Brief Articles

Amino Functionalized Novel Cholic Acid Derivatives Induce HIV-1 Replication and Syncytia Formation in T Cells

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Synthesis of C-11 azido/amino functionalized cholic acid derivatives has been achieved in excellent yields. Contrary to the previous prediction of analogous compounds to be HIV-1 protease inhibitors, in the present study these novel cholic acid derivatives induced host cell fusion during the progress of HIV-1 infection and produced multinucleated giant cells. This is the first report of syncytia induction and enhancement of viral replication in HIV-1 infected T cells by cholic acid derivatives.

Introduction

A C_2-homodimer containing two aspartate residues in the catalytic site has been reported to have therapeutic potential as a specific inhibitor of HIV-1 protease, and numerous examples of potent inhibitors of HIV-1 protease have been disclosed.[1,2] The most active inhibitors of HIV-1 protease that are competitive by nature contain[3] the partial structure **1** or the related carbonyl equivalent **2** (Figure 1). Other important structural features include the incorporation of lipophilic residues and bulky hydrophobic groups or solubility-increasing substituent at the appropriate positions.[4,5] On the basis of these important features, Marples and co-workers[6] have designed novel C-11 amino functionalized steroidal inhibitors, namely, 11-amino-12-(oxo/hydroxy)steroids **3** and **4** based on estra-1,3,5(10)-triens and bile acids with the intention to mimic the transition state of HIV-1 protease catalysis. Attempted synthesis[6] of these targeted 11-amino-12-oxygenated compounds starting from *d*-(+)-estrone and deoxycholic acid failed because of the decomposition of precursor 11α-azido ketones to the steroidal enamine.[7,8] Thus, Marples et al. did not realize the synthesis of 11-amino-12-oxosteroids with a specific stereogenic center at C-11. However, this group synthesized A/B ring cis steroidal enamine **5a**, *N*-benzyloxycarbonyl (*N*-Cbz) **5b**, *N*-pivaloyl **5c**, and *N*-ethoxycarbonyl **5d** derivatives (Figure 1). These compounds were predicted to be HIV-1 protease inhibitors based on their modest activity against HIV in cell culture.

Steroids with C-11 functionality are well-known for biological activity and are obtained in a number of naturally occurring molecules such as cortisone, hydrocortisone, and corticosterone.[9,10] Potent synthetic corticosteroids such as dexamethasone, triamcinolone, and fluticasone also possess C-11 hydroxy functionality.[11] Stereoselective C-11 functionalization in the steroids is one of the challenging targets for synthetic organic chemists because it involves severe steric interactions due to

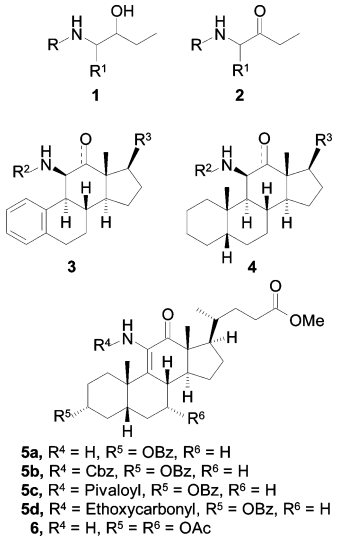

Figure 1. Amino functionalized bile acid derivatives.

C-18 and C-19 angular methyl groups. Introduction of the C-11 α-hydroxyl functionality via microbial hydroxylation by the Syntex group[12] and via long-range chemical functionalization by Breslow is well documented.[13]

Human immunodeficiency virus type 1 (HIV-1) induces host cell fusion during the progress of infection and forms multinucleated giant cells called syncytia.[14,15] Most of the T cell infecting viruses are syncytium-inducing (SI), whereas monocyte infecting viruses are non-syncytium-inducing (NSI). HIV-1 induced syncytia is the primary cause of T lymphocyte depletion and is characterized by a high rate of cell−cell fusion between infected and uninfected cells, eventually leading to cell death.[16] Syncytia are observed in "in vitro" cell culture and "in vivo" in the lymph nodes and brain regions of HIV-1 infected patients.[17] HIV-1 infected cells express the gp120 envelope protein on their surfaces, which interact with CD4 receptor and coreceptors (CXCR4 and CCR5) on uninfected bystander cells. This interaction leads to the fusion of both of these cells to form syncytium.[14]

