Structure−Activity Relationships of L-Dioxolane Uracil Nucleosides as Anti-Epstein Barr Virus Agents

Ju-Sheng Lin,[†] Toshihiko Kira,[†] Elizabeth Gullen,[†] Yongseok Choi,[‡] Fucheng Qu,[‡] Chung K. Chu,[‡] and Yung-Chi Cheng*,[†]

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510, and Center for Drug Discovery and Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, Georgia 30602

Received December 1, 1998

A series of 1,3-dioxolanyluracil analogues was prepared from the dioxolane intermediates **2**, and their anti-Epstein Barr virus (anti-EBV) activities were detemined. The potency of L-dioxolane uracil nucleosides against EBV replication is dependent on the substituents at the 5-position in the following decreasing order: I > Br > Cl > CH_3 > CF_3 > F. The most active and selective analogue was the iodo derivative (L-I-OddU) with an EC_{50} value of 0.03 μM and an EC_{90} value of 0.16 μM. There was no cytotoxicity or depletion of mitochondrial DNA in cells after exposure to L-I-OddU at 50 μM. The action against EBV replication in H1 cells is time-dependent, and EBV DNA in cells treated with L-I-OddU could rebound to pretreatment levels once the drug was removed. In view of the potent antiviral activity plus favorable toxicity profiles, L-I-OddU may be potentially useful for the treatment of EBV-related infectious diseases as well as for delaying the onset or decreasing the incidence of EBV-associated cancers.

Introduction

Epstein Barr virus (EBV) is a common pathogen of humans. It causes infectious mononucleosis and fatal acute infectious mononucleosis/X-linked lymphoproliferative syndrome (XLP) and is closely associated with several types of malignancies. These include Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, EBV-associated T-cell lymphoma, and nasal T-cell lymphoma.[1−8] Recently, it was found that most all "posttransplantation" lymphomas appear to be positive for the EBV genome, irrespective of histological appearances or clonability of lesion.[6,9−11] EBV is associated with oral hairy leukoplakia in AIDS patients, and a new EBV-related virus, human herpes type 8, has been identified to be reponsible for Kaposi's sarcoma.[12,13] To date, there are no clinically effective anti-EBV agents that are without undesirable side effects. Several nucleoside analogues have been shown to be effective against EBV replication in cell culture including DHPG,[14] D-FIAU,[15] ACV,[16] D-FMAU,[17] and *S*-oligos.[18] However, their clinical application was restricted by their lack of potency and/or undesirable toxicity.[17] Therefore, the discovery and development of new drugs, which have higher potency and selectively inhibit EBV replication, could have a major impact not only on the treatment of infectious viral diseases but also on the prevention or delay of the onset of EBV-associated malignancies.

Recently, we reported on a novel L-nucleoside analogue, L-FMAU, which had potent and selective antiviral activity against EBV replication as well as anti-hepatitis B virus (HBV) activity.[19] Since then, we have evaluated several other L-uridine and thymidine analogues for their antiviral activity against EBV. Herein, we describe the structure−activity relationships of uracil analogues, including newly synthesized L-dioxolanyluracil nucleosides.

Results

Synthesis. The synthesis of the new L-1,3-dioxolanyl nucleosides was accomplished from the key intermediates **2**, which were prepared from L-gulono-γ-lactone (**1**) in nine steps according to our previously published method.[20] The key intermediates **2** were condensed with silylated uracil derivatives in dry 1,2-dichloroethane using TMSOTf as the Lewis acid catalyst to give a α,β-mixture of L-dioxolanyl nucleosides **3**−**8** (Scheme 1). Then the combined α,β-anomers were treated directly with methanolic ammonia to form single isomers. The protected anomeric mixtures could not be separated, but the free nucleosides could be separated by silica gel column chromatography. Enantiomerically pure α- and β-L-(2*S*,4*S*)-dioxolanyluracil derivatives **9**−**20** were isolated.

Structure−Activity Relationships. The anti-EBV activity of optically active β-L-dioxolane uracil analogues was evaluated (Table 1). The potency of the anti-EBV activity is exhibited in decreasing order: L-I-OddU > L-Br-OddU > L-Cl-OddU > L-CF_3-OddU > L-F-OddU. L-I-OddU is the most potent compound among those tested with an EC_{50} value of 0.033 ± 0.018 μM. The toxicity of L-I-OddU is quite low with a selective index of >6000. The enantiomer, D-I-OddU, also showed moderate anti-EBV activity; however, it showed significantly greater cytotoxicity with an ID_{50} value of 18 μM. On the other hand, the oxathiolane derivative L-I-SddU showed neither antiviral activity nor cytotoxicity. L-Br-

* Corresponding author: Dr. Y.-C. Cheng, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06520-8066. Tel: 203-785-7118. Fax: 203-785-7129.
[†] Yale University School of Medicine.
[‡] University of Georgia.

