Articles

Nucleoside Conjugates. 15. Synthesis and Biological Activity of Anti-HIV Nucleoside Conjugates of Ether and Thioether Phospholipids[1]

Chung I. Hong,*,† Alexander Nechaev,† Alan J. Kirisits,† Rakesh Vig,† Charles R. West,† Konstantine K. Manouilov,‡ and Chung K. Chu‡

Department of Neurosurgery, Roswell Park Cancer Institute, Buffalo, New York 14263, and Department of Medicinal Chemistry, College of Pharmacy, University of Georgia, Athens, Georgia 30602

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A series of the anti-HIV nucleoside conjugates of ether (1-*O*-alkyl) and thioether (1-*S*-alkyl) lipids linked by a pyrophosphate diester bond has been synthesized as micelle-forming prodrugs of the nucleosides to improve their therapeutic efficiency. These include AZT 5′-diphosphate−*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol (**1**), 3′-azido-2′,3′-dideoxyuridine 5′-diphosphate−*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol (**2**), 2′,3′-dideoxycytidine 5′-diphosphate−*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol (**3**), and AZT 5′-diphosphate−*rac*-1-*O*-tetradecyl-2-*O*-palmitoylglycerol (**4**). The conjugates form micelles by sonication (mean diameters ranging 6.8−55.5 nm). Conjugate **1** protected 80% of HIV-infected CEM cells as low as 0.58 μm and lost the protection at 180 μM due to prevailing cytotoxicity, while the conjugate started to show the cytotoxicity at 100 μM. Pharmacokinetics studies showed a significant increase of half-life values ($t_{1/2}$) of AZT and AZddU[2] (respective $t_{1/2}$ = 5.69 and 6.5 h) after administration of conjugates **1** and **2**, while those after administration of AZT and AZddU were 0.28 and 0.89 h, respectively. The fractions of the prodrugs **1** and **2** converted to the parent compounds AZT and AZddU were 36% and 55%, respectively. The results indicate that AZT and AZddU thioether lipid conjugates **1** and **2** warrant further investigation.

In view of the etiologic role of the human immunodeficiency virus type 1 (HIV-1)[2] on AIDS, compounds that inhibit HIV-1 replication are potentially effective therapeutic agents for the treatment of AIDS. 2′,3′-Dideoxynucleosides such as AZT,[3] ddI, ddC,[4] d4T,[5] and AZddU[6] are inhibitors of HIV-1 reverse transcriptase, which is vital for viral replication. In general, these nucleosides are prodrugs and are sequentially phosphorylated to the 5′-triphosphates by host cell kinases.[7] The efficiency of this phosphorylation process can account for some of the differences in activity of these nucleosides. Membrane interactive ether lipid analogues inhibit both HIV-induced cytopathology and infectious virus multiplication by inducing defective intracytoplasmic vacuolar HIV-1 formation in T-cells.[8] Combinations of ether lipid analogue and AZT produced an apparent synergistic action in suppressing infectious HIV-1 replication.[8]

In an attempt to improve the antiretroviral activity, a variety of phospholipid analogues of the nucleosides have been synthesized independently by a number of laboratories including ours. These include the nucleoside conjugates of diacylglycerols with monophosphate[9,10] and diphosphate,[10−13] and those of ether, thioether, and amidoalkyl ether lipids with monophosphate,[14] phosphonate,[14] and diphosphate.[1] Since T-lymphocytes and monocyte/macrophages serve as important reservoirs of latent and active HIV-1 infections,[15−17] and since the lipophilic compounds such as ether lipids have a natural affinity to these cells,[18] they could provide a new possibility for the selective delivery of the antiretroviral drugs to the cells. In fact, the anti-HIV nucleoside conjugates of phospholipids encapsulated in liposome were found to be more active than the parent nucleoside in HIV-infected cells.[10] Previously, the antitumor nucleoside conjugates of ether and thioether phospholipids demonstrated a superior antitumor activity against both animal leukemia and solid tumor models *in vivo*.[19−27] And micelle formation of the conjugates further improved their antitumor activity.[28]

The favorable properties demonstrated previously by the micelle-forming antitumor nucleoside conjugates of ether and thioether phospholipids prompted us to synthesize the anti-HIV nucleoside conjugates of ether and thioether lipids with diphosphate. The conjugates have both anti-HIV nucleoside and potential antiretroviral ether or thioether lipid substituted for cytidine and diacylglyceride in naturally occurring cytidine diphosphate diacylglyceride, a precursor for membrane phosphatidylinositol and cardiolipin.[29] The rationale is that (1) the conjugate is not only a new prodrug of the nucleoside but also may generate two antiretroviral agents, the 5′-triphosphate and the ether lipid analogue, with different target sites, (2) thus, this combination could produce a synergistic action,[8] and (3) micelles formed from the conjugates could deliver the antiretoviral agents selectively to the HIV-1 infected cells.[15−17]

This paper describes the synthesis of the conjugates, particle sizes of micelles, stability, *in vitro* anti-HIV-1 activity, and preliminary pharmacokinetics in mice.

† Roswell Park Cancer Institute.
‡ University of Georgia.
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Table 1. Physical Data of anti-HIV Nucleoside Conjugates

compd	yield, %	mp, °C	solubility (mM)[a]	partition coefficient (*P*)[b]	UV_{max}, nm ($\epsilon \times 10^{-3}$) neutral	acid	base	formula	analysis
1	27	199−201	0.51	1.66	266 (8.31)	266 (8.03)	266.3 (6.13)	$C_{47}H_{85}N_5O_{12}SP_2 \cdot 2Na \cdot 3H_2O$	C, H, S, P
2	14	185−187	0.39	3.58	261 (8.40)	262.2 (7.86)	262 (5.64)	$C_{46}H_{83}N_5O_{12}SP_2 \cdot 2Na \cdot H_2O$	C, H, S,[c] P
3	14	198−200	0.51	0.59	272 (6.92)	282 (10.01)	272.5 (6.46)	$C_{46}H_{85}N_3O_{11}SP_2 \cdot 2Na \cdot 2H_2O$	C, H,[d] S, P
4	19	197−199	0.76	1.76	266.5 (7.01)	266 (6.83)	266 (5.10)	$C_{43}H_{77}O_{13}N_5P_2 \cdot 2Na \cdot 2H_2O$	C, H, P

[a] Determined by UV absorption at 261, 266, or 272 nm which is UV_{max} of the conjugate. [b] Partition coefficients (*P*) in 1-octanol/PBS (pH 7.4) at 25 °C. *P* values for AZT, AZddU, and ddC are 1.15, 0.59, and 0.21, respectively. [c] S: calcd, 3.04; found, 3.49. [d] H: calcd, 8.69; found, 9.17.

