Prodrugs of 2′,3′-Didehydro-3′-deoxythymidine (D4T): Synthesis, Antiviral Activity, and Rapid Pharmacokinetic Evaluation

DAVID R. TORTOLANI[X], JOHN W. RUSSELL, VALERIE J. WHITEROCK, MICHAEL J. M. HITCHCOCK*, ISMAIL GHAZZOULI, JOHN C. MARTIN*, MUZAMMIL M. MANSURI, AND JOHN E. STARRETT, JR.

Received September 11, 1992, from the *Bristol-Myers Squibb Company, Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492-7660.* Accepted for publication September 9, 1993®. *Current address: Gilead Sciences, Foster City, CA 94404.

Abstract □ A series of 5′-derivatives and modified pyrimidine analogues of 2′,3′-didehydro-3′-deoxythymidine (d4T, stavudine, **1**) were synthesized to determine their potential as oral prodrugs of d4T. Utilizing a screen developed for the rapid evaluation of a variety of prodrugs in mice, it was determined that 5′-acetate **2** provided comparable plasma levels of d4T after oral administration of the prodrug to that when d4T was administered alone. The relative oral bioavailability of methoxy acetate **3** and cyclohexyl carbonate **5** was 79 and 41%, respectively. Dihydropyridine ester **6** did not provide detectable levels of d4T up to 1 h after oral administration of **6**. Thiopyrimidines **8** and **9**, as well as aminopyrimidine **10** also failed to provide measurable levels of d4T after oral administration. 5′-Derivatives **3**, **5**, and **6** showed similar activity to that of d4T against HIV and MuLV, as did 5′-benzoyl-4-thio derivative **8**. However, the corresponding 4-thio 5′-alcohol **9** was inactive.

Introduction

Acquired immunodeficiency syndrome (AIDS) results from infection by the retrovirus human immunodeficiency virus (HIV).[1] A specific affinity of HIV for the helper/inducer T cells[2] leads to their depletion and predisposes the infected patient to life-threatening opportunistic infections. Evaluation of a number of 2′,3′-dideoxy nucleoside analogues as antiretroviral chemotherapeutic agents has revealed potential drug delivery problems such as rapid metabolism[3] and low bioavailability.[4] Currently, 3′-azido-3′-deoxythymidine (AZT, zidovudine), 2′,3′-dideoxyinosine (ddI, didanosine), and 2′,3′-dideoxycytidine (ddC, zalcitabine) are the only approved drugs for the treatment of HIV infection.[5-8] All of these drugs suffer from several undesired side effects. AZT causes hematological toxicity leading to anemia and leukopenia,[9] while the major toxicities seen for ddI are peripheral neuropathy and pancreatitis.[10-15] Painful peripheral neuropathy was also found to be a dose-limiting toxicity associated with ddC.[8b,16] As a result of the less than optimal properties of these drugs, there is an onging effort to identify compounds which overcome these drawbacks.

In 1987, Lin and co-workers identified 2′,3′-didehydro-3′-deoxythymidine (d4T, stavudine, **1**) as a potent nucleoside against HIV with comparable activity to AZT.[17] It was subsequently shown through a series of in vitro, biochemical, and in vivo experiments that d4T exhibited a very different phosphorylation pattern and lower toxicity relative to AZT.[18,19] At the onset of the present investigation, it was not known if the favorable toxicity and pharmacokinetic profile observed with d4T in animal models would translate to a beneficial effect in human clinical trials. As a result, we undertook a study to prepare and rapidly evaluate functionally diverse prodrugs in an effort to identify compounds which could potentially prove to be superior to d4T.

The prodrug approach has been successfully applied to a wide variety of drugs in order to improve delivery of drug and to overcome potential side effects. Use of alkyl or aryl ester prodrugs for compounds containing an alcohol or an acid functionality has been frequently utilized.[20] This process has been employed to prepare 5′-esters of AZT with the goal of overcoming the problems of rapid elimination and low permeability of AZT through the blood–brain barrier.[21] A novel prodrug approach utilizing a dihydropyridine–pyridinium salt type of redox system has been used for the specific brain delivery and sustained release of 2′,3′-dideoxynucleosides such as d4T[22] and AZT.[23] The goal of the present study was to synthesize and identify potential prodrugs of d4T that would provide appreciable plasma levels of d4T after oral administration. The study was intended to be preliminary in nature and to serve as a rapid screening method to evaluate a variety of structural classes, thereby quickly identifying compounds that warranted further investigation. A series of 5′-derivatives of d4T and pyrimidine-modified analogues were synthesized and evaluated in several biological systems.

