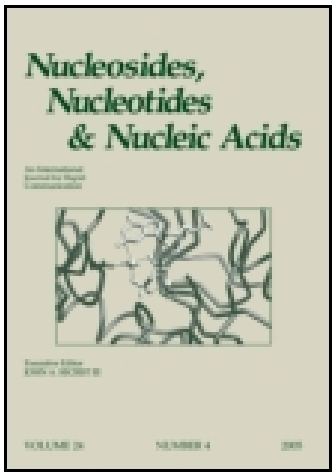

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Zhengqing You [a] , A. Moshin Mian [b] , Ram P. Agarwal [b] & Henry Joung Lee [a c]
[a] College of Pharmacy and Pharmaceutical Sciences , Florida A & M University , Tallahassee, Florida, USA
[b] Department of Medicine , University of Miami School of Medicine , Miami, Florida, USA
[c] College of Pharmacy and Pharmaceutical Sciences , Florida A & M University , Tallahassee, FL, 32304, USA
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Synthesis, Anti-HIV, and Cytotoxic Activity of New AZT Conjugates of Steroid Acids

Zhengqing You,[1] A. Moshin Mian,[2] Ram P. Agarwal,[2] and Henry Joung Lee[1,*]

[1]College of Pharmacy and Pharmaceutical Sciences, Florida A & M University, Tallahassee, Florida, USA
[2]Department of Medicine, University of Miami School of Medicine, Miami, Florida, USA

ABSTRACT

In an attempt to discover potent anti-HIV agents devoid of the serious toxicity of the current HIV-reverse transcriptase inhibitors, three steroid prodrugs of AZT have been synthesized and their anti-HIV profiles determined with CEM-SS cell line. Two of the prodrugs were active against HIV, though weaker than AZT. The third agent was totally inactive against HIV. However, it demonstrated remarkably high anti-growth activities. Further experiments established that growth inhibition of the third agent was caused by induction of apoptosis rather than general necrosis.

Key Words: AZT; Steroid; Prodrug; Anti-HIV; Cytotoxicity.

Azidothymidine (AZT), the first clinically approved agent for treatment of acquired immunodeficiency syndrome (AIDS), acts by inhibiting the reverse transcriptase of human immunodeficiency virus (HIV).[1–3] Although AZT has attained

*Correspondence: Henry Joung Lee, College of Pharmacy and Pharmaceutical Sciences, Florida A & M University, Tallahassee, FL 32304, USA; Fax: (850) 599-3323; E-mail: henry.lee@famu.edu.

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a mainstay status in the treatment of AIDS, its serious dose-related side effects have necessitated ever-growing research to find safer and more effective analogs.[4] One promising approach to improve the therapeutic index has been the prodrug strategy. This approach couples AZT with an acid to form an ester which is designed to gradually release AZT by metabolism. Since AZT has a short plasma half-life due to glucuronidation at the 5′-OH,[5] and poor cellular penetrating capability,[6] the esterification is expected to increase drug stability against metabolic destruction and improve its cellular bio-availability, thus reducing dose-related toxicity. The most studied AZT prodrugs have been carboxylic ester and phosphoester. The former includes use of bicyclams attached to an acid group,[7] retinoic acids,[8] 1,4-dihydronicotinic acids,[8] and steroid acids.[9] Some of the latter are derived from salicyl alcohols,[10] ether lipids,[11] lipophilic glycosides.[12] In design of new steroid conjugates with AZT, four rationales were employed. 1. Biologically inactive steroid acids obtained based on the antedrug concept[13] are devoid of systemic toxicity. 2. The acids are designed to improve lipophilicity and bio-availability of the resulting prodrugs. 3. Protection of the 5′-OH could increase drug half-life in vivo. 4. Binding of steroid conjugates to transcortin could further improve metabolic stability.[14] In this study, two biologically inactive steroid acids (**2**,**3**) synthesized in our lab and one commercially available, non-toxic acid (**1**, cholenic acid) were conjugated with AZT. Preliminary anti-HIV bioassays showed that the two esters (**5**,**6**) were active against HIV, while the third (**4**) was not. However, the intriguing activity/toxicity profile of the third conjugate led to an in-depth evaluation of its anti-proliferation activities. Presented in this paper are both anti-HIV results and anti-growth profile of these three compounds.