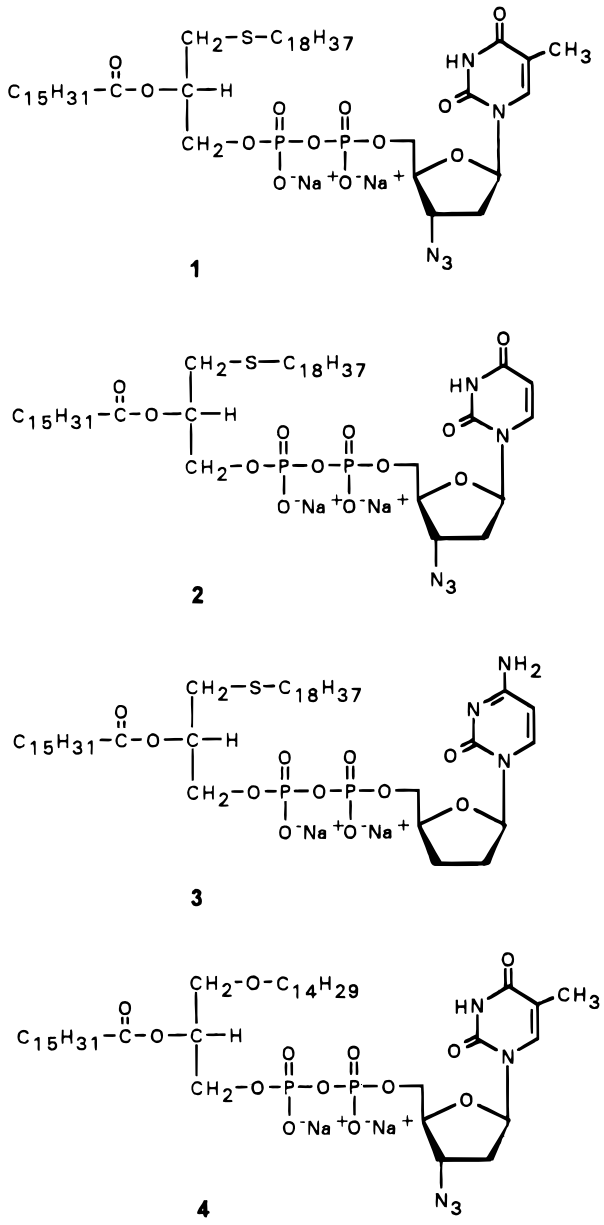

Figure 1. Structures of anti-HIV nucleoside conjugates of ether and thioether phospholipids.

Chemistry

The conjugates in Figure 1 were synthesized in an analogous manner previously described for the *ara*-C conjugates.[19,20] The nucleoside 5′-monophosphoromorpholidates[10,11] prepared from the nucleoside 5′-monophosphates were condensed with *rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol 3-phosphate[20] and *rac*-1-*O*-tetradecyl-2-*O*-palmitoylglycerol 3-phosphate[30] in pyridine, and then the conjugates were separated by column chromatography using a DE-52 cellulose (AcO^-) and 0.02 M NH_4OAc in $CHCl_3$−95% ethanol−H_2O (2:4:1) according to the literature procedures.[19,20] The overall yields were 14−27% (Table 1). Structures were verified by elemental analysis and ^1H NMR and UV spectrometry.

Table 2. Mean Particle Sizes of Micellar Formulations of the Conjugates

compd	vol-weighed nonlinear multimodal anal. (solid particles)[a] mean diameter, nm (vol %) peak 1	peak 2	peak 3
1	6.8 (98)		
2	16.4 (71)	143.1 (12)	427.9 (17)
3	55.5 (52)	215.9 (48)	
4	8.2 (82)	21.8 (14)	131.1 (4)

[a] Analyzed using photon correlation spectroscopy on NICOMP 370 submicron particle sizer with ZERO-OFF and peak at around 2 nm is an artifact.[33] Measurement of the particle sizes were performed by using the solid particles setting of the instrument. Peaks with <2% were deleted.

Water Solubility

The water solubilities of the conjugates in sterile water for injection, USP at room temperature, are listed in Table 1. Since the conjugates were sparingly soluble in water (0.39−0.76 mM), they were formulated in micellar suspensions by sonication, and the micellar suspensions were used for the biological testings such as anti-HIV screenings.

Lipophilicity

Partition coefficients (*P*) were determined for all conjugates (Table 1) using a mixture of 1-octanol and phosphate-buffered saline solution (PBS), pH 7.4, at room temperature.[31] *P* values for AZT conjugates **1** and **4** were 1.66 and 1.76, respectively, while the *P* value of AZT was 1.15. *P* values for conjugates **2** and **3** were 3.58 and 0.59, while those of the respective parent nucleosides AZddU and ddC were 0.59 and 0.21. Thus, an increase in lipophilicity was about 1.5-fold for the AZT conjugates, 6-fold for the AZddU conjugate, and 3-fold for the ddC conjugate as compared to those of the parent nucleosides.

Particle Size

Mean diameter of particles of the conjugates in micellar suspension were determined by volume-weighed nonlinear multimodal analysis using photon correlation spectroscopy[32] on a NICOMP 370 submicron particle sizer[33] (NICOMP Particle Sizing System, Santa Barbara, CA) (Table 2).[33] Mean particle sizes of the micelles were mainly (52−98%) 6.8−55.5 nm. AZT conjugates **1** and **4** formed smaller micelles (6.8−8.2 nm, >82%), while AZddU conjugate **2** and ddC conjugate **3** formed larger micelles (16.4 and 55.5 nm, >52%, respectively).

