Synthesis and Evaluation of Novel Thymidine Analogs as Antitumor and Antiviral Agents

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Two series of thymidine analogs with a hydroxyalkylammonium(amine) moiety have been synthesized and evaluated for antitumor and antiviral activities. The hydroxyalkylammonium-(amine) group was introduced at the 5′ position of the 2′-deoxyribose residue of thymidine or at a corresponding position in acyclic thymidine analogs. In order to increase the lipophilicity of these compounds and potentially enable them to cross the cell membrane, the free hydroxy group also was esterified with a long hydrocarbon chain. The hexadecanoyl analogs (compounds **1c**, **1d**, **7c**, and **7d**) showed moderate antitumor cytotoxicity against SV-28 and KB cell lines ($IC_{50} \sim 20$ μM). Compound **1d** showed moderate anti-HIV activity ($EC_{50} = 6.8$ μM), while compound **5** showed weak anti-HIV activity ($EC_{50} = 55$ μM). None of the compounds showed antiherpes simplex virus activity.

Introduction

Interest in nucleoside research has intensified since the AIDS epidemic, and many nucleoside derivatives have been synthesized. Therefore, new nucleoside analogs should be distinctly dissimilar in structure to the current families of analogs. Accordingly, we selected the β-hydroxyalkyltrimethylammonium moiety as a new template for a novel series of nucleoside derivatives. Previously, incorporation of this functionality into alkyl ether lipids has resulted in potent inhibitors of *in vitro* neoplastic cell growth,[1,2] protein kinase C activity,[3] and HIV-1 infectivity,[4] although the mechanism(s) of action is (are) not clearly understood. The effect of this moiety on nucleosides, however, has not been investigated. Therefore, its incorporation into thymidine and other nucleosides is of interest. Since thymine is a nucleic acid base unique to DNA, it often serves as a model in the design of novel nucleoside analogs targeting DNA. Previous research on thymidine analogs has produced several successful antiviral drugs, such as 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T), which, after phosphorylation, can inhibit HIV-1 reverse transcriptase. Both compounds are used currently in the treatment of AIDS. Further, other nucleic acid bases also have been used to combat AIDS, for example, inosine (in dideoxyinosine, ddI) and cytosine (in dideoxycytidine, ddC, and 2′-deoxy-3′-thiacytidine, 3TC).

We have designed two types of compounds containing the hydroxyalkylammonium moiety. In type I, it is introduced at the 5′-position of the 2′-deoxyribose residue of thymidine, and in type II, it is incorporated into an acyclic chain, which is similar to that of the antiherpes simplex virus drug acyclovir. The hydroxyalkylammonium moiety in either series of analogs will contain both a positive charge and a free hydroxy group. The positive charge may result in a favorable interaction with a potential target and provide a better inhibitor, while the free hydroxy group may offer a site for activation to the monophosphate form. This latter reaction is an obligatory step for further phosphorylation to the mono-, di-, and tri-phosphate forms.

The nucleoside sugar ring has been modified extensively.[5] One such modification is exemplified by acyclovir, an open-chain analog of guanosine, which is highly effective in the treatment of herpes simplex virus infection. Although acyclovir mimics the conformation of guanosine quite well, it is recognized preferentially by the viral thymidine kinase.[6] Since the introduction of acyclovir as an antiviral drug, many acyclic nucleoside analogs have been synthesized.[7,8] Therefore, the open-chain thymidine analogs (type II) were based on this model. A conformational search with Sybyl has shown that the flexible side chain of compound **1a** can adopt a conformation in which the distance between the terminal hydroxy group and the thymine base is similar to that in thymidine.

The chain length between the free hydroxyl and the ammonium nitrogen may affect the biological activity; thus, compounds with both two (**1a**, **1b**, **1c**) and three (**1d**, **2**, **3**) carbon chains were made. Compound **3**, which has two free hydroxyls, was synthesized to increase the possibility of phosphorylation. Hydroxyalkylamine analogs (**4** and **5**) also were synthesized to explore the effects of the quaternary ammonium moiety versus a tertiary amine group. At physiological pH, the tertiary amine will be protonated and bear a positive charge that is electrostatically similar to the ammonium analogs. At the active site of the enzyme, however, the protonated tertiary amine may be neutral since there may be no water molecules around it. Therefore, analogs **4** and **5** may indicate whether or not the positive charge

