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Design, Synthesis, Antiviral Activity, and Pre-formulation Development of Poly-*L*-Arginine-Fatty acyl Derivatives of Nucleoside Reverse Transcriptase Inhibitors

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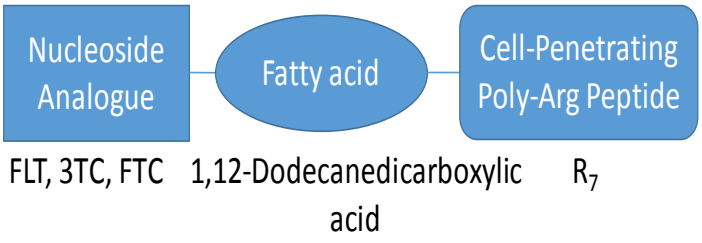
Graphical Abstract



Abstract

The objective of this work was to design conjugates of anti-HIV nucleosides conjugated with fatty acids and cell penetrating poly-*L*-arginine (polyArg) peptides. Three conjugates of polyArg cell-penetrating peptides with fatty acyl derivatives of alovudine (FLT), lamivudine (3TC), and emtricitabine (FTC) were synthesized. In general, the compounds exhibited anti-HIV activity against X4 and R5 cell-free virus with EC_{50} values of 1.5-16.6 µM. FLT-CO-$(CH_2)_{12}$-CO-$(Arg)_7$ exhibited EC_{50} values of 2.9 µM and 3.1 µM against X4 and R5 cell-free virus, respectively. The FLT conjugate was selected for further preformulation studies by determination of solution state degradation and lipid solubility. The compound was found to be stable in neutral and oxidative conditions and moderately stable in heated conditions.

Keywords: *Anti-HIV agents; Fatty acids; Lipophilicity; Nucleosides; Polyarginine; Reverse Transcriptase*

INTRODUCTION

According to UNAIDS estimation, 35.3 million people are living with Acquired Immunodeficiency Syndrome (AIDS) in 2013. High Activity Antiretroviral Therapy (HAART) involves the use of a minimum of three antiretroviral drugs including protease inhibitors, non-nucleoside reverse transcriptase inhibitors, and nucleoside reverse transcriptase inhibitors. HAART has increased the lifespan of HIV-infected patients and has converted this terminal illness into a chronic and manageable disease.[1] Although the FDA approved anti-HIV nucleosides zidovudine (AZT), lamivudine (3TC), and emtricitabine (FTC) have reduced the mortality rate in patients, their use is still complicated because of toxicity, chronic intake, development of resistant virus, and incomplete elimination of the viral reservoirs. These anti-HIV nucleosides have also limited cellular uptake due to their hydrophilic nature. Upon intracellular uptake, they need to undergo three phosphorylation steps in order to become active nucleoside triphosphate analogues.[1] Thus, novel drug delivery systems are urgently needed in order to generate nucleoside conjugates with better anti-HIV profile, such as higher cellular uptake and anti-HIV activity.

The primary objective of this work was to design conjugates of anti-HIV nucleosides conjugated with fatty acids and cell penetrating polyArg peptides. Herein, we designed polyArg-fatty acyl derivatives of anti-HIV nucleosides with the expectation to overcome their limited cellular uptake. The objective was to design multifunctional anti-HIV drug conjugates where:

1. Nucleosides act as reverse transcriptase inhibitors (NRTIs);
2. Long chain fatty acyl component acts as *N*-myristoyl transferase (NMT) inhibitor and also improves the cellular uptake; and
3. The Poly-*L*-arginine component plays a crucial role as a cell-penetrating peptide.

Long chain Carboxylic acids as NMT Inhibitors: Long chain carboxylic acids are known to inhibit NMT, which is responsible for the myristoylation of various HIV proteins, such as P17 capsid protein, $Pr55^{gag}$, $Pr160^{gag\text{-}pol}$, and $p27^{nef}$ in the virus infected host cells.[2] Myristoylation of viral proteins allows the viral protein components to become more hydrophobic.[3] Certain heteroatom-containing myristic acid analogues, such as 12-thioethyldodecanoic acid, 4-oxatetradecanoic acid and 2-methoxydodecanoic acid derivatives, inhibit HIV-1 replication in acutely infected T-lymphocytes.[4,5] 12-Thioethyldodecanoic acid was found to be moderately active against HIV infected T4 lymphocytes with an EC_{50} value of 9.4 μM.[6]

Various 5′-*O*-fatty acyl derivatives of nucleoside reverse transcriptase inhibitors (3'-azido-3'-deoxythymidine (zidovudine, AZT), 3'-fluoro-3'-deoxythymidine (alovudine, FLT), (-)-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC), 5-fluoro-(-)-2',3'-dideoxy-3'-thiacytidine (emtricitabine, FTC), 2',3'-didehydro-2',3'-dideoxythymidine (stavudine, d4T)) were found to have enhanced anti-HIV activity profiles as compared to their parent nucleosides against X4, R5 strains, and cell-associated virus, presumably due to increased cellular uptake caused by their high lipophilic profile.[6-14] These ester conjugates and other reported by others[15,16] were expected to act as bifunctional agents through intracellular hydrolysis by esterases to release nucleoside reverse transcriptase inhibitors, and the fatty acids.