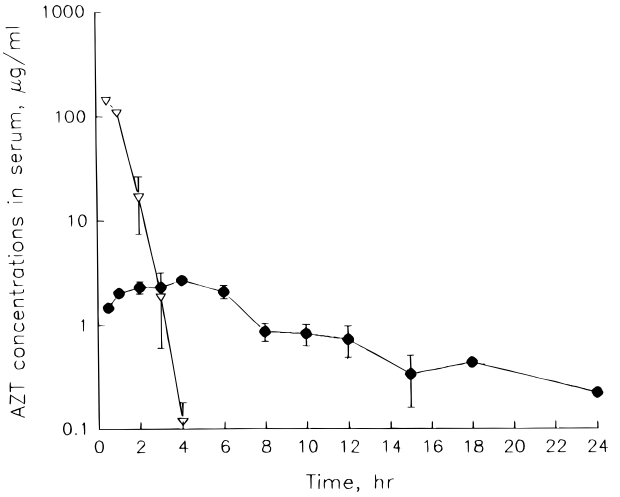

Figure 2. Mean AZT concentrations (±SD) in serum after ip administration of 100 mg/kg of AZT (▽) and 394 mg/kg (equivalent to 100 mg/kg AZT) of AZT conjugate **1** (●) to mice.

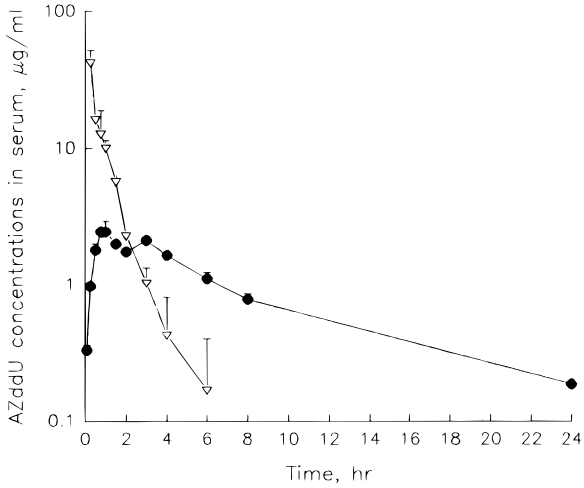

Figure 3. Mean AZddU concentrations (±SD) in serum after iv administration of 50 mg/kg of AZddU (▽) and 205 mg/kg (equivalent to 50 mg/kg AZddU) of AZddU conjugate **2** (●) to mice.

In Vitro Release of Nucleosides from Conjugates

In vitro hydrolysis of AZT and AZddU conjugates by serum and liver and brain homogenates was studied according to the procedures published previously.[34] AZT conjugate **1** and AZddU conjugate **2** were both stable in the serum at 37 °C. In liver and brain homogenates, AZT conjugate **1** released up to 10% of the parent nucleoside during the first 4 h of incubation. After that no significant release of AZT were found. AZddU conjugate **2** was more stable than the AZT conjugate. In 4 h of incubation free AZddU amounted to only 3% of the loaded value of the conjugated nucleoside.

Pharmacokinetics

In order to find the differences in pharmacokinetic parameters by route of injection of the conjugates, AZT conjugate **1** and AZddU conjugate **2** were injected ip and iv, respectively. The pharmacokinetics of AZT and AZddU in mice were determined after ip administration of 100 mg/kg of AZT and 394 mg/kg of AZT conjugate **1** (equivalent to 100 mg/kg of AZT) and after iv administration of 50 mg/kg of AZddU and 205 mg/kg of AZddU conjugate **2** (equivalent to 50 mg/kg of AZddU).[34] The serum concentrations of AZT and AZddU, as measured by HPLC, are illustrated in Figures 2 and 3, respectively. Pharmacokinetics parameters for both nucleosides are presented in Table 3. The serum concentration of the intact conjugate could not be determined by the HPLC method.

The AZT level in the serum after administration of **1** rose to a maximum 2.70 μg/mL at 4 h and slowly decreased over a period of 24 h, with a half-life of 5.69 h. In contrast, when AZT was administered, a fast decline of AZT concentrations in the serum was observed with a half-life of 0.28 h. The limit of assay quantitation was reached within 4 h. AZT conjugate **1** could extend the half-life value of the parent nucleoside AZT but not the area under concentration versus time curve (AUC) value. The fraction of conjugate **1** converted to AZT (f_m) value was only 36%.

The marked differences were observed also in disposition of AZddU after iv administration of conjugate **2** and AZddU (Figure 3). Intravenous injection of **2** resulted in a rapid rise of the nucleoside to 2.44 mg/mL at 1 h and slow decrease to 0.18 μg/mL over a period of 24 h with a half-life of 6.5 h. The f_m value of AZddU in the serum was 55%. However, iv injection of AZddU resulted in a rapid decline of the nucleoside with a half-life of 0.89 h in the serum.

Table 3. Pharmacokinetics Parameters for AZT and AZddU in Mice following a Single-Dose Administration of Conjugates **1** and **2**[a]

compd	route of administration	pharmacokinetic parameters AUC (mg·h/L)	CL_T	f_m	$t_{1/2}$ (h)
AZT conjugate **1**	ip	25.79	1.38	0.36	5.69
AZT	ip	174.7			0.28
AZddU conjugate **2**	iv	21.63	1.27	0.55	6.50
AZddU	iv	42.26			0.89

[a] Single doses of AZT conjugate **1** (394 mg/kg equivament to 100 mg/kg AZT), AZT (100 mg/kg), AZddU conjugate **2** (205 mg/kg equivalent 50 mg/kg AZddU), and AZddU (50 mg/kg) were injected ip or iv to female NIH-Swiss mice (wt 25−30 g).

Table 4. Effect of the Anti-HIV Nucleoside Conjugates on HIV-1-Infected CEM Cells

compd	concentration (μM)[a] EC_{50}[a]	IC_{50}[b]	therapeutic index TI_{50} (IC/EC)
AZT[c]	0.186	191	1027
1	<0.58	120	>207
2	17.0	380	23
3	130	2800	21
4	57	14000	246

[a] EC_{50} = concentration of drugs to protect the infected CEM cells by 50%. [b] IC_{50} = concentration of drugs to reduce the viable cell number by 50%. [c] Reference 35.