RESULTS AND DISCUSSIONS

Chemistry

AZT conjugates with steroids were synthesized through ester formation of steroid acids with AZT alcohol as shown in Sch. 1. Either 1,3-dicyclohexylcarbodiimide (DCC) or carbonyldiimidazole (CDI) was used as the coupling agent. Cholenic acid (**1**) underwent coupling with AZT only under the DCC conditions, while acid **3** yielded the desired product **6** only under the CDI conditions. Although acid **2** could form some desired product **5** under the CDI conditions, the DCC conditions gave much higher yield of **5**. Acid **1** was purchased from Steraloids Inc. (Wilton, N.H.). The two biologically inactive acids (**2** and **3**) [15,16] were prepared as shown in Sch. 2. Acid **2** was synthesized by a 4-step procedure starting from enone **9**. Nitrile **8** was formed cleanly through a one-step conversion of enone **9** with metal fulminate.[17] Mattox rearrangement and methanolysis then yielded methyl ester **7**[18] which eventually gave target acid **2** by alkaline hydrolysis. Acid **3** was synthesized from diol **11** which in turn was derived from prednisolone using a known literature procedure.[19] Acetonide **10** was obtained from diol **11** with 2,2-dimethoxypropane and p-toluenesulfonic acid. Alkaline hydrolysis then gave acid **3**.

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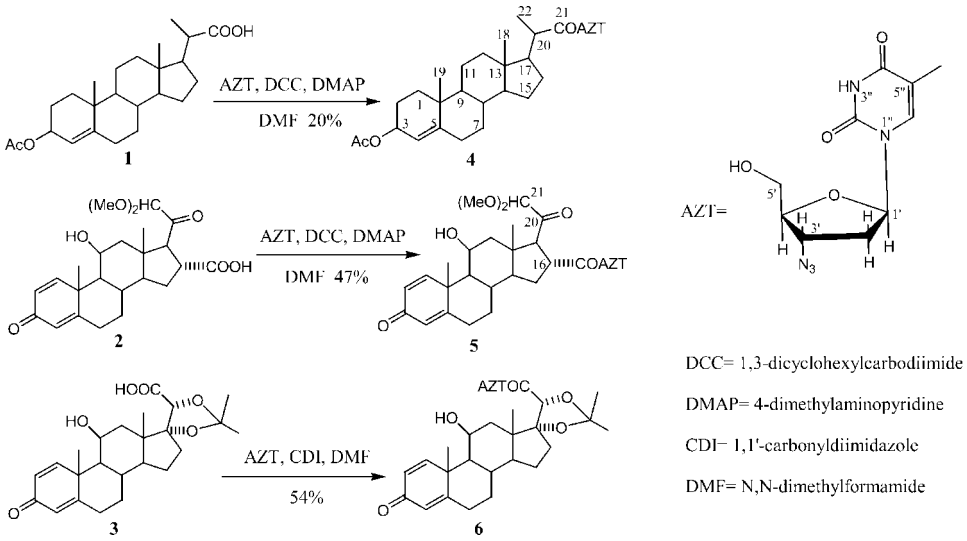
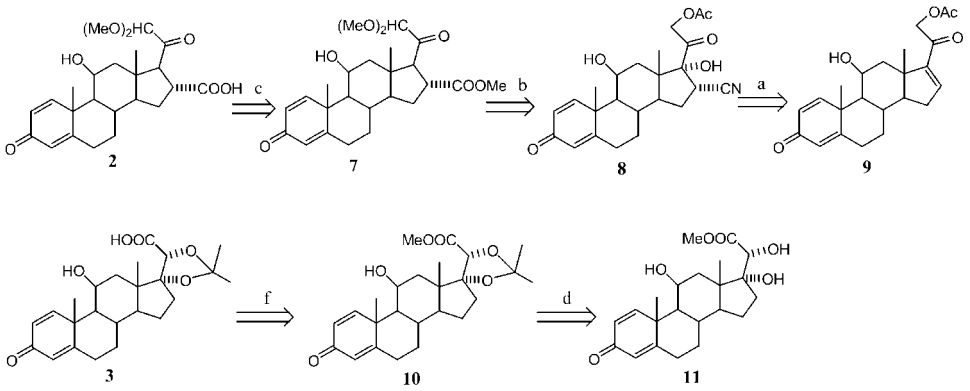


Scheme 1.