Poly-L-Arginine as a Cell Penetrating Peptide: An important subclass of these molecular transporters are CPPs, which contain guanidinium-rich transporters (GRTs). The positively charged guanidinium groups on the arginine side chains are responsible for its penetrating ability through interaction with the phospholipid bilayer. The development of theses polyarginine transporter molecules was inspired by the lead HIV-1 transcription transactivator protein (Tat) made up of repeated arginine and lysine residues.[14] This transporter is highly polar, readily soluble in water, and unlike other polar drugs travels

across the lipid bilayer membrane.[17] Studies have shown that peptides with 6-20 arginine residues pass through the membrane showing rapid uptake across the membrane and into the nucleus without any signs of acute toxicity.[17] Molecular transporters when attached to poorly bioavailable drugs could enable them to pass through the biological membrane as shown previously.[18]

Herein, we report the design, synthesis, and biological activities of three poly-*L*-arginine linked poly-Arg 1,12-dodecanedicarboxylate derivatives of anti-HIV nucleosides (FLT, 3TC, and FTC (Figure 1) and their application as multifunctional anti-HIV agents. It was expected that the conjugation of nucleosides to the poly-*L*-arginine-fatty acyl residue could result in the development of anti-HIV agents with enhanced efficacy, and/or higher uptake into infected cells.

Please insert Figure 1 here.

RESULTS AND DISCUSSION

FLT (**1**), N_4-amino protected 3TC (**8**), N_4-amino protected FTC (**9**), and 1,12-dodecandicarboxylate-polyarginine resin were used as the building blocks. N4-DMTr-3TC (**8**) and N4-DMTr-FTC (**9**) were synthesized according to the previously reported procedure by us.[12] First, *tert*-butyldimethylsilyl chloride (TBDMS–Cl) was reacted with 3TC (**2**) or FTC (**3**) in the presence of imidazole to afford 5′-*O*-TBDMS-3TC (**4**) or 5′-O-TBDMS-FTC (**5**). Next, the N4–amino group of **4** and **5** was protected with 4,4′-dimethoxytrityl (DMTr) protecting group, by reaction with DMTr-Cl in the presence of pyridine to yield **6** and **7**, respectively. Finally, TBDMS was removed by using tetrabutylammonium fluoride (TBAF) to yield N4-DMTr-3TC (**8**) and N4-DMTr-FTC (**9**) (Scheme 1).

Please insert Scheme 1 here.

Second, the polyarginine peptide (R_7) was manually synthesized by agitation of resin using nitrogen gas by using solid-phase Fmoc/*t*Bu strategy. The preloaded Fmoc-*L*-arginine(Pbf) Wang resin (**10**) was swelled in DMF followed by deprotection of Fmoc group by using 20% piperidine in DMF.

The resin was washed with DMF and coupled with Fmoc-Arg(Pbf)-OH in the presence of coupling reagents 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA) as a base, respectively, in DMF at room temperature under nitrogen. The deprotection and coupling cycles were repeated 5 more times followed by the final *N*-terminal Fmoc deprotection using 20% piperidine in DMF to yield NH_2-polyarginine Wang resin **11**. A small amount of resin cleavage confirmed the assembly of polyarginine peptide on Wang resin. 1.12-Dodecanedicarboxylic acid was coupled to NH_2-polyarginine Wang resin **11** to yield 1,12-dodecanedicarboxylate-polyarginine resin **12** in the presence of a combination of coupling reagents (HBTU, HOAt and DIC) (Scheme 2).

Peptidyl polyarginine dodecandicarboxylate resin **12** was conjugated with the nucleoside analogues (**1**, **8**, or **9**) in the presence of HBTU, HOAt DIPEA and DIC in anhydrous DMF (Scheme 2). The resin was cleaved along with the deprotection of Pbf and DMTr protective groups from the side chain of arginine by and corresponding nucleosides, respectively, using reagent R for 4 h at room temperature. The conjugates were purified by preparative reverse-phase HPLC. The purity of final products (>95%) was confirmed by analytical HPLC. The chemical structures of compounds were determined by a SELDI-TOF

mass spectrometer on a Ciphergen protein chip instrument using α-cyano-4-hydroxycinnamic as a matrix and by ^{1}H and ^{13}C NMR.

Please insert Scheme 2 here.

The anti-HIV activity of the compounds was evaluated according to the previously reported procedure.[11, 19-21] Table 1 shows the anti-HIV activities of the conjugates compared to their parent analogues. No cellular cytotoxicity was observed up to the highest tested concentration for the conjugates (EC_{50} > 30 μM). The conjugates showed no significant anti-HIV activity against cell associated virus (EC_{50} > 30 μM). All conjugates exhibited less anti-HIV activity when compared with the parent nucleoside analogues against cell free virus. FLT conjugate **13** (EC_{50} = 2.9-3.1 μM) showed less potency than its parent nucleoside (EC_{50} = 0.1-0.2 μM) against X4 and R5 cell free virus. FTC conjugate **15** (EC_{50} = 1.5-3.0 μM) showed less anti-HIV-1 activity when compared to that of FTC (EC_{50} = 0.18-0.48 μM) against X4 and R5 cell free virus. 3TC conjugate **14** showed approximately 2-fold higher inhibition against X4 virus (EC_{50} = 8.7 μM) than the R5 virus strain (EC_{50} =16.6 μM) but exhibited also less potency than that of 3TC (EC_{50} = 2.6-7.5 μM). The conjugates were less potent than their parent structures possibly due to the limited uptake. Different strains of HIV are known to have different gp120 V3 loops. Both X4 and R5 strains of HIV possess a high positive charge density on their V3 loop,[22,23] suggesting that the presence of positive charge on the conjugates could block the interaction with gp120. Thus, these conjugates exhibited less anti-HIV activity of these conjugates when compared to their parent analogs because of the limited cellular uptake.

Please insert Table 1 here.

To better understand the physicochemical properties of these compounds in designing more optimized compounds, we further explored stability and lipid solubility of these compounds. Since FLT-conjugate contains several potentially labile bonds, forced degradation of an aqueous solution of **13** at elevated temperature, in acid, base, and under oxidizing conditions, were conducted. Compound **13** was selected for further evaluation in solution state degradation studies and determination of lipid solubility (partition coefficient Thus, it was necessary to determine the relative stability of these compounds under different conditions. Furthermore, these conjugates were designed to be used in areas where shipping and storage conditions are not ideal in terms of temperature and humidity.).