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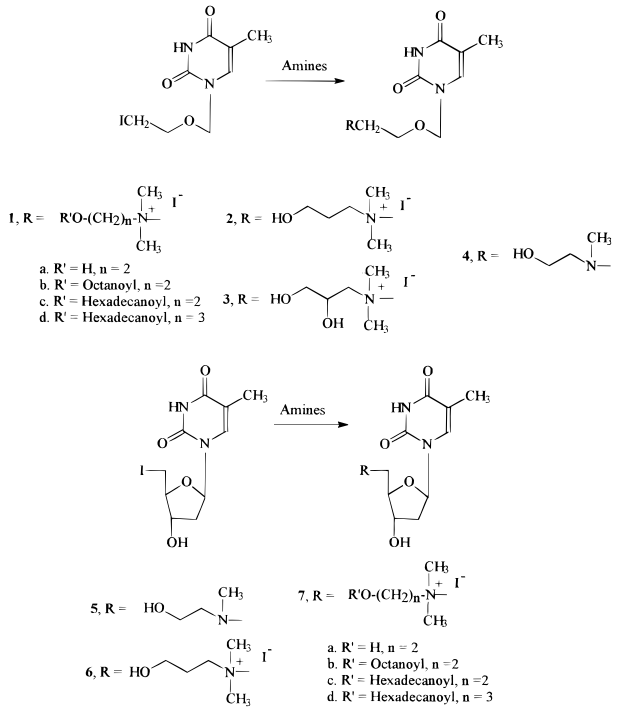
Scheme 1



at the quaternary ammonium nitrogen is important for biological activity. These compounds are polar and may not be able to cross the cell membrane unless they can be transported by an active nucleoside transport mechanism. Therefore, the free hydroxy group was esterified by two different hydrocarbon chains (octanoyl and hexadecanoyl) to produce prodrugs (**1c**, **1d**; **7c**, **7d**) with increased ability to enter the cell through passive diffusion. The choice of either the octanoyl or the hexadecanoyl derivatives to serve as model prodrugs was based on a lipophilicity consideration as well as rate of ester cleavage.[9]

Chemistry

General Method for the Synthesis of Acyclic Thymidine Analogs and Their Prodrugs (Scheme 1). The synthesis of *N*,*N*-dimethyl-*N*-(2-hydroxyethyl)-*N*-[N^1-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]-ammonium iodide (**1a**) exemplifies the preparation of compounds **1a**, **2**, **3**, and **4**. 1-[(2-Iodoethoxy)methyl]-thymine, prepared by literature procedures,[10,11] was treated with 2-(dimethylamino)ethanol to give the desired product **1a**. Compounds **2**, **3**, and **4** were obtained in a similar manner from the corresponding amines 3-(dimethylamino)-1-propanol, 3-(dimethylamino)-1,2-propanediol, and 2-(methylamino)ethanol in acetonitrile.

We also were interested in synthesizing long-chain esters of compound **1a**, since the design of a prodrug of this thymidine analog would lead to increased lipophilicity and, thus, might facilitate passive diffusion into the cell. Direct treatment of **1a** with octanoyl chloride or hexadecanoyl chloride failed to give the desired product. [Similarly, other laboratories[12] could not directly phosphorylate (2,3-distearoyloxypropyl)dimethyl-(β-hydroxyethyl)ammonium bromide. Our laboratory also has been unsuccessful in phosphorylating analogs of this compound.] Therefore, the ester prodrugs were prepared by direct reaction of an iodo nucleoside intermediate with dimethylaminoalkyl esters. The latter were prepared by first treating octanoyl chloride or hexadecanoyl chloride with 2-bromoethanol or 3-bromo-1-propanol in benzene in the presence of pyridine.[2] Reaction of the corresponding products, 2-bromoethyl octanoate, 2-bromoethyl hexadecanoate, and 3-bromopropyl hexadecanoate, with dimethylamine in acetonitrile gave 2-(dimethylamino)ethyl octanoate, 2-(dimethylamino)ethyl hexadecanoate, and 3-(dimethylamino)propyl hexadecanoate. These intermediates then were treated with 1-[(2-iodoethoxy)methyl]thymine to give the corresponding products **1b**, **1c**, and **1d**.

General Method for the Synthesis of Thymidine Analogs and Their Prodrugs (Scheme 1). The synthesis of *N*,*N*-dimethyl-*N*-(2-hydroxyethyl)-*N*-[5′-(5′-deoxythymidinyl)]ammonium iodide (**7a**) illustrates this series. 5′-Iodothymidine, prepared from thymidine and methyltriphenoxyphosphonium iodide (MTPI),[11] was reacted with the tertiary amine 2-(dimethylamino)-ethanol in acetonitrile to give the corresponding product **7a**. Compounds **5** and **6** were synthesized in the same manner using 2-(methylamino)ethanol and 3-(dimethylamino)-1-propanol. The prodrugs of **7a** were prepared as outlined for **1b**, **1c**, and **1d**. Thus, treating 5′-iodothymidine with 2-(dimethylamino)ethyl octanoate, 2-(dimethylamino)ethyl hexadecanoate, or 3-(dimethylamino)propyl hexadecanoate afforded **7b**, **7c**, and **7d**.