Biology

The ester conjugates were subjected to in-vitro anti-HIV bioassays with CD4 expressing lymphocytes (CEM-SS cell line). Drug efficacy (EC_{50}) was obtained by adding HIV and AZT conjugate to the cell to measure protection of the drug against HIV-induced cell death. The cytotoxicity (IC_{50}) data was obtained from adding only the conjugate to the cell to measure cell death caused by the agent. Therapeutic index (TI) is the ratio IC_{50}/EC_{50}. As summarized in Table 1, conjugates **5** and **6** showed TIs of 370 and 184 as compared to >150 for AZT. The two active agents have



Scheme 2. a) $Hg(CNO)_2/LiBr/Et_3N/HOAc/DMF$, 60°C, 70%; b) MeOH/HCl, −20°C, 65%; c) NaOH/MeOH/H_2O, 87%; d) $Me_2C(MeO)_2$/TsOH/DMF, 50°C, 54%; e) NaOH/MeOH/H_2O, 75%.

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Table 1. Anti-HIV activity and cytotoxicity in CEM-SS cell line.[a]

Drug	EC_{50}[b] (M)	IC_{50}[c] (M)	$TI(IC_{50}/EC_{50})$
4	Inactive	4.7×10^{-6}	
5	3.5×10^{-8}	1.3×10^{-5}	370
6	6.5×10^{-8}	1.2×10^{-5}	184
AZT	7.0×10^{-9}	$> 1 \times 10^{-6}$	> 150

[a]Numbers derived by averaging two repeat experiments.
[b]50% Effective concentration against HIV cytopathic effects.
[c]50% Inhibitory concentration for cell growth.

weaker anti-HIV potency than AZT, but appear to possess relatively low cytotoxicity (relatively high IC_{50} value). The National Cancer Institute, which performed the anti-HIV screen test with CEM-SS cells, uses AZT as a reference compound with its concentration up to 1×10^{-6} M which is considered quite high. As shown in Table 1, compounds **5** and **6** gave IC_{50} values of 1.3×10^{-5} and 1.2×10^{-5} M respectively. Conjugate **4** provided no protection against HIV-induced death, but higher cytotoxicity than **5** or **6**. The intriguing profile of conjugate **4** prompted further evaluations of its growth inhibition in comparison to **5** and **6**.

Cell growth inhibitory activity against human lymphoblasts (H9 cells) for compounds **4–6** and AZT is depicted in Fig. 1. Compound **4** was the most potent with an IC_{50} value of 2.5 μg/mL, while the values for the compounds **6** and **5** were estimated

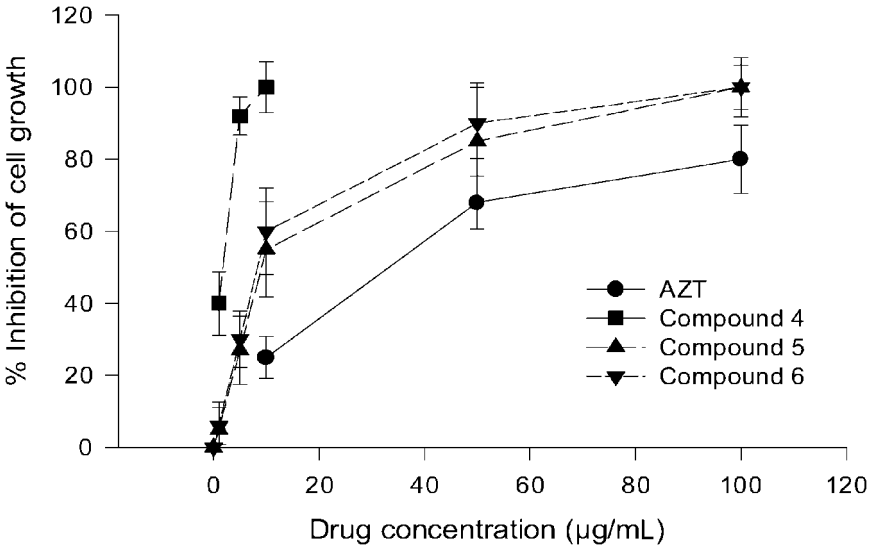

Figure 1. Cell growth inhibition by compounds **4–6**. H9 cells (5×10^4 cells/mL), in triplicate, were incubated with various concentrations of compounds **4–6**. After 72 h incubation at 37°C, trypan blue excluding cells were counted and the % cell growth inhibition was calculated from the untreated controls.