Please insert Figure 2 here.

At heated (panel B) conditions, significant degradation of the compound **13** was observed resulting in a second HPLC peak at 9 min. Under both acidic and alkaline conditions, the compound degraded to a great extent resulting in a different peak at 7 min (panels C and D). Minimal degradation of the drug was noted at neutral (panel E) and oxidative (H_2O_2) (panel F) conditions. (Figure 2). Thus, the FLT conjugate was relatively stable in neutral and oxidative conditions, but less stable in heated, acidic, and alkaline conditions.

The Log P of the compound was determined by distributing the compound between equal volume of *n*-octanol (organic) and pH 4 acetate buffer (aqueous) with stirring at room temperature for 2 days, followed by HPLC studies. The Log P of the compound was found to

be -0.34, indicating that the compound is hydrophilic, most likely due to the presence of several guanidinium moieties from hepta-*L*-arginine. FLT has a Log P value of -0.52. These data suggest that the compound is still very hydrophilic despite the presence of the long 1,12-dodecane dicarboxylic acid linker.

Various gel formulations of the compound were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of the thermo-reversible gelling (Pluronic F-127) polymer. The derivative was used for dissolution studies using four different gels with and without the thermogelling polymer. The HPC-SL formulation consisted of 2.25% w/v in water while Carbopol consisted of 0.2% w/v. For the thermogelling formulations, the HPC-SL and Carbopol gels were mixed with 20% w/v solution of Pluronic F-127(3:1 v/v ratio) in water. The formulations were then sealed in dialysis tubes, and the rate and extent of compound dissolution were determined in simulated vaginal fluid at 37°C. There was no observed drug release that could be identified by UV spectrophotometry at 220 nm and 256 nm, suggesting that the compound was either unstable in these formulations.

CONCLUSIONS

In conclusion, three poly-*L*-arginyl-1,12-dodecanedicarboxylate nucleoside conjugates of FLT, 3TC, and FTC were designed and synthesized using solid-phase chemistry. The structures of the compounds were confirmed by NMR and mass spectroscopy. The compounds were evaluated for their anti-HIV activity against cell-free and cell-associated virus. FLT conjugate **13** showed EC_{50} values of 2.9-3.1 μM against X4 and R5 virus. The compound was relatively stable in neutral and oxidative conditions, and unstable in heated, acidic, and alkaline conditions. FLT conjugate was hydrophilic as the Log P was

found to be -0.34. The derivative was evaluated in dissolution studies using four different hydrogels gels with and without a thermogelling polymer. Gel formulations of the compound were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of a thermo-reversible gelling (Pluronic F-127) polymer. The compound was unstable or did not undergo the release from the tested hydrogel formulations. These data indicate that the presence of positively-charged CPP could impede the interactions between positively charged V3 loop in gp120 and the conjugates. Further optimization of conjugated CPP-fatty acid-nucleoside conjugates is required to generate compounds with improved anti-HIV activity and optimized stability and formulation performance.

EXPERIMENTAL

Materials and Methods

Materials

Nucleosides (**1-3**) were purchased from Euro Asia Trans Continental (Bombay, India) for the nucleoside ester conjugate synthesis. [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate] (HBTU), *N*,*N*-diisopropylethylamine (DIPEA), and 1,12-dodecanedicarboxylic acid were bought from Sigma Aldrich Chemical Co. Solvents and all other reagents were purchased from Fisher Scientific. The products were purified using a Phenomenex-Gemini C18 column (10 µm, 250 × 21.2 mm) with Hitachi HPLC system using a gradient system at a constant flow rate of 10 ml/min (Table 2). The purity of the final products was confirmed by using a Hitachi analytical HPLC system on a C18 column using a gradient system (water:acetonitrile 30:70 v/v) at a constant flow rate of 1 ml/min with UV detection at 220 nm and 265 nm. The chemical structures of the final products were determined by nuclear magnetic resonance spectrometry (^{1}H NMR and ^{13}C NMR) on a

Bruker NMR spectrometer (400 MHz) and confirmed by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument using α-cyano-4-hydroxycinnamic acid as a matrix.

Please insert Table 2 here.

Chemistry

Synthesis of Polyarginine (11). The peptide synthesis was carried out in Bio-Rad polypropylene columns by shaking and mixing using a Glass Col small tube rotator or on a PS3 automated peptide synthesizer (Rainin Instrument Co., Inc.) at room temperature unless otherwise stated. Fmoc-Arg(Pbf)-Wang resin (**10**, 1.1 mmole, 2.97 g, 0.37 mmole/g) was placed in manual peptide synthesis container (250 mL) equipped with a three way stopper. Resin was swelled in *N*,*N*-dimethylformamide (DMF, 150 mL) with constant N_2 bubbling for 30 min (2 times). The Fmoc group was removed from the resin in the presence of piperidine (20% v/v in DMF, 2 × 50 mL) for 15 min. The resin was washed with DMF (5 × 50 mL). Fmoc-Arg(Pbf)-OH (3 equiv, 3.3 mmol, 2.14 g) and HBTU (3 equiv, 1.25 g, 3.3 mmol) were added to the reaction vessel, followed by the addition of DMF (25 mL) and DIPEA (6 equiv, 6.6 mmol, 1.15 mL) (Scheme 2) and bubbled with N_2 for 2.5 h. The resin was washed with DMF (5 × 25 mL) to remove unreacted starting materials, impurity, and reagents. The deprotection and coupling cycles were repeated five more times to add a total of 7 arginine residues on the resin. *N*-Terminal Fmoc was deprotected by using piperidine (20 % v/v in DMF) to afford resin **11**. Resin **11** was washed with DMF (5 × 100 mL). The structure of **11** was confirmed by cleaving a small amount of the resin in the presence of reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole, 90:5:3:2 v/v/v/v, 2 mL) for 4 h. The crude peptide was precipitated in the presence of cold diethyl ether (Et_2O, 150 mL), and separated and washed by centrifugation (3 × 15 mL) at 4000 rpm for 5 min. The crude peptide was dissolved in water (0.1%, TFA). The molecular weight of poly-L-arginine (R_7) was

confirmed by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument. MS (SELDI-TOF) (m/z): $C_{42}H_{86}N_{28}O_8$, calcd, 1111.3; found, 1111.1 $[M]^+$.