Biological Activity

The antiviral and cytotoxic activities of the conjugates are summarized in Table 4. AZT conjugate **1** protected 80% of HIV-infected CEM cells at as low as 0.58 μM (EC_{50} < 0.58 μM) and lost the protection at 180 μM due to prevailing cytotoxicity, while the conjugate started to show the cytotoxicity at 100 μM (IC_{50} = 120 μM). The anti-HIV activity of AZT in HIV-infected CEM cells reported previously[35] was ED_{50} = 0.186 μM and IC_{50} = 191 μM, and *in vitro* therapeutic index ($TI_{50} = IC_{50}/EC_{50}$) was 1027. Conjugates **2**−**4** gave EC_{50} values of 17, 130, and 57 μM, respectively. These conjugates started to

show the cytotoxicity (IC_{50}) at 380, 2800, and 14 000 μM, respectively. Only AZT conjugates **1** and **4** produced TI_{50} of >200.

Conjugate **1** was also tested for *in vivo* cytotoxicity against ip implanted L1210 lymphoid leukemia in DBA/2Ros mice.[36] A single dose administrations (ip) of 100−300 mg (95−285 μmol)/kg of **1** to the leukemic mice did not increase their life span (% ILS = 0). Toxicity as reflected in weight loss was not observed. These results indicate that the conjugate is not cytotoxic at a relatively high dose.

Since the previous antitumor nucleoside conjugates of ether and thioether phospholipids have been much more effective in *in vivo* than *in vitro* antitumor testings,[21,25] the anti-HIV nucleoside conjugates can be also effective *in vivo* anti-HIV drugs with improved therapeutic indices.

Discussion

A variety of prodrugs of anti-HIV nucleosides conjugated with lipid moieties has been reported previously.[9−14] However, the present prodrugs are anti-HIV nucleoside conjugates of ether and thioether lipids linked with diphosphate. Thus, in addition to the favorable properties demonstrated by the previous prodrugs, the present anti-HIV nucleoside conjugates may generate two antiretroviral agents, the anti-HIV nucleoside 5′-triphosphate and the ether or thioether lipid analogue, with different target sites.

It has been reported that the *in vitro* anti-HIV activity of AZT is higher than those of AZddU[6] and ddC.[35] Among conjugates **1**−**3** which contain the same thioether lipid moiety, AZT conjugate **1** was also more active than AZddU conjugate **2** and ddC conjugate **3** (Table 4). Conjugates **1** and **2** were both hydrolyzed slowly to AZT and AZddU, respectively, when they were incubated in liver and brain homogenates. There were little differences in the pharmacokinetics parameters of AZT conjugate **1** and AZddU conjugate **2** in spite of differences in particle sizes (Table 2) and route of injection of the conjugates (Table 3). These results indicate that the potency of the parent nucleoside is one of the important factors for the development of a promising anti-HIV nucleoside conjugate. In addition, the lipid moiety might also play an important role since the activity of AZT conjugate of thioether lipid, conjugate **1**, was found to be higher than that of of AZT conjugate of ether lipid, conjugate **4**. This analogy was also demonstrated previously with the ara-C conjugates. Cytoros, ara-C conjugate with thioether lipid, was more active than the analogue with the corresponding ether lipid.[20,22]

Dipalmitoylphosphatidyl-AZT (DPP-AZT) was considerably less active than AZT. But the liposome encapsulated DPP-AZT administered ip significantly increased the AUC value of AZT compared to that of the administration of AZT.[37] It has been also demonstrated that administration of micellar suspensions of dipalmitoyl-AZT and dipalmitoyl-AZddU resulted in consistently higher serum concentrations of AZT and AZddU in mice after 2−3 h with greater half-lives compared to those of the parent compounds.[38] Administration of a micellar suspension of the ara-C conjugates of thioether phospholipid into L1210 leukemic mice gave a greater retention of ara-CTP than that resulting from ara-C.[21] The favorable properties demonstrated by these previous nucleoside-phospholipid prodrugs were also found in the present conjugates (Figures 2 and 3). Conjugates **1**−**4** were formulated in micellar suspension without using liposome. The micellar formulations were quite stable in serum *in vitro* at 37 °C. However, administration of micellar formulations of AZT conjugate **1** and AZddU conjugate **2** resulted in higher serum concentration of AZT and AZddU in mice after 3−4 h with greater half-lives to the parent nucleosides. Other pharmacologically favorable properties of micellar formulation of the conjugates are the release of more drug, the same amount of drug over a longer interval, and the release of drug at a more constant rate than if micelles are absent.[39] Previously, micellar formulations of the ara-C conjugates of thioether phospholipids further improved their *in vivo* antitumor activity.[28] The micelles could also deliver the antiretroviral moieties selectively to the HIV-infected cells.[15−17] Thus, as an intact molecule, the micelle-forming conjugates act as a sustained-release form and possibly target a specific delivery system of the parent nucleosides. For the biological activity, the cleavage of the conjugate to the nucleoside and the lipid appeared to be essential. Slower release of the nucleoside from the conjugate might have resulted in an *in vitro* activity by the conjugate weaker than that of the nucleoside (Table 4). However, the conjugates would be more effective than the nucleosides against *in vivo* models as demonstrated previously by the ara-C conjugates.[21,25]

The micellar formulation can be also used for combination chemotherapy by solubilizing other water-insoluble antiretroviral agents, since a micellar suspension increases solubility of water-insoluble or sparingly soluble organic compounds, which has been applied to drug formulation.[40] Previously, combination chemotherapy of nitrosoureas solubilized in micellar suspension of the ara-C conjugates produced synergy.[22]

In summary, the convenient micellar formulation, high *in vitro* anti-HIV activity, and favorable pharmacokinetics demonstrated by AZT conjugate **1** and AZddU conjugate **2** warrant further investigation in *in vivo* HIV models and then clinical trials.