The 5′-derivatives were selected on the basis of several criteria: (1) Functionality—linkages with differing degrees of chemical and enzymatic stability that would result in different rates of hydrolysis (acetate, alkoxy acetate, carbonate, and pyridyl acetate). (2) Synthetic accessibility—derivatives that could be prepared in an expedient manner and that were compatible with d4T. (3) Pyrimidine modifications—the amino derivative was prepared based upon the known enzymatic conversion of 5-methylcytidine to thymidine, both in vitro and in vivo.[24] The replacement of oxygen for sulfur in amide systems is a significant metabolic reaction and led us to propose thiopyrimidines as potential prodrugs.[25] It was our intention to identify classes of prodrugs which provided adequate plasma levels of d4T. Future studies would be used to determine if these prodrugs showed improved elimination half-lives, CNS penetration, or reduced toxicity profiles as compared to d4T.

Chemistry

2′,3′-Didehydro-3′-deoxythymidine (d4T, **1**) was originally prepared by Horwitz *et al.* by two different routes.[26,27] One of these approaches has since been modified to prepare large quantities of d4T.[18] The prodrugs synthesized for this work comprise two groups. The first group consists of 5′-derivatives of d4T, while the second considers modifications to the pyrimidine base. The latter changes were achieved by effecting oxygen–sulfur exchange at the C-4 carbonyl moiety of the base, followed by aminolysis of the resulting thioamide.

Selected sugar-modified prodrugs were prepared as shown in Scheme 1. Each of these prodrugs is modified in the 5′-OH position starting from d4T (**1**). The 5′-*O*-acetyl ester **2** and the 5′-*O*-methoxyacetyl ester **3** were prepared by treating d4T (**1**) in pyridine with the appropriate acid chloride. The reactions were complete within 24 h to give the desired prodrugs in yields of 79% and 42%, respectively, after recrystallization from ethanol.

Treatment of **1** with excess carbonyldiimidazole in DMF at ambient temperature gave carbamate **4** in 86% yield (Scheme

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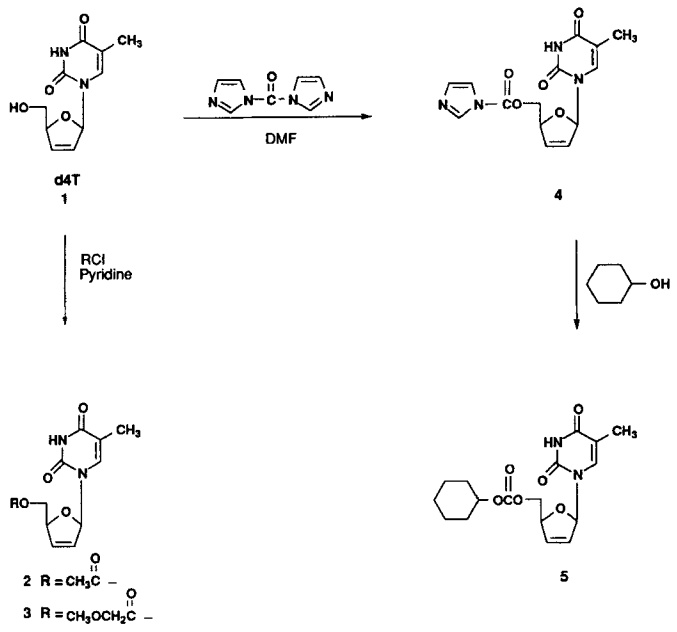
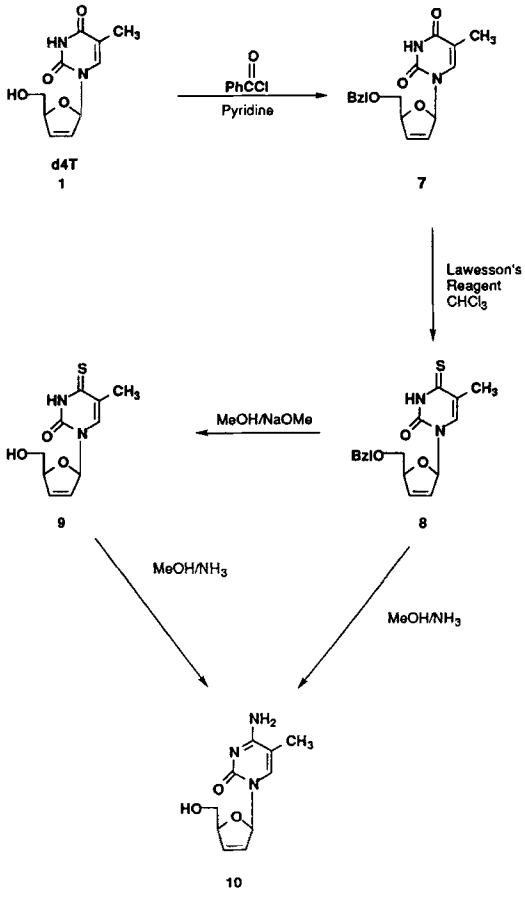