10.1021/jm9806749 CCC: $18.00
Published on Web 05/21/1999

Scheme 1. Synthesis of L-Dioxolanyluracil Analogues

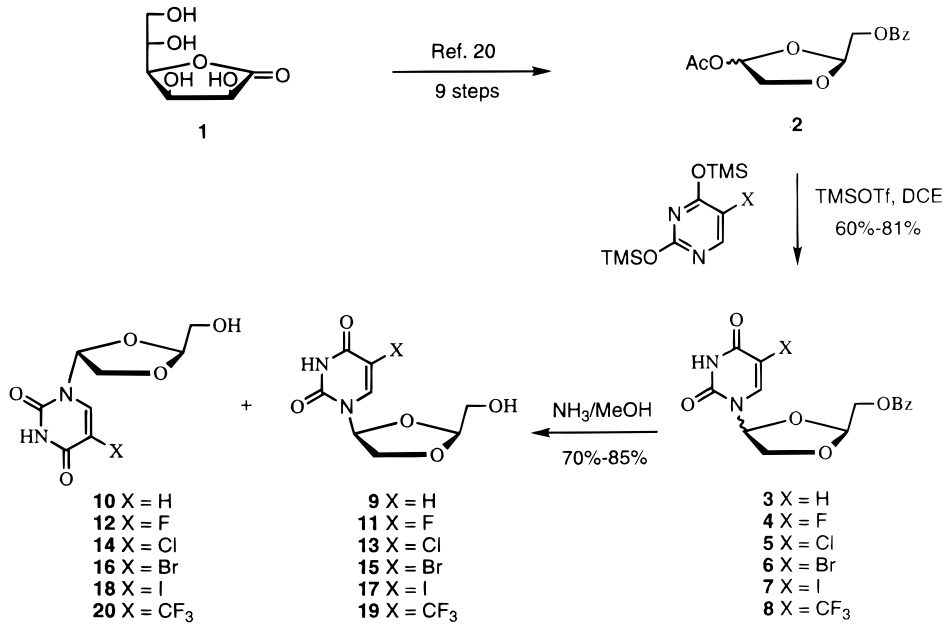
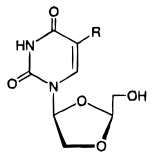


Table 1. Structure−Activity Relationship of L-Dioxolane Uracil Analogues against EBV[a]



compd	R	anti-EBV activity EC_{50} (μM)	anti-EBV activity EC_{90} (μM)	cytotoxicity ID_{50} (μM)	mt-DNA toxicity (μM)	SI
9, L-OddU	H	>50	ND	ND	ND	
11, L-F-OddU	F	50.0	ND	>100	>50	
13, L-Cl-OddU	Cl	0.6 ± 0.28	1.37 ± 0.9	>100	>50	>72
15, L-Br-OddU	Br	0.19 ± 0.065	0.48 ± 0.092	>1000	>50	>2083
17, L-I-OddU	I	0.033 ± 0.017	0.016 ± 0.075	>1000	>50	>6250
D-I-OddU	I	7.6	ND	18	ND	
L-I-SddU[b]	I	>100	ND	>100	ND	
19, L-CF_3-OddU	CF_3	4.0		>100	ND	
ACV		50		1000		
DHPG		5.0		75		
L-FMAU		5.0		1000		

[a] All the procedures are described in the Experimental Section. SI, selective index = value of ID_{50}/value of EC_{90}. [b] The O in the sugar moiety at the 2' position has been replaced by an S for this compound.

OddU also showed potent anti-EBV activity with an EC_{50} value of 0.19 ± 0.065 μM with low cytotoxicity. A typical dose−response curve of L-I-OddU and L-Br-OddU against EBV confirms that L-I-OddU is slightly more potent than L-Br-OddU. These compounds did not inhibit cell growth or decrease mt-DNA content of cells at concentrations up to 50 μM. To assess the time required to exert anti-EBV activity in culture, the cellular content of EBV DNA was studied following the addition of 10 μM L-I-OddU. EBV DNA was inhibited less than 20% following 1 day of drug exposure and approximately 80% at 1.5 days (Figure 1 A). Therefore, the exposure time of drug to the cells could be a critical factor for the anti-EBV activity of L-I-OddU. While the 5-chloro derivative **13** also showed potent anti-EBV activity (EC_{50} 0.6 μM), the 5-fluoro derivative **11** exhibited antiviral activity at a concentration 250-fold higher than that of derivative **13**. The 5-CF_3 derivative showed moderately potent anti-EBV activity. The anti-EBV activity of the newly synthesized nucleosides was also compared with known anti-EBV compounds, including ACV, DHPG, and L-FMAU (Table 1).

We identified L-I-OddU as the most potent and selective compound; therefore we studied the reversibility of EBV DNA in the cells treated with various concentrations of L-I-OddU (Figure 1B). On day 0 the drugs were removed and the cells washed, and then we monitored the amount of EBV DNA in each set of cells. The rebound times to pretreatment level in 0.4, 2, and 10 μM L-I-OddU-treated cells were day 50, day 55, and day 60, respectively. The higher the concentration of L-I-OddU used for the treatment of cells, the longer the time required for EBV DNA to rebound to the pretreatment level.

Discussion

It was demonstrated that the halogenated L-OddU analogues were a potent class of anti-EBV agents.