Experimental Section

Synthesis. Melting points were taken on a Mel-Temp capillary melting point apparatus. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Associates EM-390 or GE GN-500 MHz high-resolution NMR spectrometer. The chemical shift values were expressed in values (parts per million) relative to tetramethylsilane as an internal standard. UV absorption spectra are recorded on a Perkin-Elmer Lambda 4A spectrophotometer. AG1-X8 (Bio-Rad), Dowex 50W-X8 (Bio Rad), (diethylamino)ethylcellulose (DE-52, Whatman), and Amberlite CG-50 (Sigma) were used for column chromatography. Evaporation was performed *in vacuo* at 30 °C. TLC was performed on glass plates coated with a 0.25 mm layer of silica gel PF-254 (Brinkman) using the following solvent systems: (A) $CHCl_3$, (B) $CHCl_3$−MeOH (95:5), (C) $CHCl_3$−MeOH−H_2O−HOAc (25:15:4:2), and (D) *i*-PrOH−H_2O−concentrated NH_4OH (7:2:1). UV-absorbing compounds were detected by visualization under a UV lamp (254 nm), and phosphorus-containing compounds will be detected with a modified Dittmar−Lester spray.[41] The organic compounds were also detected by charring after spraying with the above reagent. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. When analyses are recorded only by the elemental symbols, results are within ±0.4% of the theoretical values including given numbers of H_2O of hydration

unless noted otherwise. The presence of H_2O as indicated by elemental analysis was verified by ^1H NMR.

AZTMP morpholidate,[10] *rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol 3-phosphate,[20] and *rac*-1-*O*-tetradecyl-2-*O*-palmitoylglycerol 3-phosphate[30] were prepared by a literature procedure.

AZT 5′-Monophosphate and 2′,3′-Dideoxycytidine 5′-Monophosphate. Nucleoside 5′-monophosphates were prepared by phosphorylating the nucleosides with $POCl_3$ and triethyl phosphate by literature procedures.[11,42]

3′-Azido-2′,3′-dideoxyuridine 5′-Monophosphate. This compound was prepared by a literature procedure.[43] A mixture of 0.76 g (3 mmol) of 3′-azido-2′,3′-dideoxyuridine, 2-cyanoethyl phosphate prepared from 1.94 g (6 mmol) of 2-cyanoethyl phosphate barium salt dihydrate, and 4.95 g (24 mmol) of DCC in 150 mL of anhydrous pyridine was stirred at room temperature for 5 days. Water (9 mL) was added to the mixture and stirred at room temperature for 2 h in ordre to destroy the excess DCC. After the solid was filtered, the filtrate was evaporated to dryness and the residue was stirred with 1 N NaOH (40 mL) at room temperature for 3 h. This was then neutralized with HCOOH and applied to an AG1-X8 (formate) column (3.5 × 10 cm). The column was eluted with H_2O (200 mL) and then 0.3 M triethylammonium formate (400 mL). The eluate was then passed through a Dowex 50W-X8 (H^+) column (2.5 × 30 cm), and the column was washed with H_2O until no product was detected. The eluate was evaporated to dryness, and the residue was treated with acetone. The yield was 0.565 g (56.5%). The product was homogeneous by TLC and used for the next step without analysis.

Nucleoside 5′-Monophosphoromorpholidate 4-Morpholine-*N,N′*-dicyclohexylcarboxamidinium Salt (AZddUMP- or ddCMP-morpholidate). These compounds were prepared by condensation of nucleoside 5′-monophosphate with morpholine in the presence of DCC using the published procedure.[10]

3′-Azido-3′-deoxythymidine 5′-Diphosphate−*rac*-1-*S*-Octadecyl-*O*-palmitoyl-1-thioglycerol (1). An anhydrous mixture of 1.42 g (2.0 mmol) of AZTMP morpholidate and 1.70 g (2.5 mmol) of *rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol 3-phosphate in 100 mL of anhydrous pyridine was stirred at room temperature for 7 days and then evaporated to dryness. The residue was coevaporated with toluene to remove the residual pyridine and dissolved in 200 mL of $CHCl_3$−95% ethanol−H_2O (2:4:1), and 40 mL of H_2O was added. The aqueous layer contained the unreacted morpholidate, which was recovered. The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL). The combined organic phase was extracted with 0.5 N HCl (30 mL), and the aqueous layer was extracted with $CHCl_3$ (2 × 50 mL). The organic phase was evaporated to dryness, and the residue was dissolved in 200 mL of $CHCl_3$−95% ethanol−H_2O (2:4:1). The solution was then applied to a DE-52 (acetate) column (4.5 × 20 cm). The column was eluted first with 1000 mL of the same solvent and then 7000 mL of 0.02 M ammonium acetate in the same solvent. Fractions of 950−1550 mL of 0.02 M ammonium acetate were pooled and kept at 0−3 °C overnight. The white solid (the phosphatidic acid) was removed by filtration, and the filtrate was quickly evaporated to a small volume. The white solid was filtered and washed with 50% aqueous acetone and then with acetone. The solid (NH_4 salt of the conjugate) was dissolved in $CHCl_3$−95% ethanol−H_2O (2:4:1), and the solution was passed through an Amberlite CG-50 (Na^+) column (2.5 × 10 cm). The column was eluted with the same solvent until no UV-absorbing compound was detected. The combined eluate was cooled at 0−3 °C overnight, and the white solid (more phosphatidic acid) was removed by filtration. The filtrate was evaporated to a small volume, and the product was filtered, washed with acetone, and dried *in vacuo* over P_2O_5. The conjugate as Na salt weighed 566 mg (27% yield): mp 199−201 °C dec; ^1H NMR ($CDCl_3$−CD_3OD, 1:1) δ 0.94 (6, dt, J = 7 Hz, terminal 2 CH_3), 1.34−1.43 (5, m, (CH_2)$_{15}$, (CH_2)$_{12}$), 1.57−1.73 (4, m, SCH$_2$CH_2, COCH$_2$CH_2), 2.01 (3, s, thymine CH_3), 2.41 (2, m, *H*-2′), 2.64 (2, m, CH$_2$CH_2CO), 2.77 (2, m, SCH_2CH$_2$), 2.91 (2, dd, J = 5 Hz, 1-CH$_2$), 4.07−4.14 (3, m, *H*-4′, 3-CH_2), 4.24 (2, m, *H*-5′), 4.65 (1, t, J = 3.3 Hz, *H*-3′), 5.21 (1, quintet, J = 6 Hz, 2-C*H*), 6.21 (1, t, J = 6 Hz, *H*-1′), 7.71 (1, s, thymine *H*-6). Conjugates **2**−**4** in Table 1 were prepared in an analogous manner.