Biological Results and Discussion

The antitumor activity of this series of compounds was evaluated by tissue culture assay against the SV-28 cell line, an SV40-transformed line of baby hamster kidney cells. Compounds **1c**, **1d**, **7c**, and **7d** were equipotent with IC_{50} values of 18 ± 3, 21, 23 ± 3, and 25 μM, respectively. All other compounds were inactive (no inhibition at >50 μM). Compounds **1c** and **7c** were further evaluated against the human nasopharyngeal KB cell line; the IC_{50} values were 20 ± 2 and 25 ± 5 μM, respectively.

Since all four active compounds have a hexadecanoyl ester moiety, increased lipophilicity may offer one of two possible explanations for the pharmacological activity. Compounds **1a**, **2**, and **3** were synthesized first, and their lack of antitumor activity led to the synthesis of the ester prodrugs based on the following rationale. Although a nucleoside requires a transport carrier to cross the cell membrane, compounds **1a**, **2**, and **3** may not be actively transported by this carrier, and their hydrophilic character also may impede diffusion across the cell membrane. However, the hexadecanoyl analogs (**1c**, **1d**, **7c**, **7d**) may be lipophilic enough to diffuse through the membrane. (See also the dipyridamole assay below.) Once the compounds are in the cells, the hydrocarbon ester chain is expected to be cleaved enzymatically, and the nucleoside analog released. Lengthening the alkyl chain between the sterically hindered quaternary ammonium nitrogen and the ester group might be expected to affect the rate of enzymatic hydrolysis and increase levels of the nucleoside analog inside the cell.[14] However, since the IC_{50} values of **1d** and **7d**, which have a three-carbon bridge, equal those of **1c** and **7c**, which have a two-carbon bridge, the cytotoxic effect of these hexadecanoyl esters may be independent of their rate of cleavage. A second explanation for the activity of these compounds is that **1c**, **1d**, **7c**, and **7d** may interact with the cellular membrane

without metabolic activation. Compounds with similar structures have been shown to exert cytotoxic and antiviral effects through a membrane-interactive mechanism.[16−18] The inactivity of the octanoyl analogs (**1b**, **7b**) may be due to their inability to either cross the membrane or exert a biological effect through membrane interaction.

The tertiary amine analogs (**4** and **5**), which were prepared to ascertain the role of the quaternary ammonium head, were inactive as antitumor agents. Although sterically less hindered,[9] their inactivity might be due to the protonation of the tertiary amine group at physiological pH; this charge would increase polarity and hinder these compounds from crossing the cell membrane if not transported by a nucleoside carrier protein.

To ascertain whether these thymidine analogs are actively transported across the cellular membrane, **1c** and **7c** were tested for cytotoxicity in the presence of 1 μM dipyridamole, an antithrombotic drug that inhibits nucleoside transport. If compounds **1c** and **7c** are transported by the active nucleoside transport mechanism, the presence of dipyridamole should decrease the amount of compound entering the cells and, therefore, increase the IC_{50} values. In this assay, the IC_{50} values with dipyridamole (**1c**: 22 μM, **7c**: 20 μM) were unchanged from those (**1c**: 18 $\pm$ 3 μM, **7c**: 23 $\pm$ 3 μM) without dipyridamole, which is evidence that these compounds do not enter the cell by a nucleoside transport mechanism.

In order to investigate the mechanism of action of these thymidine hexadecanoyl ester prodrugs, **1c** and **7c** were tested in the SV-28 cell assay with excess thymidine (10 μM) in the medium. Usually, nucleoside analogs exert their activity by competing with the natural substrate; thus, the presence of a large amount of the natural substrate should affect the activity of the nucleoside analog. However, 10 μM thymidine in the cell medium did not change the IC_{50} values of **1c** (24 μM, with, vs 18 μM, without) and **7c** (21 μM, with, vs 23 μM, without). This suggests that these compounds may not act as nucleoside analogs of thymidine to exert their cytotoxic effects, or they may compete with a nucleoside other than thymidine.

Compounds **1c** and **7c** also were tested for their effects on cell plating efficiency. The SV-28 cell has a plating efficiency between 50 and 60%. Drugs that target DNA or RNA usually decrease plating efficiency. At 25 μM, **1c** (47.4%) and **7c** (58.5%) did not lower cell plating efficiency after a 24 h exposure.

All compounds also were tested against the herpes simplex virus 1 (HSV-1, KOS strain) by using a plaque reduction assay. Compounds first were tested for cell toxicity against the HSV-1 virus host, Vero, cell line to determine the appropriate concentrations to be used in the antiviral assay. Consistent with the antitumor cytotoxicity assay, only **1c**, **1d**, **7c**, and **7d** inhibited the growth of Vero cells with IC_{50} values in the range of 35−40 μM after 2 days of continuous treatment. At 200 μM, **1c**, **1d**, **7c**, and **7d** were cytotoxic within a 30 min exposure based on the change in cell morphology (shrinkage and rounding) visible upon microscopic examination. Although these compounds inhibited the growth of proliferating cells, they did not exert visible cytotoxic effects on preformed cell monolayers after an incubation period of 48 h. All the compounds in this series were then tested for anti-HSV-1 activity; none showed activity at concentrations up to 100 μM.