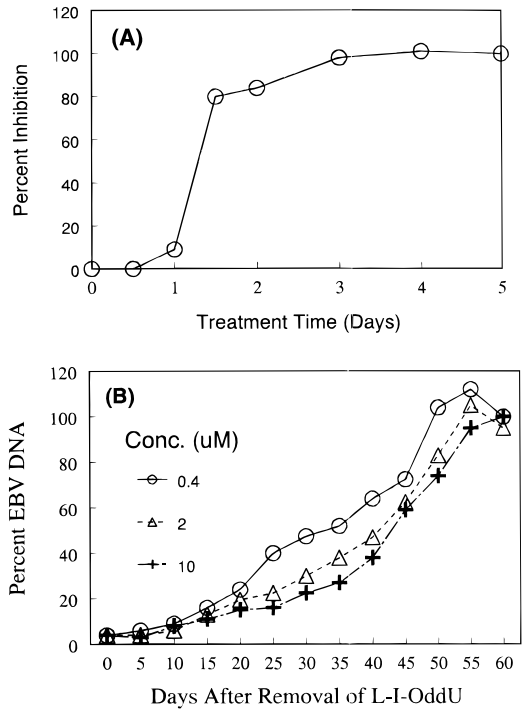


Figure 1. (A) H1 cells were exposed to 10 μM L-I-OddU for the days indicated. The samples were then prepared for Slot blot analysis. Viral inhibition was compared to untreated control cells. (B) Reversibility of the anti-EBV action of L-I-OddU in H1 cells at different dosages. Samples were prepared for Slot blot analysis and compared to untreated controls.

Among these compounds, L-I-OddU was the most potent nucleoside (Table 1). L-I-OddU also has the highest selective index (SI) in cell culture among all anti-EBV compounds reported thus far. Unlike L-FMAU, which is active against EBV and HBV,[17,19,21] L-I-OddU showed specific potency against only EBV with an EC_{50} at least 50-fold lower than that of L-FMAU. The antiviral spectrum of L-I-OddU is more specific for EBV and therefore is different from spectra of any other L-nucleoside analogues reported.[15,23−27] This also suggests that the mechanism of anti-EBV activity of L-I-OddU will require the participation of some EBV-specific proteins. It is possible that L-I-OddU could be selectively phosphorylated to L-I-OddUMP by EBV-specific thymidine kinase (TK) in EBV-producing cells, whereas the L-I-OddU will not be phosphorylated in EBV latent infected cells or uninfected cells due to the lack of EBV-specific TK. This "selective alternative substrate" approach for developing anti-HSV or anti-VZV drugs based on its viral TK has been previously proposed by our laboratory.[28] L-I-OddUMP, once formed in EBV-producing cells, may be further phosphorylated by human dTMP kinase and NDP kinase to L-I-OddUDP and L-I-OddUTP, respectively. The L-I-OddUTP formed may then act as either an alternative substrate of dTTP or a competitive inhibitor with dTTP for EBV DNA polymerase without effect on human DNA polymerases α, β, γ, and δ. We are currently testing this hypothesis in our laboratory.

The anti-EBV action of L-I-OddU in cells is not an instantaneous event (Figure 1A). The delay of the onset of action may be due to the time required for L-I-OddU to form the active metabolite. The antiviral action of L-I-OddU is reversible. Replication of EBV DNA could reappear upon removal of drug (Figure 1B). The time required to reinitiate viral DNA replication following drug removal is dependent on the concentration of the drug used in the treatment of H1 cells. The mechanism of the reversal action is unclear. The reemergence of EBV DNA is unlikely due to the appearance of drug-resistant virus, since the virus DNA reemerged from those cells can still be inhibited by L-I-OddU (data not shown).

Kaposi's sarcoma-associated herpes virus (KSHV/HHV-8) is a novel herpes virus which has been shown to be related to Kaposi's sarcoma and some tumors.[12,29,30] KSHV and EBV belong to the γ herpes virus subfamily and are similar to each other.[31,32] KSHV TK and DNA polymerase are 50.7% and 70.9% similar to EBV TK and DNA polymerase in amino acid level, respectively.[39] It is possible that L-I-OddU can also be specifically phosphorylated by KSHV TK and inhibit the KSHV replication. Recently, Kedes and Ganem reported that ACV was relatively inactive (ED_{50} 60−80 μM); however, DHPG and (*S*)-HPMPC showed potent activity against KSHV (ED_{50} 2.7−4 and 0.5−1 μM, respectively) in in vitro antiviral assay system.[33] These results are similar to the anti-EBV effect of these compounds. ACV was also less potent than DHPG or (*S*)-HPMPC in EBV.[17,34] We are currently examining the action of L-I-OddU against KSHV.

In summary, L-I-OddU is the most potent and selective anti-EBV compound without cytotoxicity discovered to date. The spectrum of activity is different from those of other antiviral L-nucleosides. This compound, therefore, should be considered as a candidate drug for the treatment or prevention of EBV-associated diseases.

Experimental Sections

Chemical Synthesis. Melting points were determined on a Mel-temp II laboratory device and are uncorrected. NMR spectra were recorded on a Bruker 400 Fourier transform spectrometer; chemical shifts are reported in parts per million (δ), and signals are quoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (doublet of doublets). UV spectra were obtained on a Beckman DU-7 spectrophotometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. TLC was performed on Uniplates (silica gel) purchased from Analtech Co. Elemental analysis was performed by Atlantic Microlab, Inc., Norcross, GA. Dry 1,2-dichloroethane, dichloromethane, acetonitrile, benzene, and pyridine were obtained by distillation from CaH_2 prior to use. Dry THF was obtained by distillation from Na and benzophenone when the solution became purple.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]uracil (9) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]uracil (10). A mixture of uracil (0.63 g, 5.6 mmol), a catalytic amount of $(NH_4)_2SO_4$, and HMDS (25 mL) was refluxed under N_2 atmosphere for 5 h. The resulting clear solution was concentrated in vacuo under anhydrous conditions to afford a colorless oil, to which a solution of the dioxolane intermediate **2** (1 g, 3.7 mmol) in dry DCE (10 mL) and TMSOTf (1.4 mL) were added. The mixture was stirred for 1 h at room temperature and quenched by adding saturated $NaHCO_3$ (10 mL). After the mixture was stirred for an additional 30 min, it was diluted with H_2O (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with saturated $NaHCO_3$ and brine, then dried, and filtered. The filtrate was concentrated to give an inseparable mixture of α- and β-anomers **3** (0.936 g, 81%), which was then treated with methanolic ammonia at room temperature for 24 h. After concentration, the residue was purified by silica gel column chromatography (1−8% MeOH