On the basis of Marples' report and in continuation of our work on the synthesis of various bile acid conjugates[18,19] as

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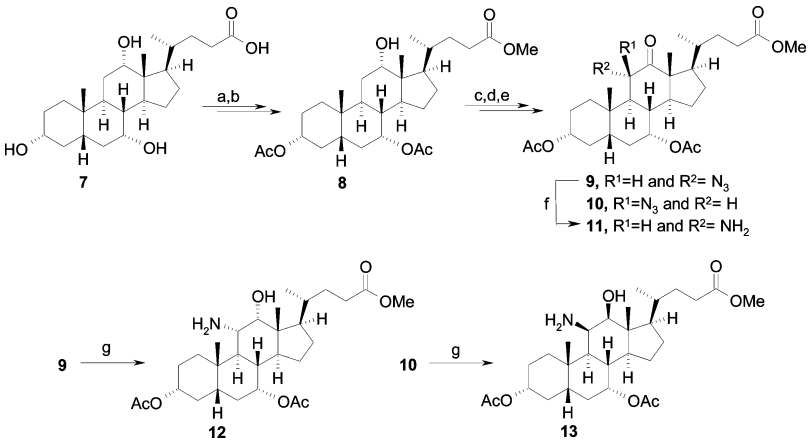
Scheme 1[a]



[a] Reagents and conditions: (a) PTSA, MeOH, 25 °C, 12 h, 98%; (b) Ac_2O, Et_3N, DMAP, CH_2Cl_2, 25 °C, 5 h, 92%; (c) CrO_3, H_2SO_4, H_2O, acetone, 0−10 °C, 5 min, 98%; (d) Br_2, BF_3-OEt_2, AcOH, 25 °C, 96 h, 93%; (e) NaN_3, DMF, 50−100 °C, 4−48 h, 64−98%; (f) H_2/Pd−C, ethyl acetate, 40 psi, 28 °C, 5 h, 95%; (g) $NaBH_4$, $CoCl_2 \cdot 6H_2O$, cetrimide, CH_2Cl_2, H_2O, CH_3OH, 25 °C, 30 min, 86% (**12**) and 91% (**13**).

novel natural product hybrids, in the present study we have tested the effect of cholic acid analogues **6**−**13** on HIV-1 replication (Figure 1 and Scheme 1). Here, we report the synthesis of two novel steroids, namely, methyl 11α-amino-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate **12** and methyl 11β-amino-3α,7α-diacetoxy-12β-hydroxy-5β-cholan-24-oate **13**. For the first time, we report that the cholic acid analogues **6**, **9**, **10**, **11** and novel compounds **12** and **13** induce HIV-1 mediated syncitia formation in HIV-1 infected T cells and thereby enhance viral replication.

Table 1. Enhancement of Syncytia Formation Due to Treatment with Cholic Acid Derivatives[a]

treatment	no. of syncytia	treatment	no. of syncytia
DMSO	85	compound **11**	170
compound **6**	214	compound **12**	173
compound **7**	69	compound **13**	235
compound **8**	92	inophyllum B	2
compound **9**	136	AZT	4
compound **10**	108		

[a] The number of syncytia was determined by flow cytometry as described in Experimental Section.

Results and Discussion

Overnight stirring of cholic acid **7** in dry methanol using a catalytic amount of PTSA followed by selective acetylation provided diacetoxy methyl ester **8** in 90% overall yield in two steps (Scheme 1). Improved yield in oxidation of compound **8** was obtained by using $CrO_3/H_2SO_4/H_2O$ in acetone at a shorter reaction time (98% yield, 5 min), whereas the literature procedure used two-phase oxidation[20] in diethyl ether (86% yield,[6] 90 min).