Synthesis of Polyarginine 1,12-Dodecanedicarboxylate (12). 1,2-Dodecanedicarboxylic acid (1.43 g, 5.5 mmol), HBTU (2.09 g, 5.5 mmol), and 1-hydroxy-7-azabenzotriazole (HOAt, 0.74 g, 5.5 mmol) were added to the reaction vessel containing polyargine resin **11** in anhydrous DMF (200 mL), followed by the addition of DIPEA (8.1 mmol, 1.42 mL) and *N*,*N*′-diisopropylcarbodiimide (DIC, 9.0 mmol, 1.4 mL) (Scheme **2**). The peptidtyl resin was bubbled with N_2 for 7 h and then washed with DMF (5 × 100 mL). The *N*-terminal Fmoc group was deprotected by using piperidine (20 % v/v in DMF) to yield **12**. Resin **12** was finally washed with DMF (5 × 150 mL). A small amount of the resin was cleaved using reagent R (TFA/thioanisole/1,2-ethanedithiol/anisole, 90:5:3:2 v/v/v/v, 2 mL) and precipitated with cold diethyl ether (200 mL) and finally centrifuged at 4000 rpm for 5 min. The residue was dissolved in water, and the formation of product **12** was confirmed with SELDI-TOF mass spectrometer. MS (SELDI-TOF) (m/z): $C_{56}H_{110}N_{28}O_{11}$, calcd, 1351.7; found, 1352.0 $[M + H]^+$.

General Procedure for the Synthesis of Polyarginine-1,12-Dodecanedicarboxylate Acylated Nucleoside Analogues (13-15). N4-4,4′-Dimethoxytrityl (DMTr)-3TC (**8**) and N4-DMTr-FTC (**9**) were synthesized as described in the previously published procedure.[12,13] Peptidyl polyarginine dodecandicarboxylate resin **12** (0.7 g), the nucleoside analogues (**1**, **8**, or **9**, 1.1 mmol), HBTU (417 mg, 1.1 mmol), and HOAt (148 mg, 1.1 mmol) were dissolved in anhydrous DMF (25 mL) in a round bottom flask (100 mL) followed by the addition of DIPEA (1.95 mmol, 342 µL) and DIC (2.5 mmol, 383 µL) (Scheme 2). The reaction vessels were flushed with N_2 and placed on a shaker at 4000 rpm to mix the reagents.

The reaction mixture was filtered off, and the resin was washed with DMF (3 × 20 mL), methanol (3 × 20 mL), and dichloromethane (DCM, 3 × 20 mL). The resin was cleaved along with the deprotection of Pbf and DMTr protective groups from the side chain of arginine by and corresponding nucleosides, respectively, using reagent R (20 mL) for 4 h at room temperature. The resin was filtered, and the filtrate was added dropwise to cold diethyl ether (50 mL) for precipitation followed by centrifugation at 4000 rpm. The precipitates were washed with diethyl ether (2 × 50 mL) to afford solid crude peptide-nucleoside conjugates (**13**-**15**). The precipitates were dried and dissolved in 50% acetonitrile and water. The conjugates were purified by preparative reverse-phase HPLC (Shimadzu LC-8A preparative liquid chromatograph) on a Phenomenex-Gemini C18 column (10 µm, 250 × 21.2 mm) at 10.0 mL/min flow rate using a gradient of 10% acetonitrile (0.1% TFA) in water (0.1% TFA) to 100% acetonitrile (0.1% TFA) (Table 2). Chromatograms were recorded at 220 and 265 nm using a UV detector. The purity of final products (>95%) was confirmed by analytical HPLC. The chemical structures of compounds were determined by a SELDI-TOF mass spectrometer on a Ciphergen protein chip instrument using α-cyano-4-hydroxycinnamic as a matrix and by ^{1}H and ^{13}C NMR.

1-[5′-O-(3′-Fluoro-2′,3′-dideoxythymidinyl)]tetradecan-1,14-dioate conjugate of PolyArg (FLT-OCO(CH_2)$_{12}$CONH-RRRRRRR-OH, 13). Yield (210 mg, 60.5%). ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.30 (s, 1H, H-6), 6.12 (dd, *J* = 5.7 and 8.6 Hz, 1H, H-1′), 5.15 (dd, *J* = 4.3 and 53.2 Hz, 1H, H-3′), 4.04-4.26 (m, 7H, -NH-C*H*-CO), 3.90-4.00 (m, 1H, H-4′), 3.52-3.66 (m, 2H, H-5′ and H-5″), 3.26-3.35 (m, 2H, =NH-CH_2), 2.95-3.10 (m, 10H, =NH-CH_2), 2.70-2.90 (m, 2H, =NH-CH_2), 2.40-2.55 (m, 1H, H-2″), 2.05-2.25 (m, 5H, CH_2O and H-2′), 1.84-1.90 (br s, 7H, 5-CH_3 and C$H_2$$CH_2$CO), 1.35-1.80 (m, 32H, =NH-CH_2-CH_2), 1.08-1.20 (br s, 16H, methylene protons). ^{13}C NMR (CD_3CN, 100 MHz, δ ppm): 175.87,

173.26, 172.79, 172.69, 156.36, 135.79, 118.26, 114.78, 84.89, 82.22, 63.19, 52.82, 51.83, 48.74, 40.22, 37.14, 33.16, 27.49, 24.07, 19.98, 19.25, 13.83, 11.43, 0.73, 0.52, 0.31, 0.11, 0.10, 0.36.MS (SELDI-TOF) (m/z): $C_{66}H_{121}FN_{30}O_{14}$, calcd, 1576.97; found, 1577.5 $[M + H]^+$.