Scheme 1



Scheme 2

1). The imidazolyl carbamate 4 was then converted to cyclohexyl carbonate 5 upon treatment with cyclohexanol at 100 °C for 18 h. Purification by column chromatography furnished 5, as an oil, in 46% yield. The dihydropyridine derivative of d4T, 6 (see Table 2 for structure), was prepared via the method of Palomino.[22]

Preparation of the modified pyrimidine nucleosides utilized the methodology shown in Scheme 2. 5′-*O*-Benzoate ester 7 was conveniently prepared in 95% yield, after recrystallization, by treating 1 with excess benzoyl chloride in pyridine. Thiation of 5′-*O*-benzoyl-2′,3′-didehydro-3′-deoxythymidine (7) with Lawesson's reagent[28] in ethanol-free chloroform heated to reflux afforded 8[29] in 80% yield after silica gel column chromatography. Treatment of thiobenzoate ester 8 with anhydrous methanol and sodium methoxide afforded 3′-deoxy-2′,3′-didehydro-4-thiothymidine (9) in virtually quantitative yield after column chromatography. 2′,3′-Didehydro-2′,3′-dideoxy-5-methylcytidine (10) could then be furnished directly from either 8 or 9. Ammonolysis of alcohol 9 in methanol in a bomb at 100 °C introduced the amino group at the C-4 position to give amine 10 in 45% yield after silica gel column chromatography. Alternatively, ammonolysis of thiobenzoate ester 8 with concomitant deprotection afforded 10 in 90% yield after purification by column chromatography.

Biological Results and Discussion

The objectives of this project were to design and synthesize prodrugs of d4T, explore their pharmacokinetic properties, and test their antiviral activity. Because we were interested in developing an assay method for the rapid screening of a number of d4T analogues, our studies focused on determining the plasma levels of d4T and not the parent prodrug. The plasma concentration of d4T and the oral bioavailability of each compound relative to d4T was determined after mice were given a single oral dose of each prodrug. The chosen dose was equivalent to 25 mg/kg of d4T. Single blood samples were then taken from each mouse (3 mice per time point) at 20, 45, and 60 min after dosing and separated by centrifugation, and then the separated plasma was assayed for d4T by a validated HPLC method.[30] The plasma concentrations measured after dosing with each drug are summarized in Table 1. The area under the curve (AUC) from 0 to 60 min was calculated for d4T after administration of d4T and each candidate prodrug. It should be noted that the data presented are estimates of relative bioavailability based upon limited data points.

The acetyl 2 and methoxyacetyl 3 achieved maximum plasma concentrations measured as d4T within 20 min, and cyclohexyl carbonate 5 reached maximum plasma concentration within 45 min (Table 1). The oral bioavailabilities relative to d4T of 2, 3, and 5 were 1.0, 0.8, and 0.4, respectively. The low relative bioavailability (0.4) of cyclohexyl carbonate 5 may be a result of limited sampling times (≤1 h). Because of the relatively flat plasma concentration vs time curve for 5, extending the sampling times could result in a larger AUC and therefore an increase in the relative bioavailability. The two 4-thio analogues, 8 and 9, and amino analogue 10 gave no measurable plasma levels of d4T at any of the time points. On the basis of comparison of log k' data in Table 2 (10 vs 1; 9 vs 2; and 8 vs 5) and structural similarity to compounds which give measurable levels of d4T, it is not unreasonable to assume that 8–10 are being absorbed. The lack of measurable d4T plasma levels with these compounds would appear to be a result of their inability to undergo biological conversion to d4T in the mouse.