The same compounds were tested for anti-HIV-1 activity and for cytotoxicity against the HIV-1 host cell (CEM-SS cell). Compounds **1c**, **1d**, **7c**, and **7d** showed some toxicity against CEM-SS cell growth with IC_{50} values of 67.3 $\pm$ 10.0, 62.4 $\pm$ 0.4, 53.0 $\pm$ 24.2, and 22.3 $\pm$ 1.9 μM, respectively. All other compounds showed no inhibition at 100 μM. Compound **1d** showed moderate anti-HIV-1 activity at a concentration (EC_{50} = 6.8 $\pm$ 1.6 μM) 10 times less than its cytotoxic IC_{50} value, while compounds **5**, **6**, and **7a** showed weak anti-HIV-1 activity by inhibiting viral multiplication by 50, 31, and 44% at 55, 100, and 100 μM, respectively. The latter three compounds have an intact sugar moiety; their polarity and probable lack of active transport might be responsible for their weak activity.

Summary

Novel thymidine and acyclic nucleoside analogs containing hydroxyalkylammonium moieties were designed, synthesized, and evaluated for *in vitro* cytotoxicity and antiviral activity. Prodrugs of both types containing hexadecanoyl ester groups were cytotoxic in SV-28, KB, Vero, and CEM-SS cells, while the parent hydroxy compounds and the octanoyl esters were not. The increased lipophilicity of the long alkyl chain may enhance the membrane permeability of these compounds. None of the compounds were active against HSV-1, and most compounds were either inactive or weakly to moderately active against HIV-1. Further structural modification and biological evaluation of this novel class of nucleoside analogs are in progress.

Experimental Section

All melting points were taken on a Thomas Hoover melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Perkin-Elmer 1320 spectrophotometer. ^{1}H NMR spectra were obtained using a Bruker AC-300 NMR. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. Due to the hygroscopic nature of the quaternary ammonium compounds, some of the structures were confirmed by high-resolution mass spectroscopy by using a VG70-SQ spectrometer. Thin-layer chromatography (TLC) was performed on 1 × 3 in. fluorescent precoated Whatman Silica Gel 60 Å TLC plates. Silica gel (70−230 mesh) from Fisher Scientific was used for column chromatography. Reagents were purchased from Aldrich. Solvents, including acetonitrile, *N*,*N*-dimethylformamide (DMF), and methylene chloride, were dried by placement over molecular sieves (4 Å) for 2 weeks before use.

2-(Dimethylamino)ethyl Octanoate. 2-Bromoethyl octanoate (0.2 g, 0.72 mmol), prepared by reaction of 2-bromoethanol and octanoyl chloride in 10:1 benzene/pyridine,[2] and 40% aqueous dimethylamine (4.2 mL, 36 mmol) were dissolved in acetonitrile (10 mL). The reaction mixture was stirred at room temperature for 48 h. After removal of acetonitrile under vacuum, $CHCl_3$ (30 mL) was added. The $CHCl_3$ layer was extracted with K_2CO_3 solution (20 mL, 0.01 N) and water (2 × 20 mL), and then was dried over anhydrous Na_2SO_4. The drying agent was suction-filtered and the $CHCl_3$ removed in vacuo. The resulting material was applied to a silica gel column (discontinuous gradient of $CHCl_3$/CH_3OH 95:5, 8:2 as eluent), affording 0.13 g of pure product: yield 77%; liquid; ^{1}H NMR ($CDCl_3$) δ 0.85−0.95 (t, CH_3−$(CH_2)_4$), 1.20−1.40 (m, CH_3−$(CH_2)_4$−), 1.55−1.70 (m, CH_2−CH_2−CO), 2.25−2.40 (t, CH_2−CH_2−CO), 2.45−2.55 (s, $(CH_3)_2$−N) 2.75−2.85 (t, O−CH_2−CH_2−N), 4.25−4.35 (t, O−CH_2−CH_2−N).

2-(Dimethylamino)ethyl hexadecanoate and 3-(dimethylamino)propyl hexadecanoate were prepared similarly from dimethylamine and the appropriate haloester.

***N,N*-Dimethyl-*N*-(2-hydroxyethyl)-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (1a).** 1-[(2-Iodoethoxy)methyl]thymine (100 mg, 0.32 mmol) and 2-(dimethylamino)ethanol (34.5 mg, 0.38 mmol) were dissolved in acetonitrile (10 mL). The reaction mixture was heated to a gentle reflux and stirred for 5 h. After cooling to room temperature, 77 mg of pure product was obtained through crystallization by slow addition of Et_2O: yield 59%; mp 158−161 °C; ^{1}H NMR (CD_3CN) δ 1.85−1.88 (s, CH_3−C5 thymine), 3.10−3.15 (s, $(CH_3)_2$−N), 3.30 (s, HO−CH_2), 3.45−3.48 (m, N−CH_2−CH_2−O), 3.55−3.58 (m, HO−CH_2−CH_2−N), 3.90−4.00 (m, HO−CH_2−CH_2−N−CH_2−CH_2−O), 5.10 (s, O−CH_2−N), 7.30 (s, H−C6 thymine). Anal. ($C_{12}H_{22}N_3O_4I$) C, H, N. Compounds **2** and **3** were prepared in an analogous manner with the appropriate amino alcohol.