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to be 10 and 12 μg/mL, respectively. AZT was apparently even weaker than **5** and **6**. Conjugate **4** was further tested against ddC resistant H9 cells (H9/ddC cells that have reduced thymidine and deoxycytidine kinase activities); HL60, HL60/araC (human promyelocytic leukemic cell lines, parental and araC resistant), H29, H29/FU (human colon adenocarcinomas, parental and FU resistant), MCF7 (human breast carcinoma) and BL5 (Burkitt lymphoma cell line). Preliminary results show that, at 10 μg/mL, it produced 85–100% growth inhibition in all of these human tumor cell lines.

The question of whether the alcohol and acid components within conjugate **4** were responsible for the observed cytotoxicity was addressed by exposure of H9 cells to AZT, cholenic acid, AZT plus cholenic acid, and **4**. As shown in Fig. 2, compound **4** gave not only far greater cytotoxicity than either AZT or cholenic acid, but also significantly higher activity than a combination of AZT plus cholenic acid, even though the mixture produced more than the additive effect of the two components. At 5 μg/mL, conjugate **4** prevented growth of H9 cells almost completely.

The effect of drug exposure time on cell growth inhibition is shown in Fig. 3. Treatment with conjugate **4** at 1 or 5 μg/mL for 4 h did not produce any inhibition, while 8 h of incubation resulted in 20–25% inhibition. Substantially more cytotoxicity was observed after 24 h of exposure at 5 μg/mL, although much longer (72 h) exposure only gave a plateau. The data appear to suggest that at least one cell cycle drug exposure be required to cause a significant growth inhibition.

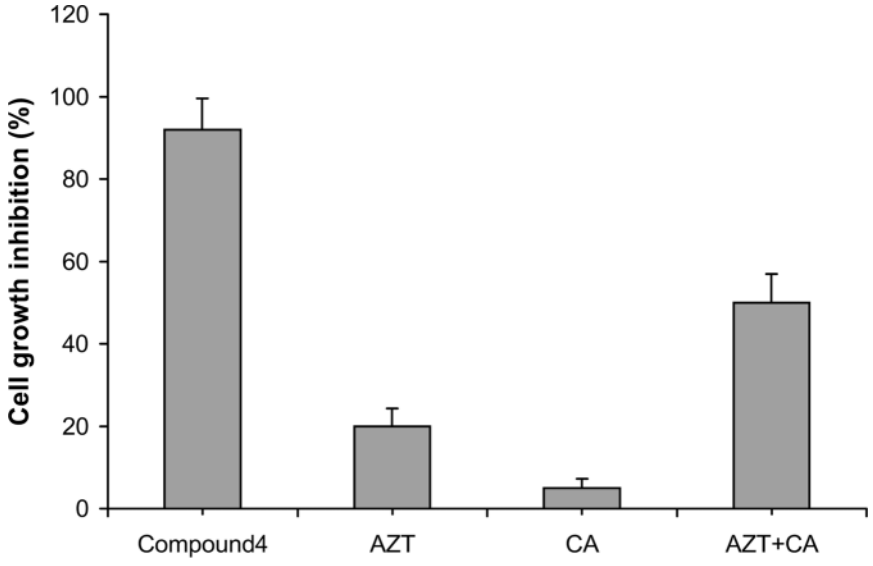

Figure 2. Cytotoxicity of compound **4** and its components (AZT and cholenic acid). H9 cells (5×10^4 cells/mL), in triplicate, were incubated with compound **4** (5 μg/mL), AZT (10 μg/mL), CA [cholenic acid (10 μg/mL)] and a combination of AZT + CA (10 μg/mL each) for 72 h at 37°C. Trypan blue excluding cells were counted and the % cell growth inhibition was calculated from the untreated controls.

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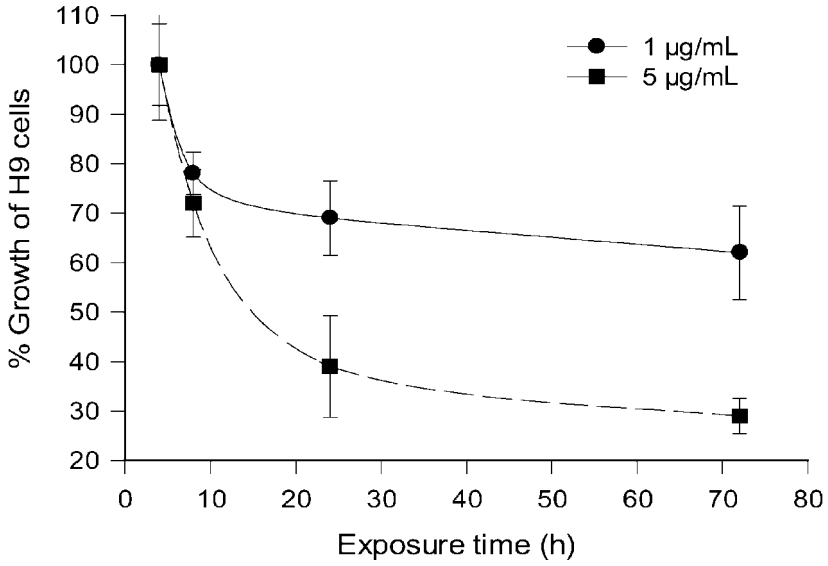