Dihydropyridine 6 was the only d4T ester evaluated which failed to provide any measurable levels of d4T. It is possible that ester 6 is not adsorbed after oral administration. Another explanation is that it is absorbed but cleaved at too slow a rate to provide detectable levels of d4T. The latter explanation is consistent with the results of Palomino et al.,[22] who found that there was no significant rate of hydrolysis of ester 6 to d4T when examined in different biological media in vitro.

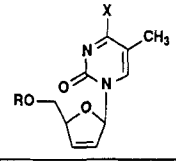
The in vitro antiviral activity against HIV and murine leukemia virus (MuLV) and the cytotoxicity are given in Table 2. D4T analogues methoxyacetyl 3, cyclohexyl carbonate 5, and dihydropyridine 6 exhibited potent anti-HIV activity, with ID_{50}'s

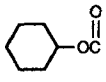
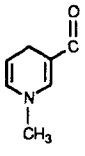
Table 1—Mean Plasma Concentration of d4T after Oral Administration of a Single 25 mg of d4T equiv/kg Dose

Compd	Vehicle[a]	Concentration of d4T (μg/mL) 20 min	45 min	60 min	$AUC_{0 \rightarrow 60\ min}$ (μg·min/mL)	Rel Bioavail
1	Water	22.7 ± 2.2[c]	11.1 ± 2.3	5.0 ± 0.8	770	1.00[e]
2	Water	20.3 ± 2.8	13.4 ± 2.3	4.3 ± 1.5	757	0.98
3	Water	19.0 ± 4.9	7.6 ± 1.9	3.8 ± 7.4	609	0.79
5	0.1% MC[b]	5.4 ± 2.4	8.0 ± 1.9	5.0 ± 0.9	319	0.41
6	0.1% MC	BDL[d]	BDL	BDL	0	0
8	4% DMSO/Water	BDL	BDL	BDL	0	0
9	4% DMSO/Water	BDL	BDL	BDL	0	0
10	0.1% MC	BDL	BDL	BDL	0	0

[a] The drug was dissolved or suspended in 2.5 mL of vehicle. [b] 0.1% methyl cellulose in distilled deionized water. [c] Mean concentration (n = 3) ± standard deviation. [d] Below detection limit. [e] The oral bioavailability of d4T after administration of d4T was assigned a value of 1.0.

Table 2—Antiviral Activity and Lipophilicity of d4T Prodrugs



Structure R	X	Compd	log k'[a]	Antiviral Activity ID_{50}/TD_{50} (μg/mL)[b] MuLV	HIV
H	OH	1 (d4T)	−0.48	0.4/>100	0.1/62
$CH_3C(=O)$	OH	2	−0.28	0.1/>100	3.2/55
$CH_3OCH_2C(=O)$	OH	3	−0.30	0.1/>100	0.4/69
	OH	5	0.55	40/>100	0.6/100
	OH	6	ND[c]	0.8/>100	<1/50
PhC(=O)	SH	8	0.57	0.1/>100	0.5/>100
H	SH	9	−0.23	>10/100	>10/48
H	NH_2	10	−0.41	>100/>100	1.8/>100

[a] The k' of each sample was determined using a Varian Micropak C-18 MCH-10 column and a 60% methanol/40% ammonium acetate (50 mmol) buffer mobile phase (pH 7). [b] 50% inhibitory dose/50% tissue culture inhibitory dose. [c] Not determined.

(50% inhibitory concentration) of 0.4, 0.6, and <1.0 μg/mL, respectively, and TD_{50}'s (50% tissue culture inhibitory dose) of 69, 100, and 50 μg/mL, respectively. The antiviral activity observed with dihydropyridine **6** may be dependent on the length of the assays (8 days for HIV and 9 days for MuLV), which may allow for substantial ester cleavage to give d4T. This is in contrast to the bioavailability study, which measured in vivo conversion to d4T in 1 h or less (dihydropyridine **6** had a relative bioavailability of 0). Acetate **2** had an ID_{50} of 3.2 μg/mL.