***N,N*-Dimethyl-*N*-(3-hydroxypropyl)-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (2):** Yield 59%; mp 178−180 °C; ^{1}H NMR (DMSO-d_6) δ 1.75−1.80 (s, CH_3−C5 thymine), 1.75−1.90 (m, HO−CH_2−CH_2−CH_2−N), 3.05−3.15 (s, $(CH_3)_2$−N), 3.40−3.50 (m, HO−CH_2−CH_2−CH_2−N), 3.50−3.60 (m, N−CH_2−CH_2−O), 3.85−3.95 (m, HO−CH_2−CH_2−CH_2−N−CH_2−CH_2−O), 4.75−4.85 (t, HO−CH_2), 5.05−5.10 (s, O−CH_2−N), 7.55−7.60 (s, H−C6 thymine), 11.35−11.40 (s, HN thymine). Anal. ($C_{13}H_{24}N_3O_4I$) C, H, N.

***N,N*-Dimethyl-*N*-(2,3-dihydroxypropyl)-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (3):** Yield 55%; mp 163−165 °C; ^{1}H NMR (DMSO-d_6) δ 1.75−1.80 (s, CH_3−C5 thymine), 3.10−3.15 (s, $(CH_3)_2$−N), 3.60−3.70 (m, CH_2−N−CH_2), 3.80−4.10 (m, HO−CH_2−CH(OH)−CH_2−N−CH_2−CH_2−O), 4.95−5.00 (t, HO−CH_2), 5.05−5.10 (s, O−CH_2−N), 5.20−5.25 (d, HO−CH_2−CH(OH)−CH_2), 7.55−7.60 (s, H−C6 thymine), 11.35−11.40 (s, HN thymine). Anal. ($C_{13}H_{24}N_3O_5I$) C, H, N.

***N*-Methyl-*N*-(2-hydroxyethyl)-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]amine (4).** 1-[(2-Iodoethoxy)-methyl]thymine (200 mg, 0.65 mmol) and 2-(methylamino)-ethanol (242 mg, 3.3 mmol) were dissolved in acetonitrile (8 mL). The reaction mixture was heated to a gentle reflux and stirred for 5 h. After evaporation of acetonitrile, $CHCl_3$ (50 mL) was added, and the solution was washed with NaOH solution (20 mL, 1 N) and water (2 × 20 mL). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4. The drying agent was suction-filtered, and the $CHCl_3$ was removed under vacuum. The resulting residue was purified by column chromatography by using a discontinuous $CHCl_3$/CH_3OH (9:1, 7:3) gradient to give 95 mg of pure product: yield 60%; mp 58−60 °C; ^{1}H NMR (CD_3OD) δ 1.85−1.92 (s, CH_3−C5 thymine), 2.25−2.35 (s, CH_3−N), 2.55−2.60 (t, HO−CH_2−CH_2−N−CH_2), 2.60−2.65 (t, HO−CH_2−CH_2−N−CH_2), 3.55−3.70 (m, HO−CH_2−CH_2−N−CH_2−CH_2−O), 5.10−5.15 (s, O−CH_2−N), 7.45−7.50 (s, H−C6 thymine). Anal. ($C_{11}H_{19}N_3O_4$) C, H, N.

***N,N*-Dimethyl-*N*-2-(octanoyloxy)ethyl-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (1b).** 1-[(2-Iodoethoxy)methyl]thymine (30 mg, 0.097 mmol) and 2-(dimethylamino)ethyl octanoate (100 mg, 3.9 mmol) were dissolved in acetonitrile (10 mL). The reaction mixture was heated to a gentle reflux and stirred for 24 h. After the removal of acetonitrile under vacuum, benzene (5 × 20 mL) was added to the residue, and the solution was decanted to remove the unreacted starting material. Pure product (40 mg) was obtained by column chromatography (discontinuous gradient of $CHCl_3$/CH_3OH 9:1, 7:3): yield 74%; mp 96−98 °C; hygroscopic; ^{1}H NMR ($CDCl_3$) δ 0.80−1.00 (t, CH_3−$(CH_2)_4$), 1.20−1.40 (m, CH_3−$(CH_2)_4$−), 1.50−1.70 (m, CH_2−CH_2−CO), 1.85−1.95 (s, CH_3−C5 thymine), 2.30−2.45 (m, CH_2−CH_2−CO), 3.30−3.60 (s, $(CH_3)_2$−N), 3.90−4.10 (m, CH_2−N−CH_2), 4.15−4.30 (m, CH_2−CH_2−O−CH_2−N), 4.50−4.65 (m, C(O)O−CH_2), 5.20−5.35 (s, O−CH_2−N), 7.35−7.40 (s, H−C6 thymine): HRMS calcd for $C_{20}H_{36}O_5N_3$ 398.2654 (M^+), found 398.2644.