Water Solubility. The conjugate (5 mg) in 5 mL of sterile water for injection, USP was shaken at room temperature using a New Brunswick Scientific Gyrotary Water Bath Shaker Model G76 at speed 6 for 2 h, and the suspension was filtered through a membrane filter (0.22 μm). Concentration of the free conjugate in the filtrate was checked by quantitative UV.

Partition Coefficient Measurements. 1-Octanol/aqueous phase partition coefficients (*P*) were determined at room temperature using the shake-flask procedure described previously.[31] The UV absorbance of both phases were measured at UV_{max} of each conjugate. The partition coefficients (*P*) were calculated from the ratio of the absorbance between the 1-octanol and aqueous phases.

Determination of Particle Sizes. Mean particle sizes of the conjugates in water suspension by sonication were determined using photon correlation spectroscopy[32] on a NICOMP 370 submicron particle sizer (NICOMP Particle Sizing Systems, Sanata Barbara, CA) with a very-high-power argon laser (500 nm, Coherent Inova 70).[33] Since the particles of the water suspensions were micelles, the measurements were performed by using the solid particles setting of the instrument (Table 2).

***In Vitro* Stability in Biological Tissues.** Brain or liver tissue of female NIH-Swiss mice (Taconic Farms, NY) was weighed and homogenized in a 1:1 (g:mL) ratio with ice cold isotonic phosphate buffer, pH 7.4. The initial solution of the conjugates (1.0 mg/mL in $CHCl_3$−methanol, 1:9) was spiked to serum or brain or liver homogenate to reach a concentration of 10 μg/mL. The mixture was incubated at 37 °C, and a 100-μL aliquot of the sample was withdrawn into a tube containing 10 μL of internal standard solution (20 μg/mL, AZddU for AZT analysis and d4T for AZddU) at the designated time (up to 10 h). Each tube was mixed with 2 M perchloric acid (50 μL) to precipitate protein (300 μL of 0.2% acetic acid in acetonitrile in case of AZddU analysis), vortexed for about 20 s, and then centrifuged at 3000*g* for 5 min. The supernatant (50−100 μL) was injected onto the HPLC system. In case of AZddU analysis the supernatant was evaporated under a nitrogen stream, and the residual film was dissolved in mobile phase for the sample injection.

Animal Studies. Female NIH-Swiss mice weighing 25−30 g were acclimatized to a 12-h light/12-h dark, constant temperature (20 °C) environment with free access to food and water for 1 week before the experiments. Micellar formulation of AZT conjugate **1** (394 mg/kg equivalent to 100 mg/kg AZT) or AZddU conjugate **2** (205 mg/kg equivalent to 50 mg/kg AZddU) was administered to mice ip and iv, respectively. Three mice were sacrificed at the designated time (up to 24 h), and AZT or AZddU in the serum and brain homogenate were analyzed by an HPLC method.

Analysis of the Samples. The solid tissues were homogenized in a 1:1 (g:mL) ratio with ice cold isotonic phosphate buffer, pH 7.4. Aliquots of serum or brain or liver homogenate were mixed with 10 μL of the internal standard solution and proteins were precipitated as described above.

The sample analyses were carried out on a Varian HPLC system (Sugarland, TX), which was equipped with a Model 2510 pump, a Model 9090 autosampler, a Model 2550 UV detector set at 260 nm, and a Model 4290 reporting integrator. Chromatography was performed on Alltech Hypersil ODS (5 μm particle size, 4.6 × 150 mm, Alltech Associates, Deerfield, IL). The mobile phase for AZT analysis consisted of 10.5% acetonitrile in acetate buffer (pH 7.0) with a flow rate of 2 mL/min. The mobile phase for AZddU analysis consisted of 5% acetonitrile in 10 mM K_2HPO_4 (pH 6.0) with a flow rate of 2 mL/min.

Standard curves were prepared for each type of sample by known amounts of AZT or AZddU to the serum or the tissues. The standard curves were linear over the concentration range 0.05−60 μg/mL in serum (μg/g in tissues). The sensitivity limit for both nucleosides was 0.1 μg/mL in both biological media.

Reproducibility of the analytical procedures was indicated by intra- and interday coefficients of variation of less than 10% for both nucleosides.[34]

Data Analysis. Mean serum and brain concentrations of the conjugate and the nucleoside versus time data were analyzed by noncompartmental technique. The area under the nucleoside serum or brain concentration versus time curve (AUC) and the first non-normalized moment (AUMC) were determined by Lagrange polynominal interpolation and integration from time zero to the last sampling time with extrapolation to time infinity using the least-squares terminal slope (λ_z).[44] Half-life was calculated from $0.693/\lambda_z$. The fraction of the conjugate converted to the parent nucleoside (f_m) was calculated from $(AUC_{p\leftarrow pd})(CL_T)/dose_{pd}$, where $AUC_{p\leftarrow pd}$ is the AUC of the parent nucleoside after administration of the conjugate ($dose_{pd}$) and CL_T is clearance of the parent nucleoside.[45]

Anti-HIV Drug Testing. Anti-HIV evaluation for the conjugates was performed at the National Cancer Institute, the National Institute of Health, by the procedures reported previously.[35]

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References

(1) This material was presented in part in April 1992 at the 203rd National Meeting of the American Chemical Society in San Francisco, CA (Abstract MEDI 18).

(2) Abbreviations: HIV-1, human immunodeficiency virus type 1; AZT, 3′-azido-3′-deoxythymidine; ddI, 2′,3′-dideoxyinosine; ddC, 2′,3′-dideoxycytidine; d4T, 2′,3′-didehydro-3′-deoxythymidine; AZddU, 3′-azido-2′,3′-dideoxyuridine; ara-C, 1-β-D-arabinofuranosylcytosine; ip, intraperitoneally; iv, intravenously; ara-CTP, ara-C 5′-triphosphate; AZTMP, AZT 5′-monophosphate; DCC, 1,3-dicyclohexylcarbodiimide; AZddUMP, AZddU 5′-monophosphate; ddCMP, ddC 5′-monophosphate.