1-[(−)-2′,3′-Dideoxy-3′-thiacytidine]tetradecan-1,14-dioate conjugate of PolyArg (3TC-OCO(CH_2)$_{12}$CONH-RRRRRRR-OH, 14). Yield (180 mg, 52.3%). ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.27 (d, *J* = 7.8 Hz, 1H, H-6), 6.42-6.48 (m, 1H, H-1′), 6.34 (d, *J* = 7.5 Hz, 1H, H-5), 5.60-5.80 (m, 1H, H-4′), 4.58-4.68 (m, 1H, -NH-C*H*-CO), 4.40-4.50 (br s, 5H, -NH-C*H*-CO), 4.30-4.40 (m 1H, -NH-C*H*-CO), 3.78 (dd, *J* = 12.8 and 5.4 Hz, 1H, H-5″), 3.61 (d, *J* = 12.8 Hz, 1H, H-5′), 3.42-3.52 (m, 1H, H-2″), 3.22-3.40 (br s, 15H, H-2′, =NH-CH_2), 2.52-5.64 (m, 2H, CH_2CONH-), 2.36-2.44 (m, 2H, CH_2COO), 1.65-2.10 (m, 32H, =NH-CH_2-CH_2), 1.30−1.50 (br s, 16H, methylene protons). ^{13}C NMR (CD_3CN, 100 MHz, δ ppm): 176.50, 176.46, 174.91, 173.47, 172.76, 172.69, 172.65, 172.59, 172.48, 162.08, 161.74, 159.40, 156.29, 148.20, 143.62, 94.00, 86.78, 84.18, 63.21, 53.17, 52.83, 52.77, 52.43, 40.17, 36.95, 35.03, 33.33, 28.51, 28.38, 28.30, 28.11, 28.02, 27.91, 27.67, 27.57, 24.96, 24.21, 24.07, 24.01, 23.92. MS (SELDI-TOF) (m/z): $C_{64}H_{119}N_{31}O_{13}S$, calcd, 1561.93; found, 1562.70 $[M + H]^+$.

1-[(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine]tetradecan-1,14-dioate conjugate of PolyArg (FTC-OCO(CH_2)$_{12}$CONH-RRRRRRR-OH, 15). Yield (130 mg, 37.5%); ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.11 (d, *J* = 6.7 Hz, 1H, H-6), 6.22-6.27 (m, 1H, H-1'), 5.44 (dd, *J* = 2.9 and 4.3 Hz, 1H, H-4'), 4.68 (dd, *J* = 12.6 and 4.4 Hz, 1H, H-5"), 4.30-4.48 (m, 7H, H-5', -NH-C*H*-CO), 4.20-4.30 (m, 1H, -NH-C*H*-CO), 3.45-4.65 (m, 1H, H-2"), 3.15-3.28 (m, 15H, H-2', CH_2NH), 2.13-2.38 (m, 4H, CH_2COO and CH_2CONH), 1.40-1.90 (m,

32H, methylene protons), 1.10-1.30 (br s, 16H, methylene proton); ^{13}C NMR (CD_3CN, 100 MHz, δ ppm): 172.88, 172.41, 171.79, 171.21, 159.47, 158.84, 158.52, 156.76, 124.80, 99.43, 86.63, 82.02, 63.77, 52.02, 40.04, 39.83, 39.62, 39.41, 39.20, 38.99, 38.78, 36.06, 35.05, 33.16, 28.87, 28.79, 28.35, 28.26.25.15, 24.86, 24.29. MS (MALDI-TOF) (m/z): $C_{64}H_{118}FN_{31}O_{13}S$, calcd, 1580.91; found, 1581.5 $[M + H]^+$.

Stability and Degradation Studies. The stability of **13** was evaluated by using HPLC. All degradation studies were carried out at a drug concentration of 1 mg/mL. The solution stability studies were conducted by using the stock solution of compound **13** in the presence of room temperature (25 °C), heat (40 °C), neutral (water), acidic (1N HCl), alkaline (1N NaOH) and oxidation (3% H_2O_2) conditions at 40 °C. The compound was incubated in the above solutions for 24 hours. All the samples were kept at room temperature for 1 h, and the analytical HPLC was run for 35 min. The solvent system used was water:acetonitrile (0.1% trifluoroacetic acid) and the HPLC was run at a flow rate of 1 mL/min at 220 nm and 256 nm wavelengths (Table 3).

Please insert Table 3 here.

Partition Coefficient (Log P). Log P HPLC studies were carried out by distributing 21.9 mg of compound **13** in 250 µL each of *n*-octanol (organic) and pH 4 acetate buffer (aqueous). The mixture was stirred for 2 days at room temperature. The analytical HPLC was run using water:acetonitrile (0.1% TFA) as a solvent system at a flow rate of 1 mL/min at 220 nm and 256 nm wavelengths for each collected fraction (Table 3).