Figure 3. Effect of compound **4** on growth as a consequence of drug exposure time. H9 cells were incubated with compound **4** (1 μg or 5 μg/mL) for 4, 8, or 24 h when the cells were washed and reincubated in fresh drug free medium for 68, 64, and 48 h respectively (final incubation time is 72 h). Another set of cells were exposed to compound **4** (1 μg or 5 μg/mL) continuously for 72 h. Cells were counted and then % growth was determined from the appropriate controls.

The role of apoptosis in the cellular cytotoxicity of compound **4** was studied and the results are summarized in Fig. 4. In the experiments, fractions of cells in various stages of apoptotic death were assessed by simultaneous Annexin V and propidium iodide staining using flow cytometry. As indicated, major fractions of H9 tumor cells treated with **4** were in early (Fig. 4C lower right quadrant) or late (Fig. 4C upper right quadrant) stages of apoptotic cell death. In contrast, such effect was significantly less on cells treated with either AZT (Fig. 4B) or cholenic acid (Fig. 4D). Compound **4** at 10 μg/mL appeared to have induced close to 90% of the cells to apoptosis, indicating that the growth inhibition was due to induction of apoptosis and not general necrosis.

EXPERIMENTAL

Chemistry

Prednisolone was purchased from The Upjohn Company, Kalamazoo, MI. 23,24-Bisnor-5-cholenic acid-3β-ol acetate was obtained from Steraloids, Inc., Newport, RI. All the other chemical reagents were ordered from Aldrich chemical company, Milwaukee, WI. Preparative flash column chromatography was performed on silica gel (200–425 mesh, Davisil G633), and the solvents used were laboratory grade (Fisher Scientific, Fair Lawn, NJ). NMR spectra were obtained

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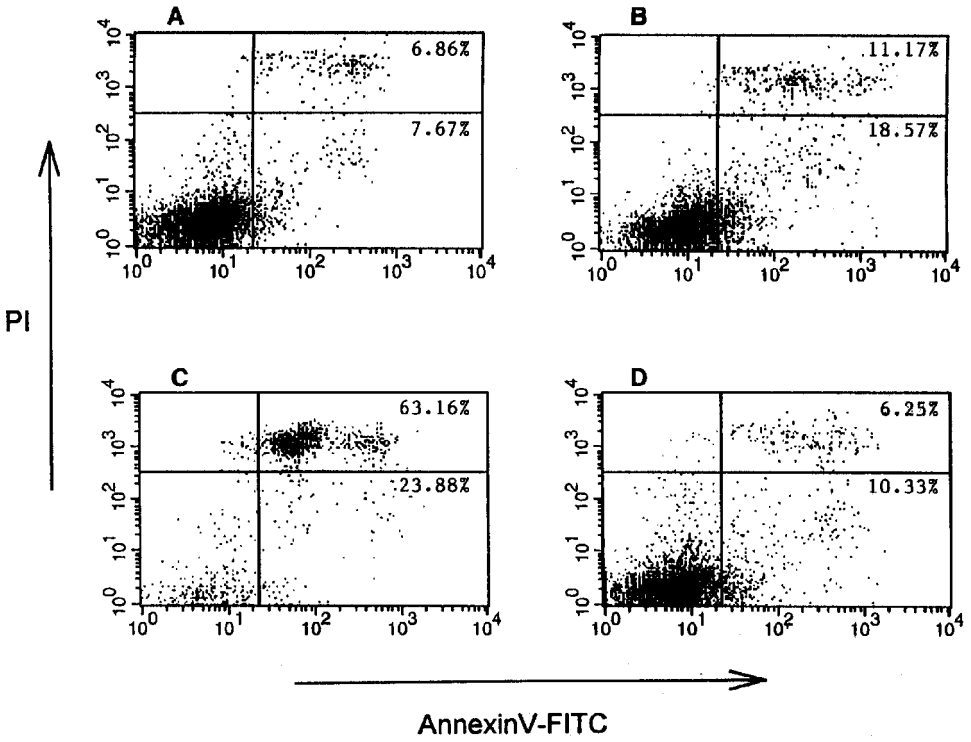

