. A. Pallansch, C. Lackman-Smith, T. L. Wallace, E. De Clercq, M. S. McGrath, and R. F. Rando.** 1995. T30177, an oligonucleotide stabilized by an intramolecular guanosine-octet, is a potent inhibitor of laboratory strains and clinical isolates of human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* **39**:2426-2435.
 20. **Parker, W. B., E. L. White, S. C. Shaddix, L. J. Ross, R. W. Buckheit, Jr., J. M. Germany, J. A. Secrist, III, R. Vince, and W. M. Shannon.** 1991. Mechanism of inhibition of human immunodeficiency virus type 1 reverse transcriptase and human DNA polymerases α , β and γ , by the 5'-triphosphates of carbovir, 3'-azido-3'-deoxythymidine, 2',3'-dideoxyguanosine, and 3'-deoxythymidine. *J. Biol. Chem.* **266**:1754-1762.
 21. **Perry, C. M., and J. A. Balfour.** 1996. Didanosine: an update on its antiviral activity, pharmacokinetic properties and therapeutic efficacy in the management of HIV disease. *Drugs* **52**:928-962.
 22. **Prisbe, E. J., H. Maag, J. P. H. Verheyden, and R. M. Rydzewski.** 1993. Structure-activity relationship among HIV inhibitory 4'-substituted nucleosides, p. 101-113. *In* C. K. Chu and D. C. Baker (ed.), *Nucleosides and nucleotides as antitumor and antiviral agents*. Plenum Press, New York, N.Y.
 23. **Reardon, J. E.** 1992. Human immunodeficiency virus reverse transcriptase: steady-state and pre-steady-state kinetics of nucleotide incorporation. *Biochemistry* **31**:4473-4479.
 24. **Riddler, S. A., R. E. Anderson, and J. W. Mellors.** 1995. Antiretroviral activity of stavudine (2',3'-didehydro-3'-deoxythymidine, D4T). *Antivir. Res.* **27**:189-203.
 25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 26. **Sarna, G. S., P. H. Hutson, M. D. Tricklebank, and G. Curzon.** 1983. Determination of brain 5-hydroxytryptamine turnover in freely moving rats using repeated sampling of cerebrovascular fluid. *J. Neurochem.* **40**:383-388.
 27. **Saunders, J., and R. Storer.** 1993. New development in RT inhibitors. *Drug News Perspect.* **5**:153-169.
 28. **Schinazi, R. F., R. M. Lloyd, Jr., M.-H. Nguyen, D. L. Cannon, A. McMillan, N. Ilksoy, C. K. Chu, D. C. Liotta, H. Z. Bazmi, and J. W. Mellors.** 1993. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob. Agents Chemother.* **37**:875-881.
 29. **Schmit, J.-C., and B. Weber.** 1998. Recent advances in antiretroviral therapy and HIV infection monitoring. *Intervirology* **40**:304-321.
 30. **Skowron, G.** 1996. ddC (zalcitabine). *Adv. Exp. Med. Biol.* **394**:257-269.
 31. **Sommadossi, J. P.** 1997. Pharmacology of HIV therapies. *Antivir. Ther.* **2** (Suppl. 3):55-60.
 32. **Stuyver, L., A. Wyseur, A. Rombout, J. Louwagie, T. Scarceq, C. Verhofstede, D. Rimland, R. F. Schinazi, and R. Rossau.** 1997. Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. *Antimicrob. Agents Chemother.* **41**:284-291.
 33. **Tisdale, M., T. Alnadaf, and D. Cousens.** 1997. Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. *Antimicrob. Agents Chemother.* **41**:1094-1098.
 34. **Tornevik, Y., and S. Eriksson.** 1990. 2',3'-Dideoxycytidine toxicity in cultured human CEM T lymphoblasts: effects of combination with 3'-azido-3'-deoxythymidine and thymine. *Mol. Pharmacol.* **38**:237-243.
 35. **Zhu, Z., H.-T. Ho, M. J. Hitchcock, and J.-P. Sommadossi.** 1990. Cellular pharmacology of 2',3'-didehydro-2',3'-dideoxythymidine (D4T) in human peripheral blood mononuclear cells. *Biochem. Pharmacol.* **39**(9):R15-R19.