(3) Mitsuya, H.; Weinhold, K. J.; Furman, P. A.; St. Clair, M. H.; Lehrman, S. N.; Gallo, R. C.; Bolognesi, D.; Barry, D. W.; Broder, S. 3′-Azido-3′-deoxythymidine (BWA509U): an antiviral agent that inhibits the infectivity and cytopathic effect on human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7096−7100.

(4) Mitsuya, H.; Broder, S. In hibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-assciated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911−1915.

(5) Balzarini, J.; Kang, G.-J.; Dalal, M.; Herdewijin, P.; De Clercq, E.; Broder, S.; Johns, D. G. The anti-HTLV-III (anti-HIV) and cytotoxic activity of 2′,3′-didehydro-2′,3′-dideoxyribonucleosides: a comparison with their parental 2′,3′-dideoxyribonucleosides. *Mol. Pharmacol.* **1987**, *32*, 162−167.

(6) Lin, T.-S.; Guo, J.-Y.; Schinazi, R. F.; Chu, C. K.; Xiang, J.-N.; Prusoff, W. H. Synthesis and antiviral activity of various 3′-azido analogues of pyrimidine deoxyribonucleosides against human immunodeficiency virus (HIV-1, HTLV-III/LAV). *J. Med. Chem.* **1988**, *31*, 336−340.

(7) Furman, P. A.; Fyfe, J. A.; St. Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; Lehrman, S. N.; Bolognesi, D. P.; Broder, S.; Mitsuya, H.; Barry, D. W. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *83*, 8333−8377.

(8) Kucera, L. S.; Iyer, N.; Leake, E.; Raben, A.; Modest, E. J.; Daniel, L. W.; Piantadosi, C. Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation. *AIDS Res. Human Retroviruses* **1990**, *6*, 491−501.

(9) Steim, J. M.; Camaioni, N.; Sarin, C.; Sun, P. S.; Sehgal, R. K.; Turcotte, J. G. Lipid conjugates of antiretroviral agents. I. Azidothymidine-monophosphate-diglyceride: anti-HIV activity, physical properties and interaction with plasma protein. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 451−457.

(10) Hostetler, K. Y.; Stuhmiller, L. M.; Lenting, H. B.; van den Bosch, H.; Richman, D. D. Synthesis and antiretroviral activity of phospholipid analogs of azidothymidine and other antiviral nucleosides. *J. Biol. Chem.* **1990**, *265*, 6112−6117.

(11) van Wijk, G. M. T.; Hostetler, K. Y.; van den Bosch, H. Antiviral nucleoside diphosphate diglycerides: improved synthesis and facilitated purification. *J. Lipid Res.* **1992**, *33*, 1211−1219.

(12) van Wijk, G. M. T.; Hostetler, K. Y.; Suurmeijer, C. N. S. P.; van den Bosch, H. Synthesis, characterization and some properties of dideoxynucleoside analogs of cytidine diphosphate diacylglycerol. *Biochim. Biophys. Acta* **1992**, *1165*, 45−52.

(13) Hostetler, K. Y.; Richman, D. D.; Carson, D. A.; Stuhmiller, L. M.; van Wijk, G. M. T.; van den Bosch, H. Greatly enhanced inhibition of human immunodeficiency virus type 1 replication in CEM and HT4-6C cells by 3′-deoxythymidine diphosphate dimyristoylglycerol, a lipid prodrug of 3′-deoxythymidine. *Antimicrob. Agents Chemother.* **1992**, *36*, 2025−2029.

(14) Piantadosi, C.; Marasco, C. J.; Morris-Natschke, S. L.; Meyer, K. L.; Gumus, F.; Surles, J. R.; Ishaq, K. S. Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity. *J. Med. Chem.* **1991**, *34*, 1408−1414.

(15) Fox, C. H.; Tenner-Racz, K.; Racz, P.; Firpo, A.; Pizzo, P. A.; Fauci, A. S. Lymphoid germinal centers are reservoirs of human immunodeficiency virus type 1 RNA. *J. Infect. Dis.* **1991**, *164*, 1051−1057.

(16) Pantaleo, G.; Graziosi, C.; Demarest, J. F.; Butini, L.; Montroni, M.; Fox, C. H.; Orenstein, J. M.; Kotler, D. P.; Fauci, A. S. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **1993**, *362*, 355−358.

(17) Embretson, J.; Zupancic, M.; Ribas, J. L.; Burke, A.; Racz, P.; Tenner-Racz, K.; Haase, A. T. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **1993**, *362*, 359−362.

(18) Modest, E. J.; Berens, M. E.; Noseda, A.; Piantadosi, C. In *The Pharmacological Effects of Lipids III. The Role of Lipids in Carcinogenesis and Therapy*; Kabara, J. J., Ed.; American Oil Chemists' Society: Champaign, IL, 1989; pp 330−337.

(19) Hong, C. I.; An, S.-H.; Buchheit, D. J.; Nechaev, A.; Kirisits, A. J.; West, C. R.; Berdel, W. E. Nucleoside conjugates. 7. Synthesis and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugates of ether lipids. *J. Med. Chem.* **1986**, *29*, 2038−2044.

(20) Hong, C. I.; Kirisits, A. J.; Nechaev, A.; Buchheit, D. J.; West, C. R. Nucleoside conjugates. 11. Synthesis and antitumor activity of 1-β-D-arabinofuranosylcytosine and cytidine conjugates of thioether lipids. *J. Med. Chem.* **1990**, *33*, 1380−1386.

(21) Hong, C. I.; Bernacki, R. J.; Hui, S.-W.; Rustum, Y.; West, C. R. Formulation, stability, and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugate of thioether phospholipid. *Cancer Res.* **1990**, *50*, 4401−4406.

(22) Hong, C. I. Ara-C conjugates of ether and thioether phospholipids. *Drugs Future* **1990**, *15*, 245−253.