Figure 4. Effect of compounds on the induction of apoptosis as measured by Annexin V staining. H9 cells treated with (A) PBS (controls); (B) AZT (10 μg/mL); (C) compound **4** (10 μg/mL); and (D) cholenic acid (10 μg/mL) for 48 h, were stained with Annexin V-FITC and propidium iodide. Percentages of apoptotic cells are shown in the right corners. Annexin V-FITC signal is shown on the X axis; propidium iodide (PI) signal is shown on the Y axis. Ten thousand events were collected for each sample.

with a Brucker HX-300 spectrometer and the chemical shifts reported in parts per million (ppm) down field from tetramethylsilane as an internal standard. Elemental analysis was carried out by Galbraith Laboratories, Knoxville, TN. Melting points were determined on a Thomas Hoover Capillary Melting Point Apparatus and uncorrected.

3′-Azido-3′-deoxythymidine-5′-[3β-acetyloxy-22,23-bisnor-5-cholenate] (4). A mixture of acid **1** (200 mg, 0.51 mmol), AZT (160 mg, 0.59 mmol), DCC (140 mg, 0.68 mmol) and DMAP (14 mg, 0.11 mmol) in dry DMF (2 mL) was stirred under a nitrogen atmosphere for 3 days and then poured in water (100 mL). The resulting aqueous suspension was extracted with ethyl acetate/hexanes (2/1, 300 mL). The organic solution was condensed via rotary evaporation and the resulting residue purified by flash chromatography (3/1 benzene/acetone) to yield, after evaporation of solvent, **4** as a white solid: 66 mg (20%). Proton NMR (DMSO-d6): 11.355 (1H, s, NH), 7.459 (1H, d, J = 1Hz, H-6″), 6.105 (1H, t, J = 6.8 Hz, H-1′), 5.326 (1H, m, H-6),

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4.43 (2H, m), 4.213 (2H, m, H-5′), 3.958 (1H, m, H-3′), 1.969 (3H, s, Ac), 1.786 (3H, d, J = 1Hz, Me-5″), 1.121 (3H, d, J = 6.8 Hz, H-22), 0.963 (3H, s, Me), 0.648 (3H, s, Me). Carbon-13 NMR (75.47 MHz, DMSO-d6): 175.4, 169.71, 163.61, 150.35, 139.46, 136.01, 121.99, 109.77, 83.58, 80.48, 79.16, 73.16, 62.96, 60.15, 55.58, 52.42, 49.34, 41.92, 41.85, 37.65, 36.47, 36.06, 35.54, 31.33, 31.20, 27.32, 26.71, 23.88, 21.04, 20.47, 18.93, 16.83, 12.12, 11.70. Analysis Calculated for $C_{34}H_{47}N_5O_7$: C64.03%, H7.43%, N10.98%. Found: C63.82%, H7.61%, N10.80%.