Recently, we have reported[21] the synthesis of methyl 11-amino-3α,7α-diacetoxy-12-oxo-5β-chol-9,11-en-24-oate **6**, methyl 11α-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **9**, methyl 11β-azido-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **10**, and methyl 11α-amino-3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate **11**. Attempted catalytic hydrogenation of 11β-azido **10** to its amine functionality was not realized (Scheme 1). Reduction of 11α-amino-12-oxo **11** with $NaBH_4$ produced mixture of 12-hydroxy epimers, and we failed to isolate the desired 11α-amino-12α-hydroxy **12**. However, the desired **12** has been synthesized in a single step from the 11α-azido-12-oxo **9** using $NaBH_4/CoCl_2 \cdot 6H_2O$[22] in $CH_2Cl_2-H_2O-CH_3OH$ and cetyltrimethylammonium bromide (CTABr) as a phase-transfer catalyst. This is a unique reaction in which the azide and the carbonyl functionalities are reduced to its amino alcohol in a single step. Use of this reagent has not been exploited earlier in steroid chemistry. In a similar fashion, treatment of 11β-azido-12-oxo **10** with $NaBH_4/CoCl_2 \cdot 6H_2O$ in $CH_2Cl_2-H_2O-CH_3OH$ and CTABr as a phase-transfer catalyst afforded stereospecifically the 11β-amino-12β-hydroxy **13** in excellent yield. Compounds **6**−**13** were subjected to biological screening against HIV-1 replication.

Enhancement of Syncytia Formation and Viral Replication. CEM-GFP cells were infected with HIV-1_{NL4-3} virus at 0.1 multiplicity of infection (MOI), and cells were cultured in 24-well plates. Cholic acid analogues were tested for their effect on viral replication at a similar, noncytotoxic concentration of 1 μg/mL. Visualization of the progression of infection using GFP fluorescence observed under a fluorescence microscope indicated an increase in syncytia formation in size and number compared to DMSO-treated (vehicle control) infected cells. Treatment of uninfected cells with these compounds did not induce any change in cellular morphology or behavior.

The number of syncytia was quantified using forward scatter (FSC) versus DNA content (FL-2), obtained by flow cytometry as described previously.[23] When we compared the syncytia formation in cholic acid analogue treated HIV-1 infected CEM-GFP cells with control cells, a further increase in the number of syncytia was observed with **6** and **9**−**13**, whereas **7**- and **8**-treated infected cells showed very little or no increase in number of syncytia (Table 1). Maximum syncytia formation was observed with **13**-treated infected cells, the microscopic and flow cytometric data for which is presented in parts A and B of Figure 2 compared to DMSO- and AZT-treated infected cells. We then analyzed the virus released in the culture supernatants on day 5 postinfection by p24 antigen ELISA, which is a sensitive method to quantitate HIV-1. This property of inducing syncytia formation was proportional with an increase in virus production in the respective treatments of cholic acid derivatives **6** and **9**−**13**, whereas **7** and **8** did not increase virus replication (Figure 2C). About 1.5- to 2.5-fold increase in p24 levels was observed with syncytia-inducing compounds. Finally, increase in viral gene expression was monitored by reverse transcription polymerase chain reaction (RT-PCR), using RNA isolated from infected cells treated with various compounds. The RT-PCR data (Figure 2D) clearly correlates with syncytia formation (Table 1) and p24 ELISA results (Figure 2C) showing increased p24 expression in **6** and **9**−**13** treatments but very little or no increase in **7** and **8** treatments.