Among the pyrimidine-modified derivatives, amine **10** demonstrated moderate HIV activity (ID_{50} of 1.8 μg/mL). The activity of 5′-*O*-benzoyl-2′,3′-didehydro-3′-deoxy-4-thiothymidine (**8**) (ID_{50} of 0.5 μg/mL) was surprising in light of the inactivity of corresponding 5′-alcohol, 2′,3′-didehydro-3′-deoxy-4-thiothymidine (**9**) (ID_{50} > 10 μg/mL).[31] Neither of these thiols is converted to d4T in the mouse (vide supra). From the log k' values (Table 2), it is evident that benzoate **8** (log k' = 0.57) is much more lipophilic than alcohol **9** (log k' = −0.23). The lipophilicity of benzoate **8** could enhance the cellular membrane penetration; however, alcohol **9** has similar lipophilicity to acetate **2** (log k' = −0.23 vs log k' = −0.28), and as such **9** should be expected to enter the cell at a rate comparable to that of acetate **2**, assuming uptake by passive diffusion. Another possible explanation for the antiviral activity of benzoate **8** compared to alcohol **9** is that benzoate **8** is a better substrate for enzymatic conversion of the thiopyrimidine to pyrimidine. Subsequent cleavage of the benzoyl ester would provide d4T, which would account for the antiviral activity. Alternatively, alcohol **9** could be metabolized within the cell to inactive components at a rate faster than benzoate **8**. Further studies would be needed to determine the exact mechanism of action of thiopyrimidine benzoate ester **8** and aminopyrimidine **10**, i.e., whether they are acting as prodrugs of d4T or active in their own right.

In the MuLV assay, esters **2** and **3** (ID_{50} of 0.1 μg/mL each) showed activity similar to d4T (ID_{50} of 0.4 μg/mL), as did dihydropyridine **6** (ID_{50} of 0.8 μg/mL). Carbonate **5** was less active (ID_{50} of 40 μg/mL) than d4T. Similar to the HIV results, 4-thio-5′-benzoate **8** demonstrated antiviral activity against MuLV (ID_{50} of 0.1 μg/mL), yet the corresponding alcohol **9** showed no inhibition at concentrations less than 10 μg/mL. In contrast to the HIV results, amine **10** was inactive against MuLV at concentrations less than 100 μg/mL.

Subsequent to the completion of this study, human clinical trials were conducted with d4T (Stavudine) which demonstrated a favorable pharmaceutical profile for the parent nucleoside.[32] Although it may be possible to improve the central nervous system (CNS) penetration or plasma half-life of d4T with a prodrug, the efficacy observed with the parent compound (d4T) does not warrant further investigation of prodrugs at this time.

In conclusion, it appears that 5′-d4T derivatives are converted to d4T in the mouse after oral administration at the relative rate of **2**, **3** > **5** >>> **6**. Neither thiols **8** or **9** nor amine **10** were detected to be converted to d4T in vivo. All of the 5′-d4T derivatives demonstrated antiviral activity against both HIV and MuLV, although none of the compounds (including d4T) showed any activity against herpes simplex viruses (HSV-1 and HSV-2; data not shown).

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Column chromatography was performed on flash silica gel (40-μm particle size, Baker). Elemental analyses were performed by the analytical department, Bristol-Myers Squibb Co., Wallingford. ^1H NMR spectra were recorded on a AM360 Bruker NMR spectrometer, Varian Gemini 300 NMR spectrometer, or Varian VXR200 spectrometer with TMS as an internal standard; chemical shifts are recorded in parts per million. Mass spectra were obtained on a Kratos MS25 (FAB) or a Finnegan 4500 (EI and CI). D4T (**1**) was prepared as previously described.[18]

5′-*O*-Acetyl-2′,3′-didehydro-3′-deoxythymidine (2)—To a stirred solution of 10.0 g (44.6 mmol) of d4T (**1**) in 100 mL of pyridine was added 4.10 mL (58.0 mmol) of acetyl chloride. The mixture was stirred at 50