Compounds **1c** and **1d** were prepared in a similar manner by using the appropriate amino ester.

***N,N*-Dimethyl-*N*-2-(hexadecanoyloxy)ethyl-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (1c):** yield 49%; wt 203 mg; mp 144−146 °C; ^{1}H NMR ($CDCl_3$) δ 0.80−1.00 (t, CH_3−$(CH_2)_{12}$), 1.20−1.40 (m, CH_3−$(CH_2)_{12}$−), 1.50−1.70 (m, CH_2−CH_2−CO), 1.85−1.95 (s, CH_3−C5 thymine), 2.30−2.45 (m, CH_2−CH_2−CO), 3.30−3.60 (s, $(CH_3)_2$−N), 3.90−4.10 (m, CH_2−N−CH_2), 4.15−4.30 (m, CH_2−CH_2−O−CH_2−N), 4.50−4.65 (m, C(O)O−CH_2), 5.20−5.35 (s, O−CH_2−N), 7.35−7.40 (s, H−C6 thymine), 9.95−10.25 (s, HN thymine). Anal. ($C_{28}H_{52}O_5N_3I$) C, H, N.

***N,N*-Dimethyl-*N*-3-(hexadecanoyloxy)propyl-*N*-[*N*^{1}-(5-methyl-2,4-dioxopyrimidinyl)methoxyethyl]ammonium Iodide (1d):** yield 29%; wt 120 mg; mp 133−135 °C; ^{1}H NMR ($CDCl_3$) δ 0.80−1.00 (t, CH_3−$(CH_2)_{12}$), 1.20−1.40 (m, CH_3−$(CH_2)_{12}$−), 1.50−1.70 (m, CH_2−CH_2−CO), 1.85−1.95 (s, CH_3−C5 thymine), 2.15−2.28 (m, O−CH_2−CH_2−CH_2−N), 2.30−2.40 (m, CH_2−CH_2−CO), 3.30−3.50 (s, $(CH_3)_2$−N), 3.65−3.72 (m, CH_2−N−CH_2−CH_2−O), 3.90−4.03 (m, CH_2−N−CH_2−CH_2−O), 4.15−4.35 (m, CH_2−CH_2−O−CH_2−N; C(O)O−CH_2), 5.20−5.35 (s, O−CH_2−N), 7.35−7.40 (s, H−C6 thymine), 9.80−9.85 (s, HN thymine); HRMS calcd for $C_{29}H_{54}O_5N_3$ 524.4064 (M^+), found 524.4043.

Compounds **5** and **6** were prepared by using the same procedure as given for the synthesis of **7a**.

***N*-Methyl-*N*-(hydroxyethyl)-*N*-[5′-(2′,5′-dideoxy)thymidinyl]amine (5):** Yield 55%; wt 94 mg; hygroscopic; ^{1}H NMR (CD_3OD) δ 1.88−1.95 (s, CH_3−C5 thymine), 2.20−2.35 (m, H_α−C2′), 2.48−2.60 (m, H_β−C2′), 2.68−2.70 (d, HO−C3′), 2.81−2.90 (s, N−CH_3), 3.40−3.54 (m, HO−CH_2−CH_2−N−CH_2), 3.75−3.80 (m, HO−CH_2−CH_2−N−CH_2), 3.82−3.91 (m, HO−CH_2), 4.22−4.31 (H−C4′), 4.35−4.42 (H−C3′), 6.18−6.26 (t, H−C1′), 7.50−7.55 (s, H−C6 thymine); HRMS calcd for $C_{13}H_{22}O_5N_3$ 300.1559 (MH^+), found 300.1562.

***N,N*-Dimethyl-*N*-(3-hydroxypropyl)-*N*-[5′-(5′-deoxythymidinyl)]ammonium Iodide (6):** Yield 54%; wt 0.35 g; hygroscopic; ^{1}H NMR (CD_3OD) δ 1.90−1.95 (s, CH_3−C5 thymine), 1.95−2.10 (HO−CH_2−CH_2−CH_2), 2.20−2.35 (m, H_α−C2′), 2.42−2.55 (m, H_β−C2′), 2.85−2.90 (d, HO−C3′), 3.00−3.03 (t, HO−CH_2), 3.20−3.35 (s, N−$(CH_3)_2$), 3.52−3.62 (m, HO−CH_2−CH_2−CH_2−N−CH_2), 3.85−3.95 (m, HO−CH_2−CH_2−CH_2−N−CH_2), 4.00−4.05 (m, HO−CH_2), 4.22−4.31 (H−C4′), 4.35−4.42 (H−C3′), 6.18−6.26 (t, H−C1′), 7.50−7.55 (s, H−C6 thymine); HRMS calcd for $C_{15}H_{26}O_5N_3$ 328.1872 (M^+), found 328.1882.