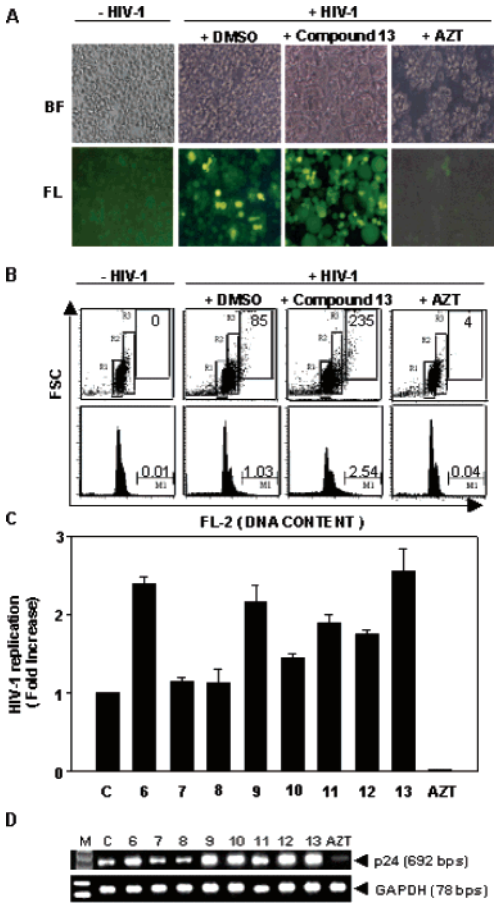


Figure 2. (A) Cholic acid derivative (**13**) induced syncytia formation in HIV-1 infected CEM-GFP cells as visualized by fluorescence microscopy: bright field (BF) and fluorescence (FL) microscopic images of uninfected (−HIV-1) and infected (+HIV-1) CEM-GFP cells in control (+DMSO), **13** (+compound **13**), and anti-HIV-1 drug AZT (+AZT) treatments. (B) Quantification of HIV-1 induced syncytia. Syncytia are quantified in uninfected (−HIV-1) and infected (+HIV-1) control (+DMSO), **13** (+compound **13**), and anti-HIV-1 drug AZT (+AZT) treated CEM-GFP cells by flow cytometry as described in Experimental Section. (C) Effect of cholic acid derivatives on HIV-1 replication in HIV-1_{NL4-3} infected CEM-GFP cells. HIV-1 released into the culture supernatants of DMSO treated (C) and cholic acid derivatives **6**−**13** treated (**6**−**13**), and anti-HIV-1 drug azidothymidine (AZT): C, 1.00 ± 0.00; **6**, 2.39 ± 0.09; **7**, 1.14 ± 0.04; **8**, 1.14 ± 0.16; **9**, 2.16 ± 0.22; **10**, 1.46 ± 0.04; **11**, 1.89 ± 0.1; **12**, 1.76 ± 0.05; **13**, 2.56 ± 0.28; AZT, 0.02 ± 0.06. (D) RT-PCR analysis of viral p24 expression in cholic acid derivative treated HIV-1 infected CEM-GFP cells. p24 transcripts were amplified by RT-PCR as described in Experimental Section: M, marker; C, DMSO control; **6**−**13**, cholic acid analogues; AZT, anti-HIV-1 drug azidothymidine treatments. GAPDH is used as the internal control.

Our primary object was to understand the relationship between molecular structure and biological activity. From Figure 2 and Table 1, it is clear that the increase in viral count is directly proportional to the syncytia formed in T cells. The 11β-amino-12β-hydroxy **13** induces the maximum viral replication in T cells followed by 11-imino-12-oxo **6**, 11α-azido-12-oxo **9**, 11α-amino-12-oxo **11**, 11α-amino-12α-hydroxy **12**, and 11β-azido-12-oxo **10**, whereas cholic acid **7** and diacetoxymethyl cholate **8** are almost acting as control samples. From the current set of data and from a comparison of the structural features of **6**−**13** with respect to the biological activity, it is clear that C-11 functionalization by amine or azide functionality is responsible for the observed enhancement of viral replication with induction of syncytium formation. In addition, the stereochemistry seems to influence the induction of syncytia formation as observed for epimers **12**/**13** and **9**/**10**. Moreover, when the biological results of **5a**−**d** were compared with that of **6**, the presence of the C-7 acetoxy group and the free primary amino functionality at C-11 seems to be the important features for enhancing HIV-1 replication.