3′-Azido-3′-deoxythymidine-5′-[21,21-dimethoxy-3,20-dioxo-11β-hydroxy-1,4-pregnadiene-16α-carboxylate] (5). Methyl ester **7** was obtained through one-step conversion of enone **9** to hydroxynitrile **8** followed by methanolysis.[17,18] Ester **7** (800 mg, 1.8 mmol) was dissolved in methanol (10 mL) and aqueous NaOH (4%, 10 mL) added. The mixture was stirred for 20 min and then treated with aqueous HCl (5%) to reach a pH 3. The methanol was removed by rotary evaporation, and the remaining aqueous mixture extracted with ethyl acetate (100 mL). The organic solution was washed with water (20 mL) and then dried over sodium sulfate. The dried solution was condensed and the residue chromatographed (9/1 EtOAc/hexanes) to give acid **2** as a white powder, after evaporation of solvent: 700 mg (86%). Proton NMR (DMSO-d6): 12.1 (1H, br s, COOH), 7.31 (1H, d, J = 9.8 Hz, H-1), 6.15 (1H, dd, J = 9.8, 2.0 Hz, H-2), 5.90 (1H, br s, H-4), 4.63 (1H, m), 4.53 (1H, s, H-21), 4.21 (1H, m), 3.29 (3H, s, MeO), 3.26 (3H, s, MeO), 3.09 (1H, d, J = 9.3 Hz, H-17), 1.37 (3H, s, H-19), 0.81 (3H, s, H-18). Acid **2** (200 mg, 0.46 mmol) was mixed with AZT (165 mg, 0.62 mmol), DCC (140 mg, 0.68 mmol) and DMAP (10 mg, 0.08 mmol) in dry DMF (2 mL). The mixture was stirred under a nitrogen atmosphere for 4 days and then poured in water (100 mL). The aqueous mixture was extracted with EtOAc/hexanes (3/1, 300 mL), and the organic layer washed with water (3 × 80 mL). The organic solution was then condensed and the resulting residue allowed to pass flash column (EtOAc) to yield conjugate **5** as a white solid after evaporation of solvent: 150 mg (47%). Proton NMR (CDCl3): 9.382 (1H, s, NH), 7.160 (1H, d, J = 10.1 Hz, H-1), 7.077 (1H, br d, J = 1 Hz, H-6″), 6.149 (1H, dd, J = 10.1, 1.8 Hz, H-2), 5.959 (1H, t, J = 6.4 Hz, H-1′), 5.899 (1H, br s, H-4), 4.364 (1H, s, H-21), 4.324 (1H, m, H-11), 4.235 (1H, dd, J = 12.1, 5.1 Hz, H-5′), 4.104 (1H, dd, J = 12.1, 4.0 Hz, H-5′), 4.05 (1H, m, H-4′), 3.90 (1H, m, H-3′), 3.485 (1H, m, H-16), 3.267 (3H, s, MeO), 3.256 (3H, s, MeO), 3.157 (1H, d, J = 9.0 Hz, H-17), 1.861 (3H, d, J = 1 Hz, Me-5″), 1.329 (3H, s, H-19), 0.827 (3H, s, H-18). Carbon-13 NMR (75.47 MHz, CDCl3): 203.91, 186.54, 175.06, 169.83, 163.82, 156.14, 150.11, 135.47, 127.80, 122.40, 111.44, 103.54, 85.50, 81.47, 69.63, 63.62, 61.17, 60.57, 56.01, 55.19, 54.66, 54.36, 47.31, 45.31, 43.94, 41.46, 37.18, 33.54, 31.74, 30.95, 29.17, 20.94, 16.63, 12.48. Analysis Calculated for $C_{34}H_{43}N_5O_{10}/0.5\,H_2O$: C59.12%, H6.42%, N10.14%. Found: C58.85%, H6.57%, N10.23%.

3′-Azido-3′-deoxythymidine-5′-[11β-hydroxy-17,20α-isopropylidenedioxy-3-oxo-1,4-pregnadiene-21-oate] (6). Diol **11** was obtained by literature procedure.[19] It (200 mg, 0.5 mmol) was mixed with 2,2-dimethoxypropane (3 g, 28 mmol) and $TsOH.H_2O$ (20 mg, 0.1 mmol) in anhydrous DMF (3 mL), and stirred under a nitrogen atmosphere at 40°C for 2 days. The resulting mixture was then diluted with