in $CHCl_3$) to afford β-isomer **9** (288 mg, 45.7%) as a white solid and α-isomer **10** (245 mg, 38.9%) as a white foam. β-Isomer **9**: mp 182−184 °C; $[\alpha]^{25}_D$ +20.9 (*c* 0.33, MeOH); UV (H_2O) λ_{max} 261.0 nm (ε 10 400) (pH 7), 260.5 nm (ε 7 930) (pH 11), 261.0 nm (ε 10 700) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.32 (s, 1H, NH, D_2O exchangeable), 7.80 (d, *J* = 8.2 Hz, 1H, H-6), 6.19 (d, *J* = 4.9 Hz, 1H, H-4′), 5.61 (d, *J* = 8.2 Hz, 1H, H-5), 5.18 (t, *J* = 5.9 Hz, 1H, OH, D_2O exchangeable), 4.90 (t, *J* = 2.8 Hz, 1H, H-2′), 4.02−4.05 (m, 2H, H-5′), 3.62 (d, *J* = 2.75 Hz, 2H, H-6′). Anal. Calcd for $C_8H_{10}O_5N_2$: C, H, N. α-Isomer **10**: $[\alpha]^{25}_D$ −3.28 (*c* 0.72, MeOH); UV (H_2O) λ_{max} 262.0 nm (ε 10 700) (pH 7), 261.5 nm (ε 8 250) (pH 11), 261.5 nm (ε 11 200) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.37 (br s, 1H, NH, D_2O exchangeable), 7.58 (d, *J* = 7.9 Hz, 1H, H-6), 6.13 (dd, *J* = 5.4, 3.0 Hz, 1H, H-4′), 5.61 (d, *J* = 7.9 Hz, 1H, H-5), 5.43 (t, *J* = 3.6 Hz, 1H, H-2′), 5.01 (t, *J* = 6.0 Hz, 1H, OH, D_2O exchangeable), 4.26 (dd, *J* = 9.4, 5.4 Hz, 1H, H-5′), 4.05 (dd, *J* = 9.4, 3.0 Hz, 1H, H-5′), 3.42 (m, 2H, H-6′). Anal. Calcd for $C_8H_{10}O_5N_2$: C, H, N.