Conclusion

C-11 functionalized cholic acid analogues have been synthesized that are not easily accessible. Surprisingly, these compounds were found to enhance HIV-1 replication with induction of syncytia formation. The increase in virus production was also found to be proportional to the increase in syncytia formation. Because syncytium is formed by cell−cell fusion, it will be interesting to study the molecular basis of this induction. The syncytia-inducing property of cholic acid analogues may also be useful for screening the efficacy of compounds that inhibit syncytia induction and also for screening the efficacy of the existing and novel antiretroviral drugs by artificial enhancement of HIV-1 replication and syncytia formation.

Experimental Section

Chemistry. TLC was performed on precoated silica gel F-254 plates (0.25 mm, E. Merk), and product(s) and starting material(s) were detected by viewing under UV light or treating with an ethanolic solution of phosphomolybdic acid or ninhydrin followed by heating. Column chromatography was performed on neutral deactivated aluminum oxide. Optical rotations were obtained on Bellingham & Stanley ADP-220 polarimeter. Specific rotations ($[\alpha]_D$) are reported in deg/dm, and the concentration (*c*) is given in g/100 mL in the specific solvent. Infrared spectra were recorded on a Schimadzu 8400 series FTIR instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AC-200 spectrophotometer at 200.13 and 50.32 MHz, respectively. The chemical shifts are given in ppm relative to tetramethylsilane. Mass specta were recorded on a LC−MS/MS-TOF API QSTAR PULSAR spectrometer, and samples were introduced by the infusion method using the electrospray ionization technique. Elemental analyses were performed by use of a CHNS-O EA 1108 elemental analyzer, Carloerba Instrument (Italy) or Elementar vario EL (Germany), and were within ±0.4% of calculated values. In the standard workup, organic layers were dried with Na_2SO_4 and concentrated in a vacuum.

General Procedure. Reduction of 11-azido-12-oxo compounds **9** and **10** to 11-amino-12-hydroxy compounds **12** and **13** is indicated below.

Methyl 11α-Amino-3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate (12). Azido compound **9** (100 mg, 0.18 mmol) was dissolved in a mixture of MeOH (2 mL) and CH_2Cl_2 (1 mL). To this, $CoCl_2 \cdot 6H_2O$ (4.3 mg, 0.018 mmol) in water (1 mL) and cetyltrimethylammonium bromide (CTABr, 10 mg, 10%) were added, and mixture was stirred for 15 min at 25 °C. Finally solid $NaBH_4$ (21 mg, 0.54 mmol) was added slowly in fractions for 5 min. The entire mixture was stirred for 30 min at 25 °C. Water (10 mL) was added, and the mixture was extracted with EtOAc (3 × 25 mL) and washed with water (2 × 25 mL) and brine (1 × 20 mL). The organic extract was dried over Na_2SO_4 and evaporated in a vacuum. The residue was chromatographed on the neutral deactivated alumina column (CH_2Cl_2−MeOH, 20:1) to afford 82 mg (86%) of **12** as colorless foam: $[\alpha]^{26}_D$ + 9.76 (*c* 0.82, $CHCl_3$); IR (Nujol) 3433, 3361, and 1735 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.67 (3H, s), 0.99 (3H, d, J = 6 Hz), 1.08 (3H, s), 2.03 (3H, s), 2.06 (3H, s), 3.44 (1H, bs), 3.63 (1H, m), 3.67 (3H, s), 4.64 (1H, m), 4.86 (1H, bs); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 11.44, 17.02, 21.24, 21.43, 23.02, 23.29, 27.04, 27.37, 29.14, 29.46, 30.82, 31.42, 34.89, 35.49, 37.74, 38.53, 40.50, 42.65, 45.71, 47.39, 50.64, 51.30, 53.01, 70.91, 73.84, 77.28, 170.32, 170.41, 174.46; MS *m*/*z* ($C_{29}H_{47}NO_7$, 521) 522.32 [30%, $(M + H)^+$], 256.26 [100%]. Anal. Calcd for $C_{29}H_{47}NO_7$: C, 66.77; H, 9.08; N, 2.69. Found: C, 66.39; H, 9.38; N, 3.10.