EtOAc/hexanes (1/1, 250 mL) and washed with saturated aqueous $NaHCO_3$ (2×10 mL) and water (15 mL). The organic solution was condensed and the resulting residue chromatographed (3/2 EtOAc/hexanes) to give acetonide **10** as a white solid, after evaporation of solvent: 120 mg (54%). Proton NMR ($CDCl_3$): 7.268 (1H, d, J = 10.1 Hz, H-1), 6.261 (1H, dd, J = 10.1, 1.9 Hz, H-2), 6.006 (1H, br s, H-4), 4.567 (1H, s, H-20), 4.445 (1H, m, H-11), 3.774 (3H, s, MeO), 1.495 (3H, s, Me), 1.440 (3H, s, Me), 1.368 (3H, s, Me), 0.945 (3H, s, H-18). Acid **3** was obtained, as a white solid, from acetonide **10** with the procedure for preparation of acid **2**, and a yield of 75% achieved. This acid (**3**, 120 mg, 0.28 mmol) was mixed with CDI (100 mg, 0.61 mmol) in anhydrous DMF (1.5 mL) and stirred under a nitrogen atmosphere for 20 min. AZT (130 mg, 0.49 mmol) was then added. Stirring continued (N_2) for 3 days and the mixture was poured in water (70 mL). The aqueous mixture was extracted with EtOAc/hexanes (2/1, 150 mL). The organic solution was condensed and the residue chromatographed (EtOAc) to yield conjugate **6** as a white solid, after evaporation of solvent: 95 mg (52%). Proton NMR (CDCl3): 9.456 (1H, s, NH), 7.187 (1H, d, J = 10.1 Hz, H-1), 7.084 (1H, br d, J = 1 Hz, H-6″), 6.153 (1H, dd, J = 10.1, 1.7 Hz, H-2), 5.925 (1H, t, J = 6.4 Hz, H-1′), 5.902 (1H, br s, H-4), 4.464 (1H, s, H-20), 4.40 (1H, H-11), 4.31 (2H, m, H-5′), 4.103 (1H, m, H-4′), 3.98 (1H, m, H-3′), 1.827 (3H, s, Me-5″), 1.384 (3H, s, Me), 1.344 (3H, s, Me), 1.212 (3H, s, Me), 0.954 (3H, s, H-18). Carbon-13 NMR (75.47 MHz, CDCl3): 186.64, 170.82, 170.35, 163.94, 156.42, 150.08, 135.69, 128.28, 122.27, 111.54, 111.23, 94.79, 86.16, 81.48, 77.54, 69.98, 63.83, 60.54, 55.31, 50.80, 46.22, 44.10, 39.25, 37.16, 33.57, 31.95, 31.45, 27.79, 27.36, 23.71, 20.97, 17.84, 12.53. Analysis Calculated for $C_{34}H_{43}N_5O_9/0.5H_2O$: C60.52%, H6.57%, N10.38%. Found: C60.82%, H6.77%, N9.94%.

Biology

Anti-HIV assay was carried out with CD4 expressing lymphocytes (CEM-SS cell line) through the following standard procedure. Agent is dissolved in dimethyl sulfoxide and diluted 1:100 in cell culture medium before preparing serial half-log10 dilutions. CD4 expressing lymphocytes are added and after a brief interval HIV-1 is added, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound serve as a toxicity control, and infected and uninfected cells without the compound serve basic controls. Cultures are incubated at 37°C in a 5% carbon dioxide atmosphere for 6 days. The tetrazolium salt, XTT, is added to all wells, and cultures are incubated to allow formazan color development be viable cells. Individual wells are analyzed spectrophotometrically to quantitate formazan production, and in addition are viewed microscopically for detection of viable cells and confirmation of protective activity. Drug-treated virus-infected cells are compared with drug-treated non-infected cells and with other appropriate controls (untreated infected and untreated non-infected cells, drug-containing wells without cells, etc.) on the same plate. Data are reviewed in comparison with other tests done at the same time and a determination about activity is made.

Growth inhibition assays were performed in triplicate by seeding 5×10^4 cells/mL in 24-well plates (Costar). Test compounds were added, and the

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trypan blue-excluding cells were counted after 72 h of incubation at 37°C. IC_{50} values were calculated from growth inhibition curves as described previously.[20,21]

Apoptosis experiments were carried out by treating H9 cells (1×10^6) with compound **4** (10 μg/mL), AZT (10 μg/mL) or cholenic acid (10 μg/mL) for 48 h. Cells were collected by centrifugation, washed with growth medium, and stained with Annexin V-FITC and propidium iodide (apoptosis dilution kit; Sigma), according to the manufacturer's protocol, and the fluorescence was analyzed via flow cytometry.[22]

SUMMARY

Three new steroidal conjugates of AZT have been synthesized and their anti-HIV profiles in CEM-SS cell line evaluated. Two of them (**5** and **6**) were active against HIV-1, though weaker than AZT. The weaker activity does not necessarily mean that this prodrug approach has failed, since the test was only conducted in an in vitro system which may be lacking in esterase enzymes that are abundant in the in vivo system, thus the prodrugs may be insufficiently cleaved to yield the active drug AZT. The third conjugate (**4**) exhibited higher cytotoxicity but no anti-HIV activity. Further experiments established that compound **4** is a highly potent anti-growth agent which inhibited growth by induction of apoptosis rather than general necrosis.

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We would like to thank the National Cancer Institute for performing the anti-HIV screening assays. Special thanks are also extended to Drs. Tieran Han and Yolong Yang for technical assistance on growth inhibition experiments. Financial support for this project has been provided by NIH grants AM 21627, RR 08111, RR 02660 and AI 20360.

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