Gel Formulations. Vaginal gel formulations of compound **13** were manufactured using non-ionic (HPC-SL) and anionic (Carbopol) polymers with and without the inclusion of thermo-reversible gelling (Pluronic F-127) polymer. The HPC-SL formulation consisted of 2.25% w/v in water while Carbopol consisted of 0.2% w/v. For thermogelling formulations, the aforementioned HPC-SL and Carbopol gels were mixed with a 20% solution of Pluronic F-127 (3:1 v/v ratio). A drug load comprising 1.6% (w/v) of FLT conjugate was loaded in the gels and was sealed in SpectraPor dialysis tubing with MWCO of 3500 Da. The tubes were suspended in 70 ml of dissolution media (Table 4) held at 37 ± 0.5 °C with a stirring speed of 75 rpm using a 0.5 inch stir bar. The dissolution media consisted of a simulated vaginal fluid (Table 4). These dissolution samples were transferred to UV 96 well plates (Costar®,) and were then analyzed using a SpectraMax M2 UV detector (Molecular Devices, PA, USA).

Please insert Table 4 here.

Anti-HIV Assays. The anti-HIV activity of the compounds was evaluated according to the previously reported procedure.[11, 19-21] Compound anti-HIV activity was evaluated in single-round (MAGI) infection assays using X4 (IIIB) and R5 (BaL) HIV-1 and P4R5 cells expressing CD4 and coreceptors. In summary, P4R5MAGI cells were cultured at a density of 1.2×10^4 cells/well in a 96 well plate approximately 18 h prior to infection. Cells were incubated for 2 h at 37 ºC with purified, cell-free HIV-1 laboratory strains IIIB or BaL (Advanced Biotechnologies, Inc., Columbia, MD) in the absence or presence of each agent. After 2 h, cells were washed, cultured for an additional 46 h, and subsequently assayed for HIV-1 infection using the Galacto-Star β-Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems, Bedford, MA). Reductions in infection were calculated as a percentage relative to the level of infection in the absence of agents, and 50%

inhibitory concentrations (EC_{50}) were derived from regression analysis. Each compound concentration was tested in triplicate wells. Cell toxicity was evaluated using the same experimental design but without the addition of virus. The impact of compounds on cell viability was assessed using an MTT (reduction of tetrazolium salts) assay (Invitrogen, Carlsbad, CA).

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FIGURES, SCHEMES, AND TABLES

Figure 1. Poly-*L*-arginine linked fatty acylated nucleosides.

Figure 2. Degradation Studies of FLT conjugate **13**. **A:** Standard **13,** Retention time 9.7 min**;** **B:** Heat: 1 mL stock + 1 mL methanol at 40 °C; **C:** Acid: 1 mL stock + 1 mL 1N HCl at 40°C; **D:** Base: 1 mL stock + 1 mL 1N NaOH at 40 °C; **E**: Oxidation: 1 mL stock + 1 mL H_2O_2 at 40 °C; **F**: Water: 1 mL stock + 1 mL H_2O at 40 °C.

Scheme 1. Synthesis of N4-DMTr protected of FTC and 3TC nucleosides.[12]

Scheme 2. Synthesis of hepta-*L*-arginyl-1,12-dodecanedicarboxylate-nucleoside conjugates **13-15**.

Table 1. Anti-HIV activity of dicarboxylic acid ester conjugates of nucleoside conjugates (**1-7**).

Table 2. HPLC method used for purification of the final compounds.

Table 3. HPLC method used for stability and degradation studies.

Table 4. Dissolution media composition for (6 liters).

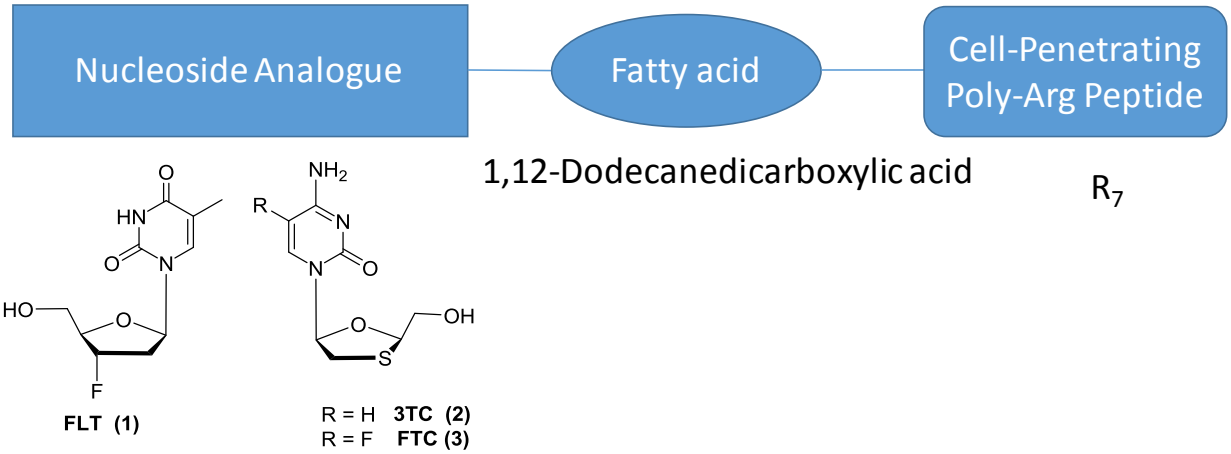


Figure 1. Poly-*L*-arginine linked fatty acylated nucleosides.