A similar method was applied to synthesize the other 5-substituted nucleosides described below.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-fluorouracil (11) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-fluorouracil (12). β-Isomer **11**: mp 164−165 °C; $[\alpha]^{25}_D$ +7.7 (*c* 0.25, MeOH); UV (H_2O) λ_{max} 268.0 nm (ε 9 970) (pH 7), 268.0 nm (ε 7 820) (pH 11), 268.0 nm (ε 10 100) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.91 (s, 1H, NH, D_2O exchangeable), 8.30 (d, *J* = 7.2 Hz, 1H, H-6), 6.24 (d, *J* = 5.4 Hz, 1H, H-4′), 5.38 (t, *J* = 5.9 Hz, 1H, OH, D_2O exchangeable), 4.97 (s, 1H, H-2′), 4.35 (d, *J* = 9.9 Hz, 1H, H-5′), 4.11 (dd, *J* = 9.9, 5.5 Hz, 1H, H-5′), 3.73 (d, *J* = 5.6 Hz, 2H, H-6′). Anal. Calcd for $C_8H_9FO_5N_2$: C, H, N. α-Isomer **12** (foam): $[\alpha]^{25}_D$ +5.8 (*c* 0.29, MeOH); UV (H_2O) λ_{max} 268.5 nm (ε 10 200) (pH 7), 268.0 nm (ε 8210) (pH 11), 268.5 nm (ε 12 200) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.90 (s, 1H, NH, D_2O exchangeable), 7.88 (d, *J* = 6.8 Hz, 1H, H-6), 6.10 (m, 1H, H-4′), 5.49 (t, *J* = 3.8 Hz, 1H, H2′), 5.00 (s, 1H, OH, D_2O exchangeable), 4.29−4.03 (m, 2H, H-5′), 3.33 (m, 2H, H-6′). Anal. Calcd for $C_8H_9FO_5N_2$: C, H, N.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-chlorouracil (13) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-chlorouracil (14). β-Isomer **13**: mp 186−188 °C; $[\alpha]^{25}_D$ +2.98 (*c* 0.26, MeOH); UV (H_2O) λ_{max} 275.0 nm (ε 9 020) (pH 7), 274.5 nm (ε 7 410) (pH 11), 276.0 nm (ε 10 400) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.86 (s, 1H, NH, D_2O exchangeable), 8.37 (s, 1H, H-6), 6.17 (d, *J* = 5.2 Hz, 1H, H-4′), 5.37 (t, *J* = 5.6 Hz, 1H, OH, D_2O exchangeable), 4.92 (s, 1H, H-2′), 4.34−4.02 (m, 2H, H-5′), 3.66 (m, 2H, H-6′). Anal. Calcd for $C_8H_9ClO_5N_2$: C, H, N. α-Isomer **14**: $[\alpha]^{25}_D$ +5.3 (*c* 0.72, MeOH); UV (H_2O) λ_{max} 276.0 nm (ε 9 450) (pH 7), 274. 5 nm (ε 8 280) (pH 11), 276.5 nm (ε 9 510) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.67 (s, 1H, NH, D_2O exchangeable), 7.68 (s, 1H, H-6), 5.90 (d, *J* = 5.2 Hz, 1H, H-4′), 5.31 (t, *J* = 3.5 Hz, 1H, H-2′), 4.76 (s, 1H, OH, D_2O exchangeable), 4.10 (dd, *J* = 9.6, 2.6 Hz, 1H, H-5′), 3.92 (dd, *J* = 9.6, 5.4 Hz, 1H, H-5′), 3.24 (m, 2H, H-6′). Anal. Calcd for $C_8H_9ClO_5N_2$: C, H, N.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-bromouracil (15) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-bromouracil (16). β-Isomer **15**: mp 220−223 °C; $[\alpha]^{25}_D$ +4.6 (*c* 0.28, MeOH); UV (H_2O) λ_{max} 277.0 nm (ε 7 230) (pH 7), 276.0 nm (ε 6 980) (pH 11), 278.5 nm (ε 9 940) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.83 (s, 1H, NH, D_2O exchangeable), 8.45 (s, 1H, H-6), 6.16 (d, *J* = 5.1 Hz, 1H, H-4′), 5.36 (t, *J* = 5.6 Hz, 1H, OH, D_2O exchangeable), 4.92 (s, 1H, H-2′), 4.34−4.01 (m, 2H, H-5′), 3.66 (m, 2H, H-6′). Anal. Calcd for $C_8H_9BrO_5N_2$·0.7MeOH: C, H, N. α-Isomer **16**: $[\alpha]^{25}_D$ +3.7 (*c* 0.29, MeOH); UV (H_2O) λ_{max} 279.0 nm (ε 11 800) (pH 7), 276.0 nm (ε 8 440) (pH 11), 279.0 nm (ε 11 800) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.88 (s, 1H, NH, D_2O exchangeable), 7.92 (s, 1H, H-6), 6.07 (dd, *J* = 5.4, 3.2 Hz, 1H, H-4′), 5.50 (t, *J* = 4.0 Hz, 1H, H-2′), 5.01 (br s, 1H, OH, D_2O exchangeable), 4.27 (m, 2H, H-5′), 3.41 (m, 2H, H-6′). Anal. Calcd for $C_8H_9BrO_5N_2$: C, H, N.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (17) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (18). β-Isomer **17**: mp 223 °C dec; $[\alpha]^{25}_D$ +7.6 (*c* 0.25, MeOH); UV (H_2O) λ_{max} 285.0 nm (ε 6 630) (pH 7), 279.0 nm (ε 5 590) (pH 11), 287.0 nm (ε 7 350) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.64 (s, 1H, NH, D_2O exchangeable), 8.42 (s, 1H, H-6), 6.16 (dd, *J* = 5.1, 1.3 Hz, 1H, H-4′), 5.30 (br s, 1H, OH, D_2O exchangeable), 4.93 (s, 1H, H-2′), 4.31−4.04 (m, 2H, H-5′), 3.66 (m, 2H, H-6′). Anal. Calcd for $C_8H_9IO_5N_2$: C, H, N. α-Isomer **18**: mp 222 °C dec; $[\alpha]^{25}_D$ −6.8 (*c* 0.29, MeOH); UV (H_2O) λ_{max} 287.5 nm (ε 8 950) (pH 7), 278.5 nm (ε 6 380) (pH 11), 287.5 nm (ε 9 050) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.54 (s, 1H, NH, D_2O exchangeable), 7.75 (s, 1H, H-6), 5.92 (dd, *J* = 5.6, 2.5 Hz, 1H, H-4′), 5.32 (t, *J* = 4.0 Hz, 1H, H-2′), 4.78 (t, *J* = 5.6 Hz, 1H, OH, D_2O exchangeable), 4.12 (dd, *J* = 8.0, 4.0 Hz, 1H, H-5′), 3.95 (dd, *J* = 8.0, 4.0 Hz, 1H, H-5′), 3.25 (m, 2H, H-6′). Anal. Calcd for $C_8H_9IO_5N_2$: C, H, N.