(23) Hong, C. I.; West, C. R.; Bernacki, R. J.; Tebbi, C. K.; Berdel, W. E. 1-β-D-Arabinofuranosylcytosine conjugates of ether and thioether phospholipids. A new class of ara-C prodrug with improved antitumor activity. *Lipids* **1991**, *26*, 1437−1444.

(24) Berdel, W. E.; Okamoto, S.; Danhauser-Riedl, S.; Hong, C. I.; Winton, E. F.; West, C. R.; Rastetter, J.; Vogler, W. E. Therapeutic activity of 1-β-D-arabinofuranosylcytosine conjugates of lipids in WEHI-3B leukemia in mice. *Exp. Hematol.* **1989**, *17*, 364−367.

(25) Berdel, W. E.; Danhauser, S.; Schick, H. D.; Hong, C. I.; West, C. R.; Fromm, M.; Fink, U.; Reichert, A.; Rastetter, J. Antineoplastic activity of conjugates of lipids and 1-β-D-arabinofuranosylcytosine. *Lipids* **1987**, *22*, 943−946.

(26) Berdel, W. E.; Danhauser, S.; Hong, C. I.; Schick, H. D.; Reichert, A.; Busch, R.; Rastetter, J.; Vogler, W. R. Influence of 1-β-D-arabinofuranosylcytosine conjugates of lipids on the growth and metastasis of Lewis lung carcinoma. *Cancer Res.* **1988**, *48*, 826−829.

(27) Herrmann, R.; Berdel, W. E. Therapeutic activity of a thioether-lipid conjugate of 1-β-D-arabinofuranosylcytosine in human colorectal xenografts. *Cancer Res.* **1992**, *52*, 1865−1867.

(28) Hong, C. I.; Nechaev, A.; Kirisits, A. J.; Vig, R.; West, C. R. Nucleoside conjugates. 13. Synthesis and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugates of thioether lipids with improved water solubility. *J. Med. Chem.* **1993**, *36*, 1785−1790.

(29) Hauser, G.; Eichberg, J. Identification of cytidine diphosphate-diglyceride in the pineal gland of the rate and its accumulation in the presence of DL-propranolol. *J. Biol. Chem.* **1975**, *250*, 105−112.

(30) Hong, C. I.; Nechaev, A.; Kirisits, A. J.; Vig, R.; Hui, S.-W.; West, C. R. Nucleoside conjugates. 14. Synthesis and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugates of ether lipids with improved water solubility. *J. Med. Chem.* **1995**, *38*, 1629−1634.

(31) Kerr, S. G.; Kalman, T. I. Highly water-soluble lipophilic prodrugs of the anti-HIV nucleoside analogue 2′,3′-dideoxycytidine and its 3′-fluoro derivative. *J. Med. Chem.* **1992**, *35*, 1996−2001.

(32) *Dynamic Light Scattering, Applications of Photon Correlation Spectroscopy*; Pecora, R., Ed.; Plenum Press: New York, 1985.

(33) Nicoli, D. F.; McKenzie, D. C.; Wu, J.-S. Application of dynamic light scattering to particle size analysis of macromolecules. *Am. Lab.* **1991**, *23*, 32−40.
(34) Doshi, K. L.; Gallo, J. M.; Boudinot, F. D.; Schinazi, R. F.; Chu, C. K. Comparative pharmacokinetics of 3′-azido-3′-deoxythymidine (AZT) and 3′-azido-2′,3′-dideoxyuridine (AZddU) in mice. *Drug Metab. Dispos.* **1989**, *17*, 590−594.
(35) Weislow, O. S.; Kiser, R.; Fine, D. L.; Bader, J.; Shoemaker, R. H.; Boyd, M. R. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J. Natl. Cancer Inst.* **1989**, *81*, 577−586.
(36) Hong, C. I. Unpublished results.
(37) Hostetler, K. Y.; Richman, D. D.; Sridhar, C. N.; Felgner, P. L.; Felgner, J.; Ricci, J.; Gardner, M. F.; Selleseth, D. W.; Ellis, M. N. Phosphatidylazidothymidine and phosphatidyl-ddC: assessment of uptake in mouse lymphoid tissues and antiviral activities in human immunodefieciency virus-infected cells and Rauscher leukemia virus-infected mice. *Antimicrob. Agents Chemother.* **1994**, *38*, 2792−2797.
(38) Monouilov, K. K.; Fedorov, I. I.; Boudinot, F. D.; White, C. A.; Kotra, L. P.; Schinazi, R. F.; Hong, C. I.; Chu, C. K. Lymphatic targeting of anti-HIV nucleosides: distribution of 3′-azido-3′-deoxythymidine (AZT) and 3′-azido-2′,3′-dideoxyuridine (AZdU) after administration of dipalmitoylphosphatidyl prodrugs to mice. *Antiviral Chem. Chemother.* **1995**, *6*, 230−238.
(39) Johnson, K. A.; Westermann-Clark, G. B.; Shah, D. O. Transport of micelle-solubilized steroids across microporous membranes. *J. Pharm. Sci.* **1987**, *76*, 277−285.
(40) Florence, A. T. Drug solubilization in surfectant system. *In Techniques of Solubilization of Drugs*; Yalkowsky, S. H., Ed.; Marcel Dekker: New York, 1981; pp 15−89.
(41) Ryu, E. K.; MacCoss, M. Modification of the Dittmer-Lester reagent for detection of phospholipid derivatives on thin-layer chromatograms. *J. Lipid Res.* **1979**, *20*, 561−563.
(42) Yoshikawa, M.; Kato, T.; Takenishi, T. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* **1967**, 5065−6068.
(43) Tener, G. M. 2-Cyanoethyl phosphate and its use in the synthesis of phosphate esters. *J. Am. Chem. Soc.* **1961**, *83*, 159−168.
(44) Rocci, M. L., Jr.; Jusko, W. J. LAGRAN program for area and moments in pharmacokinetic analysis. *Comp. Prog. Biomed.* **1983**, *16*, 203−216.
(45) Gibaldi, M.; Perrier, D. Clearance concepts. *Pharmacokinetics*, 2nd ed.; Marcel Dekker Inc.: New York, 1982; pp 319−353.

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