***N,N*-Dimethyl-*N*-(2-hydroxyethyl)-*N*-[5′-(5′-deoxythymidinyl)]ammonium Iodide (7a).** A solution of 5′-iodothymidine (0.5 g, 1.4 mmol) and 2-(dimethylamino)ethanol (5 mL, 49 mmol) in DMF (30 mL) was heated to 50 °C for 3 h. After DMF was removed under high vacuum, $CHCl_3$ (2 × 20 mL) was added to dissolve the unreacted starting material. After the $CHCl_3$ was decanted, the residue was purified via column chromatography ($CHCl_3$/CH_3OH discontinuous gradient, 6:4 to 3:7): yield 52%; wt 0.32 g; hygroscopic; ^{1}H NMR (CD_3OD) δ 1.90−2.00 (s, CH_3−C5 thymine), 2.20−2.35 (m, H_α−C2′), 2.42−2.55 (m, H_β−C2′), 2.85−2.90 (d, HO−C3′), 3.20−3.35 (s, N−$(CH_3)_2$), 3.55−3.65 (t, HO−CH_2−CH_2−N−CH_2), 3.85−3.95 (d, HO−CH_2−CH_2−N−CH_2), 4.00−4.05 (m, HO−CH_2), 4.22−4.31 (H−C4′), 4.35−4.42 (H−C3′), 6.18−6.26 (t, H−C1′), 7.50−7.55 (s, H−C6 thymine); HRMS calcd for $C_{14}H_{24}O_5N_3$ 314.1716 (MH^+), found 314.1731.

Compounds **7b**−**7d** were prepared from 5-iodothymidine and the appropriate amino ester by using the same procedure as for the preparation of **1b**.

***N,N*-Dimethyl-*N*-[2-(octanoyloxy)ethyl]-*N*-[5′-(2′,5′-dideoxy)thymidinyl]ammonium Iodide (7b):** Yield 36%; wt 0.12 g; mp 103−106 °C; hygroscopic; ^{1}H NMR ($CDCl_3$) δ 0.70−0.78 (t, CH_3−$(CH_2)_4$), 1.11−1.23 (m, CH_3−$(CH_2)_4$−), 1.42−1.53 (m, CH_2−CH_2−CO), 1.70−1.75 (s, CH_3−C5 thymine), 2.11−2.23 (m, H_α−C2′), 2.18−2.25 (t, CH_2−CH_2−CO), 2.32−2.45 (m, H_β−C2′), 3.12−3.18 (s, $(CH_3)_2$−N), 3.22 (s, HO), 3.65−3.72 (m, O−CH_2−CH_2−N−CH_2), 3.75−3.82 (m, H−C4′), 3.95−4.12 (m, O−CH_2−CH_2−N−CH_2), 4.29−4.42 (m, H−C3′; C(O)O−CH_2), 6.02−6.10 (t, H−C1′), 7.40−7.43 (s, H−C6 thymine); HRMS calcd for $C_{22}H_{38}O_6N_3$ 440.2761 (M^+), found 440.2759.

***N,N*-Dimethyl-*N*-[2-(hexadecanoyloxy)ethyl]-*N*-[5′-(2′,5′-dideoxy)thymidinyl]ammonium Iodide (7c):** Yield 30%; wt 0.12 g; mp 148−150 °C; hygroscopic; ^{1}H NMR ($CDCl_3$) δ

0.70−0.78 (t, CH_3−$(CH_2)_{12}$), 1.11−1.23 (m, CH_3−$(CH_2)_{12}$−), 1.42−1.53 (m, CH_2−CH_2−CO), 1.70−1.75 (s, CH_3−C5 thymine), 2.11−2.23 (m, H_α−C2′), 2.18−2.25 (t, CH_2−CH_2−CO), 2.32−2.45 (m, H_β−C2′), 3.12−3.18 (s, $(CH_3)_2$−N), 3.22 (s, *H*O), 3.65−3.72 (m, O−CH_2−CH_2−N−CH_2), 3.75−3.82 (m, *H*−C4′), 3.95−4.12 (m, O−CH_2−CH_2−N−CH_2), 4.29−4.42 (m, *H*−C3′; C(O)O−CH_2), 6.02−6.10 (t, *H*−C1′), 7.40−7.43 (s, *H*−C6 thymine). Anal. ($C_{30}H_{54}O_6N_3I$) C, H, N.