(2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-(trifluoromethyl)uracil (19) and (2*S*,4*S*)-1-[2-(Hydroxymethyl)-1,3-dioxolan-4-yl]-5-(trifluoromethyl)uracil (20). β-Isomer **19**: mp 200−201 °C; $[\alpha]^{25}_D$ −5.9 (*c* 0.27, MeOH); UV (H_2O) λ_{max} 261.0 nm (ε 10 700) (pH 7), 259.0 nm (ε 7 330) (pH 11), 267.0 nm (ε 11 000) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.88 (s, 1H, NH, D_2O exchangeable), 8.84 (s, 1H, H-6), 6.17 (d, *J* = 4.9 Hz, 1H, H-4′), 5.40 (t, *J* = 5.5 Hz, 1H, OH, D_2O exchangeable), 4.94 (s, 1H, H-2′), 4.39 (d, *J* = 9.8 Hz, 1H, H-5′), 4.08 (dd, *J* = 9.9, 5.3 Hz, 1H, H-5′), 3.70 (d, *J* = 5.4 Hz, 2H, H-6′). Anal. Calcd for $C_9H_9F_3O_5N_2$·0.2EtOH: C, H, N. α-Isomer **20** (foam): $[\alpha]^{25}_D$ 2.59 (*c* 0.26, MeOH); UV (H_2O) λ_{max} 260.5 nm (ε 12 200) (pH 7), 259.5 nm (ε 6 750) (pH 11), 261.0 nm (ε 12 300) (pH 2); ^{1}H NMR (DMSO-d_6) δ 11.76 (br s, 1H, NH, D_2O exchangeable), 7.80 (s, 1H, H-6), 5.91 (dd, *J* = 5.3, 3.1 Hz, 1H, H-4′), 5.32 (t, *J* = 3.6 Hz, 1H, H-2′), 4.82 (t, *J* = 5.9 Hz, 1H, OH, D_2O exchangeable), 4.18 (dd, *J* = 9.4, 5.3 Hz, 1H, H-5′), 4.00 (dd, *J* = 9.4, 3.1 Hz, 1H, H-5′), 3.31 (m, 2H, H-6′). Anal. Calcd for $C_9H_9F_3O_5N_2$·0.1H_2O: C, H, N.

Biological Evaluation. DHPG was a gift from Syntex, Inc., Palo Alto, CA. [α-^{32}P]dCTP was purchased from Amersham. RPMI 1640 medium and dialyzed fetal bovine serum were purchased from JRH Biosciences, Lenexa, KS. Labeling kit was purchased from Stratogene, La Jolla, CA.

Cell Culture. A high-yield EBV-producing cell line H1 which was derived from human P3HR1 cells[22] was used in this study. More than 95% EBV DNA cells exist in replicating linear form. Cells were cultured in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μg/mL kanamycin and were grown at 37 °C in a humidified incubator containing 5% CO_2.

Exposure of H1 Cells to Compounds. H1 cells were maintained in a logarithmic phase of growth for 2 days prior to the initiation of treatment. The H1 cells were seeded in 24-well plates at a density of 2 × 10^5 cells/well in 2 mL of fresh medium with or without the compound treatment and were incubated at 37 °C for 5 days. The cells were then pelleted by centrifugation, washed, and prepared for quantitation by Slot blot analysis.

EBV DNA Detection. Slot blot assay was performed as described previously[17] with some modification. A total of 4 × 10^5 H1 cells treated with different compounds at various concentration were lysed in 400 μL of 10 mM Tris-HCl (pH 7.5) solution by freezing and thawing three times. The cell lysate was treated with RNase A (at a final concentration of 50 μg/mL) at 55 °C for 2 h. Equal volume of 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) was added to each sample. After heating for 10 min in a water bath at 100 °C, the samples were spotted on positively charged nylon membrane using a manifold connected with vacuum system. The spotted samples were denatured by washing with 0.4 N NaOH/10 mM EDTA (pH 8.2). Then, [α-^{32}P]dCTP-labeled *Eco*RI C fragment of EBV DNA was used as a probe for DNA hybridization. Autoradiographic results were analyzed by Personal Densitometer SI (Molecular Dynamics Inc., Sunnyvale, CA). The same membrane was rehybridized with human Alu DNA *Bam*HI fragment after stripping the EBV *Eco*RI probe. The amount of EBV DNA in treated H1 cells was determined according to the ratio of EBV DNA to Alu DNA as compared with nontreated control H1 cells. The same

membrane can be reused for assessment of toxicity of compound to mitochondrial DNA by rehybridization with mitochondrial DNA probe after removing Alu DNA probe.

Cytotoxicity. An evaluation of the toxicity of compounds against H1 cells was carried out as previously described.[35] H1 cells were seeded in 24-well plates at a density of 2×10^5 cells/well in 2 mL of medium and incubated with various concentrations of each compound for 3 days and then counted for cell number. ID_{50} was defined as the inhibitory dose that caused a 50% reduction in cell number compared with the untreated control.

Reversibility of EBV Replication after Removal of Compounds. H1 cells were seeded in a 24-well plate at a density of 2×10^5 cells/well in 2 mL of fresh RPMI 1640 medium and treated with various concentrations of compounds. After incubation for 5 days, 4×10^5 cells were taken from each well and tested for inhibitory action of compounds by the Slot blot method. Cells (2×10^5) from each well were taken and washed with cold PBS. After removal of compounds, fresh compound-free medium was added for passage in a new 24-well plate. Then, every 5 days, 4×10^5 cells were taken from each well to measure the percentage of inhibition as compared with that of control, and 2×10^5 cells were taken each time for use in time course study until the amounts of EBV DNA recovered to the pretreatment level.

Acknowledgment. This work was supported by Grant CA-63477 from the National Cancer Institute and Grant AI-33655 from the National Institute of Allergy and Infectious Diseases, NIH.

References

(1) Alan, B.; Kieff, E. Epstein-Barr Virus. In *Virology*, 3rd ed.; Fields, B. N., Kimpe, D. M., Howly, P. M., Eds.; Lippincott-Raven Publishers: Philadelphia, PA, 1996; Chapter 75, pp 2397−2446.