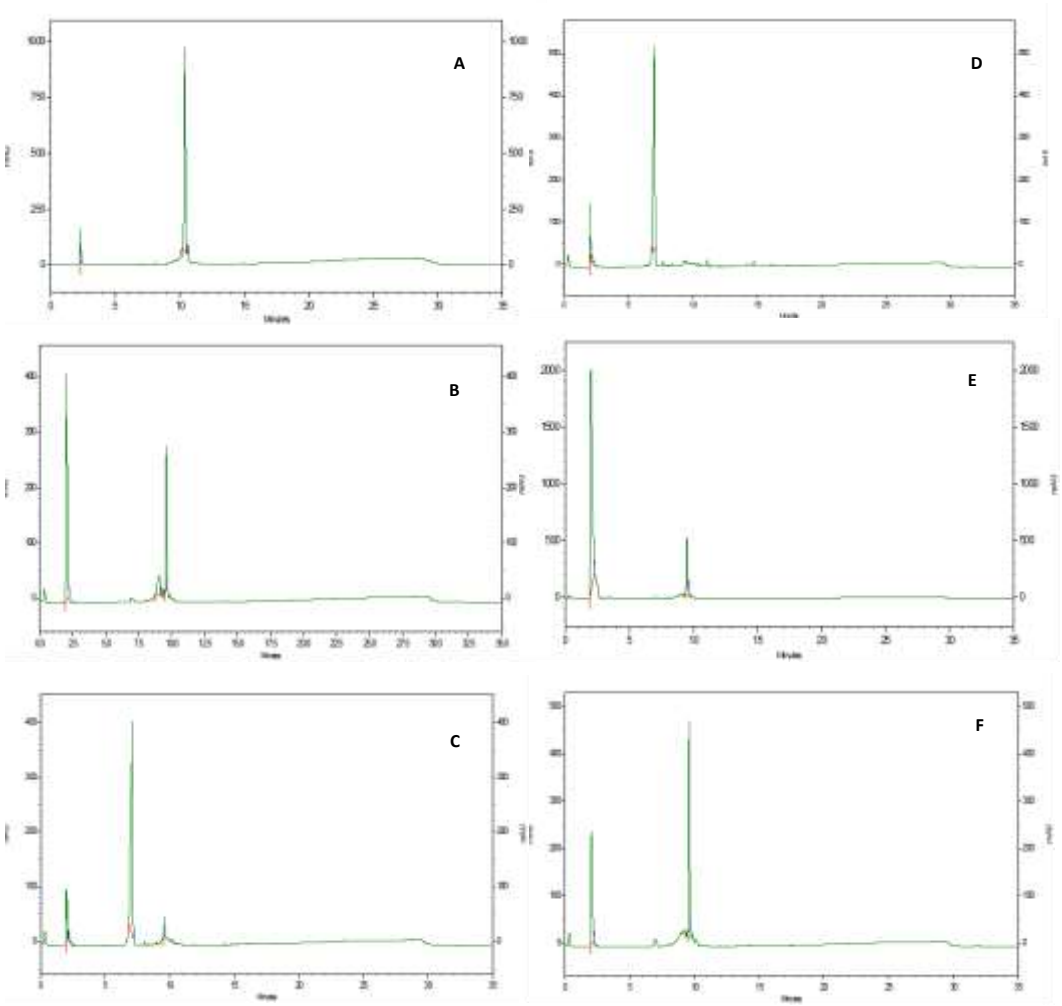
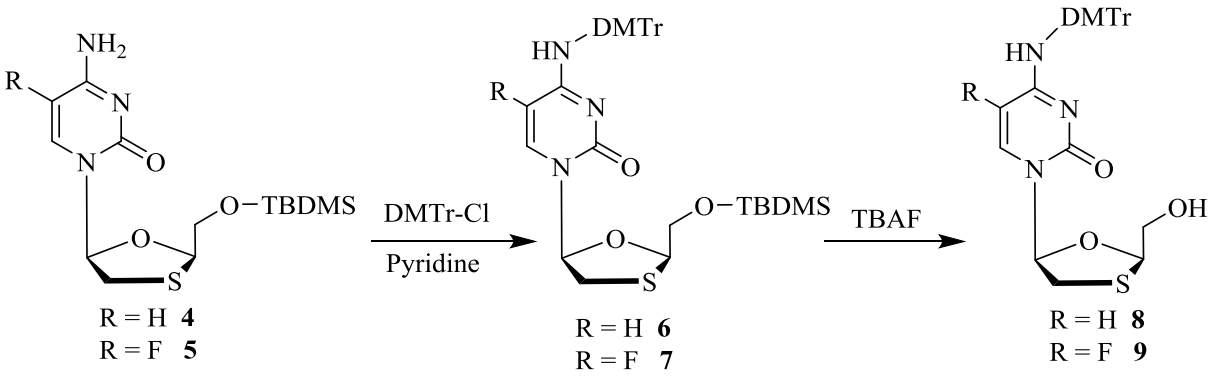
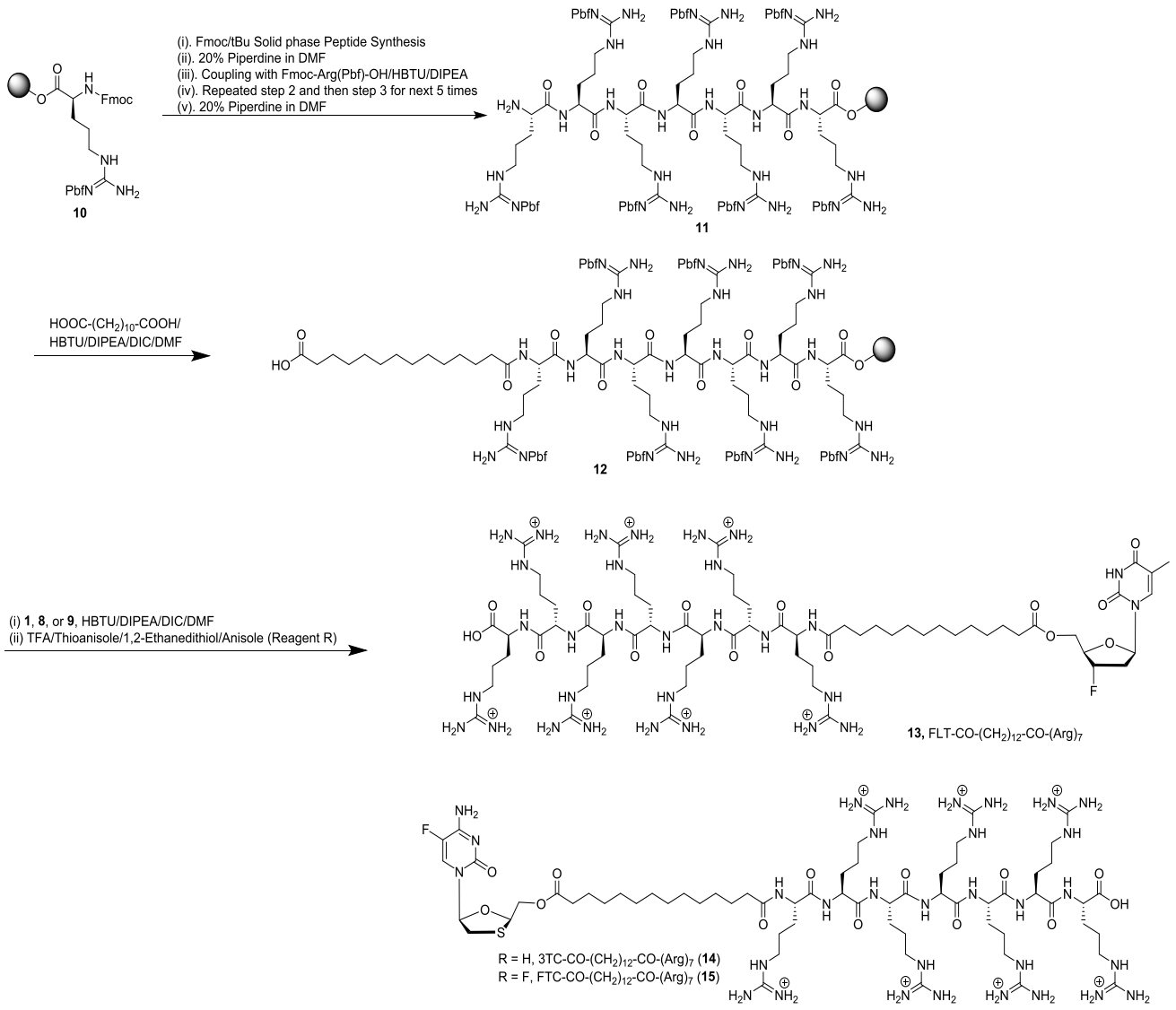