***N,N*-Dimethyl-*N*-[3-(hexadecanoyloxy)propyl]-*N*-[5′-(2′,5′-dideoxy)thymidinyl]ammonium Iodide (7d):** Yield 36%; wt 0.14 g; very hygroscopic; ^{1}H NMR ($CDCl_3$) δ 0.70−0.78 (t, CH_3−$(CH_2)_{12}$), 1.11−1.23 (m, CH_3−$(CH_2)_{12}$−), 1.42−1.53 (m, CH_2−CH_2−CO), 1.70−1.75 (s, CH_3−C5 thymine), 2.18−2.35 (t, CH_2−CH_2−CO), 2.28−2.68 (m, H_2−C2′; O−CH_2−CH_2−CH_2−N), 3.22−3.42 (s, $(CH_3)_2$−N), 3.58−3.72 (m, O−CH_2−CH_2−N−CH_2; *H*−C4′), 4.12−4.30 (m, O−CH_2−CH_2−N−CH_2), 4.48−4.56 (m, *H*−C3′; C(O)O−CH_2), 6.02−6.10 (t, *H*−C1′), 7.50−7.53 (s, *H*−C6 thymine), 10.20−10.25 (s, *H*N thymine); HRMS calcd for $C_{31}H_{56}O_6N_3$ 566.4169 (M^+), found 566.4178.

Biological Evaluations

Cytotoxicity Assay with the SV-28 and KB Tumor Cell Lines. Preliminary biological screening for antitumor activity was done with SV-28 cells. SV-28 cells are baby hamster kidney cells transformed by the oncogenic simian virus SV40. Cells were grown in MEM medium with 5% fetal calf serum. Cells were seeded at a density of 3×10^3 cells/cm^2. About 24 h later, 10, 20, and 50 μM of the test compound was added, and cells were incubated for another 48 h to allow three doublings of the control cells. The cells then were counted with a hemocytometer. Each compound was tested on at least two separate occasions. IC_{50} is the concentration of the test compound which reduced the cell count to 50% compared with the control culture. For compounds showing cytotoxicity, the experiments were repeated 3 times to determine the standard deviation. Compounds **1c** and **7c** also were tested against the KB cell line by the same assay. The doubling time for KB cells, however, is slightly longer than that for SV-28 cells.

SV-28 Cell Plating Efficiency Assay. The cell plating efficiency assay was performed to study the cells' ability to reproduce after exposure to the cytotoxic compounds. On day 1, the SV-28 cells were seeded at a density of 3×10^3 cells/cm^2. After 24 h of incubation, the test compound was added at about the IC_{50} concentration. On day 3, the cells were counted with a hemocytometer, diluted 100 times, and seeded into 75 cm^2 flasks. On day 9, the cells were fixed and stained with 0.5% methylene blue in 50% ethanol, and the number of colonies was counted. This number divided by the number of cells plated on day 3 gives the plating efficiency.

Antiherpes Simplex Virus Plaque Reduction Assay. Confluent monolayers of Vero (African green monkey kidney) cells were inoculated with HSV-1(KOS) in RPMI-1640 medium supplemented with 5% calf serum. After 30 min of adsorption at 37 °C, the medium was replaced with a drug overlay, which contained 2.5 mL of RPMI-1640 medium with 0.8% (carboxymethyl)-cellulose, 0.5% calf serum, and added test compounds at various concentrations (10, 20, 50, 100 μM). Each treatment was done in triplicate. After a 2 day incubation, the cells were fixed and stained with 1% crystal violet (w/v) in 50% ethanol. The number of plaques was counted, and the concentration of test compound that reduced the plaque number to half compared to untreated control cultures was the IC_{50} value. Acyclovir (50 μM) was used as a positive control. Stock solutions of drugs were dissolved in DMSO and stored at −20 °C.

HIV-1 Syncytial Plaque Assay. A 96-well plate was treated with 100 μL of poly(L-lysine) solution for 30 min at room temperature and washed 3 times with 0.01 M phosphate-buffered 0.85% (w/v) sodium chloride (PBS) as previously described.[4] Aliquots of CEM-SS cells in log-phase growth were washed 3 times with PBS and suspended at a density of 50 000 CEM-SS cells per 50 μL of RPMI-1640 medium without serum. The CEM-SS cells were transferred to each poly(L-lysine) precoated well. After 30 min incubation at 37 °C in a humidified atmosphere containing 5% CO_2, the cell monolayer was inoculated with 50 μL of HIV-1 diluted in growth medium. After 1 h for virus attachment, the cell monolayer was overlaid with 100 μL of growth medium containing serial concentrations of test compound. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 days, each well received a second 100 μL overlay of growth medium with or without the same concentration of test compound, and incubation was continued for an additional 2 days. On day 5, syncytial plaques were microscopically observed to be light-refractive, large, multicellular foci (10−25 nuclei per syncytium) that appeared as either brown and granular or clear. Plaques were counted with the aid of a 10× gridded ocular lens, and the concentration of test compound required to inhibit 50% of the plaque formation (EC_{50}) relative to a untreated control culture was determined.

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