(2) Crawford, S.-L.; Thomas, J. A. Epstein-Barr virus associated lymphomas. In *The Epstein-Barr Virus and Associated Diseases*; Tutsz, T., Pagano, J. S., Ablashi, D. V., De The, G., Lanoir, G., Pearson, G. R., Eds.; Colloques Inserm 225; 1993; pp 397−404.

(3) Pallesen, G.; Hamilton-Dutoit, S. J.; Zhou, X. The association of Epstein-Barr virus (EBV) with T cell lymphoproliferation and Hodgkin's disease: two new developments in the EBV field. *Adv. Cancer Res.* **1993**, *62*, 179−239.

(4) Shibata, D.; Weiss, L. M. Epstein-Barr virus associated gastric adenocarcinoma. *Am. J. Pathol.* **1992**, *140*, 769−774.

(5) Van Grop, J.; Weiping, L.; Jacobse, K.; Liu, Y.-H.; Li, F.-Y.; De Weger, R. A.; Li, G. Epstein-Barr virus in nasal T-cell lymphomas (polymorphic reticulosis/midline malignant reticulosis) in Western China. *J. Pathol.* **1994**, *173*, 81−87.

(6) Weiss, L. M.; Movahed, L. A. *In situ* demonstration of Epstein-Barr viral genomes in viral associated B cell lymphoproliferations. *Am. J. Pathol.* **1989**, *134*, 651−659.

(7) Yong, L. S.; Dawson, C. W.; Clark, D.; Rupani, H.; Busson, P.; Tursz, T.; Johnson, A.; Rickinson, A. B. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. *J. Gen. Virol.* **1988**, *69*, 1051−1065.

(8) Zeng, Y. Seroepidemiological studies on nasopharyngeal carcinoma in China. *Adv. Cancer Res.* **1985**, *44*, 121−138.

(9) Litter, E.; Baylis, S. A.; Zeng, Y.; Conway, M.; Mackett, M.; Arrand, J. R. Diagnosis of nasopharyngeal carcinoma by means of recombinant Epstein-Barr virus proteins. *Lancet* **1991**, *337*, 685−689.

(10) Shapiro, R. S.; McClain, K.; Frizzera, G.; Gajl-Peczalska, K. J.; Kersey, J. H.; Blazar, B. R.; Arthur, D. C.; Patton, D. F.; Greenberg, J. S.; Burke, B.; Ramsay, N. K. C.; McGlave, P.; Filipovich, A. H. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. *Blood* **1988**, *71*, 1234−1243.

(11) Zutter, M. M.; Martin, P. J.; Sale, G. E.; Shulman, H. M.; Fisher, L.; Thomas, E. D.; Durman, D. M. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. *Blood* **1988**, *72*, 520−529.

(12) Chang, Y.; Cesarman, E.; Pessin, M. S.; Lee, F.; Culpepper, J.; Knowles, D. M.; Moore, P. S. Identification of herpes virus-like DNA sequence in AIDS-associated Kaposi's sarcoma. *Science* **1994**, *266*, 1865−1869.

(13) Greenspan, J. S.; Greenspan, D.; Lannette, E. T.; Abrams, D. I.; Conant, M. A.; Peterson, V.; Freese, U. K. Replication of Epstein-Barr virus within the epithelial cells or oral "hairy" leukoplakia, an AIDS-associated lesion. *N. Engl. J. Med.* **1985**, *313*, 1564−1571.

(14) Cheng, Y.-C.; Huang, E.-S.; Lin, J. S.; Mar, E.-C.; Pagano, J. S.; Dutschman, G. E.; Grill, S. P. Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine against herpes virus *in vitro* and its mode of action against herpes simplex virus type I. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2767−2770.

(15) Lin, J. C.; Smith, M. C.; Pagano, J. C. Comparative efficacy and selectivity of some nucleoside analogs against Epstein-Barr virus. *Antimicrob. Agents Chemother.* **1985**, *27*, 971−973.

(16) Colby, B. M.; Shaw, J. E.; Elion, G. B.; Pagano, J. S. Effect of Acyclovir [9-(2-hydroxyethoxymethyl)guanine] on Epstein-Barr virus DNA replication. *J. Virol.* **1980**, *34*, 560−568.

(17) Yao, G.-Q.; Liu, S.-H.; Chou, E.; Kukhanova, M.; Chu, C. K.; Cheng, Y.-C. Inhibition of Epstein-Barr virus replication by a novel L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil. *Biochem. Pharmacol.* **1996**, *51*, 941−947.

(18) Yao, G.-Q.; Grill, S.; Egan, W.; Cheng, Y.-C. Potent inhibition of Epstein-Barr virus by phosphorothioate oligodeoxynucleotides without sequence specification. *Antimicrob. Agents Chemother.* **1993**, *37*, 1420−1425.

(19) Chu, C. K.; Ma, T.; Shanmuganathan, K.; Wang, C.; Xiang, Y.; Pai, S. B.; Yao, G.-Q.; Sommadossi, J.-P.; Cheng, Y.-C. Use of 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil as a novel antiviral agent for hepatitis B virus and Epstein-Barr virus. *Antimicrob. Agents Chemother.* **1995**, *39*, 979−981.

(20) Kim, H. O.; Schinazi, R. F.; Shanmuganathan, K.; Jeong, L. S.; Beach, J. W.; Nampalli, S.; Cannon, D. L.; Chu, C. K. L-β-(2*S*,4*S*)- and L-α-dioxolanyl nucleosides as potential anti-HIV agents: asymmetric synthesis and structure-activity relationships. *J. Med. Chem.* **1993**, *36*, 519−528.