Figure 2. Degradation Studies of FLT conjugate **13**. **A:** Standard **13,** Retention time 9.7 min**;** **B:** Heat: 1 mL stock + 1 mL methanol at 40 °C; **C:** Acid: 1 mL stock + 1 mL 1N HCl at 40°C; **D:** Base: 1 mL stock + 1 mL 1N NaOH at 40 °C; **E**: Oxidation: 1 mL stock + 1 mL H_2O_2 at 40 °C; **F**: Water: 1 mL stock + 1 mL H_2O at 40 °C.



Scheme 1. Synthesis of N4-DMTr protected of FTC and 3TC nucleosides.[12]



Scheme 2. Synthesis of hepta-*L*-arginyl-1,12-dodecanedicarboxylate-nucleoside conjugates **13-15**.

Table 1. Anti-HIV activity of dicarboxylic acid ester conjugates of nucleoside conjugates (**1-7**).

Compound	Chemical Name	Cytotoxicity	Cell- Free Virus		Cell-Associated Virus
		CTS[a]	X4[c]	R5[d]	CTC[e]
		EC_{50}[b](μM)	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)
FLT (**1**)	3'-Fluoro-2',3'-deoxythymidine	>100	0.2	0.1	>100
3TC (**2**)	(-)-2',3'-Dideoxy-3'-thiacytidine	>100	7.5	2.6	18.4
FTC (**3**)	(-) 2',3'–Dideoxy-5-fluoro-3'-thiacytidine	>100	0.48	0.18	21.9
13	FLT-CO-$(CH_2)_{12}$-CO-R_7	>30	2.9	3.1	>30
14	3TC-CO-$(CH_2)_{12}$-CO-R_7	>30	8.7	16.6	>30
15	FTC-CO-$(CH_2)_{12}$-CO-R_7	>30	3.0	1.5	>30

[a]Cytotoxicity assay (MTS); [b]50% Effective concentration; [c]Single-round infection assay (lymphocytotropic strain, X4); [d]Single-round infection assay (monocytotropic strain, R5); [e]Cell-associated transmission assay (X4).

Table 2. HPLC method used for purification of the final compounds.

Time (min)	**Water Concentration A (%)**	**Acetonitrile Concentration B (%)**	**Flow rate (mL/min)**
0.00	100.0	0.0	1.0
1.00	100.0	0.0	10.0
5.0	90.0	10.0	10.0
25.0	70.0	30.0	10.0
35.0	60.0	40.0	10.0
60.0	0.0	100.0	10.0
65.0	0.0	100.0	10.0
70.0	100.0	0.0	1.0

Table 3. HPLC method used for stability and degradation studies.

Time (min)	Water Concentration A (%)	Acetonitrile Concentration B (%)	Flow rate (mL/min)
0.00	100.0	0.0	1.0
1.00	100.0	0.0	1.0
20.0	0.0	100.0	1.0
25.0	0.0	100.0	1.0
30.0	100.0	0.0	1.0
35.0	100.0	0.0	1.0

Table 4. Dissolution media composition for (6 liters).

Chemicals	Acetic acid	Urea	Glucose	Lactic acid	Glycerol	Potassium hydroxide	Calcium hydroxide	Sodium chloride	pH
Weight (grams)	6	24	30	12	0.96	8.4	1.3	21	4.2