°C for 1 h, cooled, and diluted with 1 L of toluene. The solvents were removed in vacuo. The yield of product, recrystallized from ethanol, was 9.3 g (78%): ^{1}H NMR (360 MHz, $CDCl_3$) 9.01 (1H, s, NH), 7.23 (1H, s, H6), 6.99 (1H, d, J = 1.1 Hz, H1'), 6.24 (1H, d, J = 5.8 Hz, H2'), 5.88 (1H, d, J = 5.5 Hz, H3'), 5.02 (1H, s, H4'), 4.3 (2H, m, H5'), 2.07 (3H, s, CH_3 acetate), 1.90 (3H, s, CH_3); MS m/z (relative intensity) 533 (M^+, 3), 347 (40), 287 (50), 155 (40), 127 (100), 81 (80); IR (KBr, cm^{-1}) 3010, 1740, 1700, 1470, 1260, 1230. Anal. ($C_{12}H_{14}N_2O_5$) C, H, N.

2',3'-Didehydro-3'-deoxy-5'-*O*-(methoxyacetyl)thymidine (3)—To a solution of 4.50 g (20.1 mmol) of d4T (1) in 50 mL of pyridine was added 2.45 mL (26.8 mmol) of methoxyacetyl chloride. The reaction mixture was stirred at 22 °C for 24 h, diluted with 200 mL of toluene, and concentrated to dryness in vacuo. The residue was recrystallized from ethanol. NMR analysis of the product indicated the presence of pyridine hydrochloride. The solid was suspended in 100 mL of water, collected by filtration, and washed with absolute ethanol to give 2.50 g (42%) of 3: ^{1}H NMR (300 MHz, $CDCl_3$) 9.27 (1H, s, NH), 7.14 (1H, d, J = 1.3 Hz, H6), 6.96 (1H, s, H1'), 6.23 (1H, d, J = 6.0 Hz, H2'), 5.88 (1H, d, J = 6.1 Hz, H3'), 5.01 (1H, s, H4'), 4.3 (2H, m, H5'), 4.02 (2H, m, OCH_2), 3.39 (3H, s, OCH_3), 1.88 (3H, s, CH_3); MS m/z (relative intensity) 297 (M^+ + H, 40), 251 (20), 207 (40), 155 (50), 127 (100), 81 (100); IR (KBr, cm^{-1}) 3200–3000, 1760, 1700, 1480, 1260. Anal. ($C_{13}H_{16}N_2O_6$) C, H, N.

2',3'-Didehydro-3'-deoxy-5'-*O*-(1-imidazolylcarbonyl)-thymidine (4)—To a solution of 4.50 g (0.0201 mol) of d4T (1) in 50 mL of DMF was added 5.67 g (0.035 mol) of carbonyldiimidazole. The reaction mixture was stirred at 22 °C for 45 min, during which time a thick white precipitate formed. The solid was collected and washed with 5 mL of DMF. The filtrate was cooled to 0 °C for 16 h, and the resulting solid was collected and washed with cold DMF. The combined solid was dried in a vacuum desiccator for 16 h at 0.1 mm to give 5.50 g (86%) of imidazoate which contained about 5% imidazole (determined by NMR analysis): ^{1}H NMR (300 MHz, d_6-DMSO) 8.22 (1H, s, imid. H), 7.55 (1H, s, imid. H), 7.11 (1H, s, imid. H), 7.11 (1H, s, H6), 6.77 (1H, d, J = 1.9 Hz, H1'), 6.49 (1H, d, J = 6.0 Hz, H2'), 6.04 (1H, d, J = 5.9 Hz, H3'), 5.02 (1H, s, H4'), 4.5 (2H, m, H5'), 1.53 (3H, s, CH_3); MS m/z (relative intensity) 319 (M^+ + H, 100), 207 (10), 149 (20), 127 (20), 81 (25); IR (KBr, cm^{-1}) 3085, 1760, 1700, 1690, 1670, 1415, 1260.