(21) Pai, S. B.; Liu, S.-H.; Zhu, Y.-L.; Cheng, Y.-C. Inhibition of hepatitis B virus by a novel L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil. *Antimicrob. Agents Chemother.* **1996**, *40*, 380−386.

(22) Yao, G.-Q.; Tsai, C.-H.; Cheng, Y.-C. Characterization of sublines of Epstein-Barr virus producing HR-1 cells and its implication in virus propagation in culture. *Virus Gene* **1995**, *9*, 247−255.

(23) Chang, C.-N.; Doong, S.-L.; Zhou, J. H.; Beach, J. W.; Jeong, L. S.; Chu, C. K.; Tsai, C.-H.; Cheng, Y.-C. Deoxycytidine deaminase-resistant stereoisomer is the active form of (±)-2′,3′-dideoxy-3′-thiacytidine in the inhibition of hepatitis B virus replication. *J. Biol. Chem.* **1992**, *267*, 13938−13942.

(24) Coates, J. A. V.; Cammack, N.; Jenkinson, H. J.; Mutton, I. M.; Pearson, B. A.; Storer, R.; Cameron, J. M.; Penn, C. R. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH-189) both inhibit human immunodeficiency virus replication *in vitro*. *Antimicrob. Agents Chemother.* **1992**, *36*, 202−205.

(25) Lin, T.-S.; Luo, M.-Z.; Liu, M.-C.; Pai, S. B.; Dutschman, G. E.; Cheng, Y.-C. Synthesis and biological evaluation of 2′,3′-dideoxy-L-pyrimidine nucleosides as potential antiviral agents against human immunodeficiency virus (HIV) and hepatitis B virus (HBV). *J. Med. Chem.* **1994**, *37*, 798−803.

(26) Lin, T.-S.; Luo, M.-Z.; Liu, Y.-L.; Gullen, E.; Dutschman, G. E.; Cheng, Y.-C. Design and synthesis of 2′,3′-didehydro-β-L-5-fluorocytidine (β-L-Fd4C), two exceptionally potent inhibitors of human hepatitis B virus (HBV) and potent inhibitors of human immunodeficiency virus (HIV) *in vitro*. *J. Med. Chem.* **1996**, *39*, 1757−1759.

(27) Schinazi, R. F.; McMillan, A.; Cannon, D.; Mathis, R.; Lloyd, R. M.; Peck, A.; Sommadossi, J.-P.; St. Clair, M.; Wilson, J.; Furman, P. A.; Painter, G.; Choi, W.-B.; Liotta, D. C. Selective inhibition of human immunodeficiency virus by racemates and enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. *Antimicrob. Agents Chemother.* **1992**, *36*, 2423−2431.

(28) Cheng, Y.-C.; Domin, B. A.; Sharma, R. A.; Bobek, M. Antiviral action and cellular toxicity of four thymidine analogues: 5-ethyl-, 5-vinyl-, 5-propyl-, and 5-allyl-2′-deoxyuridine. *Antimicrob. Agents Chemother.* **1976**, *10*, 119−122.

(29) Gessain, A.-J.; Brière, J.; Angelin-Duclos, C.; Valensi, F.; Bèral, H. M.; Davi, F.; Nicola, M.-A.; Sudaka, A.; Fouchard, N.; Gabarre, J.; Troussard, X.; Dulmet, E.; Audouin, J.; Diebold, J.; de Thé, G. Human herpes virus 8 (Kaposi's sarcoma herpes virus) and malignant lymphoproliferations in France: a molecular study of 250 cases including two AIDS-associated body cavity based lymphomas. *Leukemia* **1997**, *11*, 266−272.

(30) Siad, J. W.; Tasaka, T.; Takeuchi, S.; Asou, H.; De Vos, S.; Cesarman, E.; Knoeles, D. M.; Koeffler, H. P. Primary effusion lymphoma in women: report of two cases of Kaposi's sarcoma herpes virus-associated effusion-based lymphoma in human immunodeficiency virus-negative women. *Blood* **1996**, *88*, 3124−3128.

(31) Moore, P. S.; Gao, S.-J.; Domingquez, G.; Cesarman, E.; Lungu, O.; Knowles, D. M.; Garber, R.; Pellett, P. E.; McGeoch, D. J.; Chang, Y. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. *J. Virol.* **1996**, 549−558.
(32) Russo, J. J.; Bohenzky, R. A.; Chien, M.-C.; Chen, J.; Yan, M.; Maddalena, D.; Parry, J. P.; Peruzzi, D.; Edelman, I. S.; Chang, Y.; Moore, P. S. Nucleotide sequence of the Kaposi's sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14862−14867.
(33) Kedes, D. H.; Ganem, D. Sensitivity of Kaposi's sarcoma-associated herpesvirus replication to antiviral drugs. *J. Clin. Invest.* **1997**, *99*, 2082−2086.
(34) Lin, J.-C.; DeClercq, E.; Pagano, J. S. Novel acyclic adenosine analogs inhibit Epstein-Barr virus replication. *Antimicrob. Agents Chemother.* **1987**, *31*, 1431−1433.
(35) Doong, S.-L.; Tsai, C.-H.; Schinazi, R. F.; Liotta, D. C.; Cheng, Y.-C. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-didieoxy-3′-thiacytidine and related analogues. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8495−8499.

JM9806749