5'-*O*-[(Cyclohexyloxy)carbonyl]-2',3'-didehydro-3'-deoxythymidine (5)—A suspension of 1.00 g (3.14 mmol) of imidazolyl d4T 4 in 20 mL of cyclohexanol was heated at 100 °C for 18 h, after which time the reaction was homogeneous. The reaction mixture was cooled, and the solvents were removed in vacuo at 50 °C (0.1 mm). The residue was purified on a 50-mm flash chromatography column, eluting with 2% MeOH–CH_2Cl_2 (1000 mL), 5% MeOH–CH_2Cl_2, (500 mL), and then 10% MeOH–CH_2Cl_2 (500 mL). The fractions containing the desired product were combined, and the solvent was removed to give an oil which still contained cyclohexanol by NMR. The oil was suspended in 30 mL of water, and the solvents were removed by freeze-drying (0.005 mm) for 16 h to give 0.51 g (46%) of 5: ^{1}H NMR (200 MHz, $CDCl_3$) 8.20 (1H, s, NH), 7.45 (1H, s, H6), 7.08 (1H, m, H1'), 6.30 (1H, m, H2'), 5.85 (1H, m, H3'), 4.05 (1H, m, H4'), 4.6 (1H, m, CHO), 4.4 (2H, m, H5'), 2.1–1.0 (13H, m, CH_3 + cyclohexyl CH_2); MS m/z (relative intensity) 351 (M^+ + H, 55), 207 (50), 155 (40), 127 (50), 81 (100); IR (KBr, cm^{-1}) 2975, 1750, 1700, 1280, 1260. Anal. ($C_{17}H_{22}N_2O_6$) C, H, N.

5'-*O*-Benzoyl-2',3'-didehydro-3'-deoxythymidine (7)—To a solution of 10.0 g (44.6 mmol) of d4T (1) in 290 mL of pyridine was added 8.2 mL (71.4 mmol) of benzoyl chloride. The mixture was heated at 50 °C for 6 h and cooled, and half of the volatiles were removed in vacuo. The residue was poured into 800 mL of ice water, and the resulting precipitate was collected, washed with ice water, and air-dried. The solid was recrystallized from ethanol to give 12.87 g (88%) of title product: ^{1}H NMR (300 MHz, d_6-DMSO) 11.35 (1H, s, NH), 7.91 (2H, d, J = 7.8 Hz, Ph), 7.65 (1H, t, J = 7.2 Hz, Ph), 7.51 (2H, d, J = 7.8 Hz, Ph), 7.09 (1H, s, H6), 6.78 (1H, s, H1'), 6.50 (1H, d, J = 6.1 Hz, H2'), 6.02 (1H, d, J = 6.1 Hz, H3'), 5.08 (1H, s, H4'), 4.48 (2H, m, H5'), 1.33 (3H, s, CH_3); MS m/z (relative intensity) 329 (M^+ + H, 12), 155 (45), 127 (100), 81 (85); IR (KBr, cm^{-1}) 3100-3000, 1710, 1460, 1280. Anal. ($C_{17}H_{16}N_2O_5$) C, H, N.

5'-*O*-Benzoyl-2',3'-didehydro-3'-deoxy-4-thiothymidine (8)—A suspension of 5-*O*-benzoyl-2',3'-didehydro-3'-deoxythymidine (7) (12.6 g, 38.45 mmol) and 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide (Lawesson's reagent; 10.87 g, 26.9 mmol) in ethanol-free chloroform (270 mL) was heated to reflux for 4 h. The homogeneous reaction mixture was concentrated and purified by flash column chromatography using methylene chloride/ethyl acetate (9:1) as eluent. The product was collected as a solid (10.56 g, 80%): mp 171–173 °C; ^{1}H NMR (300 MHz, d_6-DMSO) 12.76 (1H, s, NH), 7.90 (2H, m, ortho Ph), 7.64 (1H, m, para Ph), 7.50 (2H, m, meta Ph), 7.22 (1H, s, H6), 6.74 (1H, d, H1'), 6.52 (1H, d, H3'), 6.04 (1H, d, H2'), 5.12 (1H, s, H4'), 4.45 (2H, m, H5'), and 1.53 (3H, s, CH_3). Anal. ($C_{17}H_{16}N_2O_4S$) C, H, N.

2',3'-Didehydro-3'-deoxy-4-thiothymidine (9)—A solution of 5'-*O*-benzoyl-2',3'-didehydro-3'-deoxy-4-thiothymidine (8) (3.09 g, 9 mmol) in anhydrous methanol (145 mL) was warmed, and sodium methoxide (0.54 g, 10 mmol) was added. The solution was heated to reflux for 30 min. The pH of the solution was monitored and kept at pH 8. The reaction mixture was allowed to cool and then concentrated. The product was purified by flash column chromatography, using methylene chloride/methanol (4:1) as eluent. The product was collected as a solid (2.1 g, 99%): mp 127–129 °C; ^{1}H NMR (300 MHz, d_6-DMSO) 12.7 (1H, s, NH), 7.8 (1H, s, H6), 6.76 (1H, m, H1'), 6.38 (1H, m, H3'), 5.90 (1H, m, H2'), 5.03 (1H, t, OH), 4.77 (1H, s, H4'), 3.58 (2H, m, H5'), and 1.88 (3H, s, CH_3). Anal. ($C_{10}H_{12}N_2O_3S$) C, H, N.

2',3'-Didehydro-2',3'-dideoxy-5-methylcytidine (10)—*Method A*—5'-*O*-Benzoyl-2',3'-didehydro-3'-deoxy-4-thiothymidine (8) (0.35 g, 1.0 mmol) was treated with methanolic ammonia (50 mL) in a sealed tube at 100 °C for 4 h. The reaction mixture was concentrated and the residue purified by flash column chromatography eluting with 25% MeOH/CH_2Cl_2. The appropriate fractions were combined and evaporated to provide a solid (0.21 g, 92%).

Method B—2',3'-Didehydro-3'-deoxy-4-thiothymidine (9) (0.5 g, 2.08 mmol) was treated with methanolic ammonia (15 mL) in a sealed tube at 100 °C for 5 h. The reaction mixture was concentrated and the residue purified by flash column chromatography eluting with 25% MeOH/CH_2Cl_2. The product was collected as a solid (0.22 g, 48%): mp 140–141 °C; ^{1}H NMR (300 MHz, d_6-DMSO) 7.55 (1H, s, H6), 7.33 (1H, s, NH), 6.91 (1H, m, H1'), 6.84 (1H, s, NH), 6.34 (1H, m, H3'), 5.88 (1H, m, H2'), 5.01 (1H, t, OH), 4.76 (1H, m, H4'), 3.59 (2H, m, H5'), 1.81 (3H, s, CH_3); MS m/z (relative intensity) 251 (25), 224 (M^+ + H, 15), 126 (100), 81 (60); IR (KBr, cm^{-1}) 3600–2900, 1670, 1600, 1520, 1490; mass spectrum exact mass 224.1031 (−.427 ppm error).

Testing and Evaluation of Compounds against Retroviruses—The compounds were evaluated for antiviral activity against murine leukemia virus (MuLV) strains by using the UV-XC plaque assay.[33]

The HIV in vitro assay was done as follows. The anti-HIV/LAV activity was measured in cultures of CEM-F cells. The CEM cells were infected with approximately 30 $TCID_{50}$ (50% tissue culture infectious dose) of HIV (LAV strain). The cells were then incubated for 45 min at 37 °C. The test compounds in culture medium were added at various concentrations to the infected cells and then incubated for a further 8 days. The culture media supernatant was then assayed for p-24 gag protein by an enzyme capture assay (ELISA). The antiviral activity was expressed as the dose that inhibits 50% of the p-24 expression (ID_{50} in μg/mL).

Oral Bioavailability Studies—d4T and the prodrugs studied were dissolved in a suitable vehicle or suspended in 0.1% aqueous methyl cellulose to provide a solution or suspension which contained the equivalent of 2.5 mg of d4T/mL. Mice were dosed at a volume of 10 mL/kg to provide a dose of 25 mg d4T equiv/kg. For each compound evaluated, nine mice were given a single oral dose of drug. Single blood samples were taken from each mouse (three mice per time point) at 20, 45, and 60 min after dosing. The plasma was separated by centrifugation and then assayed for d4T by a validated HPLC method.[30]

log *k'* Determination—The k' of each sample was determined using a Varian Micropak C-18 MCH column (30 cm × 4 mm) and a 60% methanol/40% ammonium acetate (50 mmol) buffer mobile phase (pH 7). Four standards were run: benzyl alcohol, propiophenone, benzophenone, and phenyl ether. All samples had a retention time (t_R) under 30 min and gave sharp peaks. Each compound was analyzed five times, and the k' for the seven samples and three standards were calculated using $NaNO_3$ to determine void time. The k' values were tabulated and averaged and their logarithms reported.

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