Methyl 11β-Amino-3α,7α-diacetoxy-12β-hydroxy-5β-cholan-24-oate (13). In a similar way the crude amino alcohol was subjected to column chromatographic purification to afford 87 mg (91%) of pure **13** as a foamy solid: $[\alpha]^{26}_D$ +18.52 (*c* 0.54, $CHCl_3$); IR (Nujol) 3421, 3357, and 1733 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 0.73 (3H, s), 1.05 (3H, d, $J = 6$ Hz), 1.21 (3H, s), 2.04 (3H, s), 2.06 (3H, s), 3.30 (1H, m), 3.67 (3H, s), 3.75 (1H, bs), 4.62 (1H, m), 4.99 (1H, m); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 10.51, 20.61, 21.41, 21.55, 22.94, 23.41, 27.04, 29.42, 29.68, 30.67, 32.24, 32.44, 33.07, 34.49, 34.75, 35.09, 37.42, 43.94, 47.30, 48.69, 51.56, 53.49, 57.99, 70.92, 73.61, 77.17, 170.09, 170.57, 174.97; MS *m/z* ($C_{29}H_{47}NO_7$, 521) 522.32 [6%, $(M + H)^+$], 256.26 [100%]. Anal. Calcd for $C_{29}H_{47}NO_7$: C, 66.77; H, 9.08; N, 2.69. Found: C, 66.55; H, 9.06; N, 2.76.

Biology. Cell Culture. CEM-GFP, a $CD4^+$ reporter T cell line with green fluorescent protein (GFP) under the control of the HIV-1 long terminal repeat (LTR) promoter, was obtained from the NIH AIDS repository[24] and was grown in RPMI 1640 medium (Invitrogen) supplemented with 2 mmol of L-glutamine, 10% fetal calf serum, 100 U of penicillin, 100 μg of streptomycin sulfate, and 500 μg of G418 antibiotic per milliliter and was maintained at 37 °C with 5% CO_2 in a water-jacketed incubator (Forma Scientific).

HIV-1 Infection of CEM-GFP Cells and Quantitation of Virus in Culture Supernatants. The 5×10^6 CEM-GFP cells were infected with a T cell tropic viral strain HIV-1_{NL4-3} at a multiplicity of infection of 0.1 for 4 h at 37 °C in the presence of 1 μg/mL polybrene. Cells were washed twice with serum-free RPMI and resuspended in complete medium and seeded at 2×10^5 (cells/mL)/well in a 24-well plate, and compounds were added to the respective wells, keeping anti-HIV-1 drugs AZT (5μmol) and inophyllum B (1μg/mL) as inhibitor controls. Infected cells were maintained for 5−7 days at 37 °C in 5% CO_2 and were tested for virus released into the culture supernatant and syncytia formation keeping DMSO-treated wells as control. Progression of infection and syncytia formation was monitored by green fluorescence as observed under an Olympus IX-70 fluorescence microscope using 488 nm excitation and 510 nm emission filters. Virus released into the culture supernatants was quantitated by use of an HIV-1 p24 antigen ELISA kit (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer's protocol.

Quantification of Syncytia Formation. Syncytia formation was analyzed using GFP fluorescence and flow cytometric analysis with propidium iodide (PI) staining using a FACS Vantage (Becton-Dickinson). The PI emission signals, forward light scatter (FSC), and side light scatter (SSC) signals were determined, and the number of syncytia was analyzed using Cell Quest software as described previously.[23]

Reverse Transcription Polymerase Chain Reaction. Compound-treated HIV-1_{NL4-3} infected CEM-GFP cells were lysed in Trizol (Invitrogen), and RNA was isolated according to the manufacturer's protocol. An amount of 5 μg of RNA was used in a reverse transcription reaction to synthesize cDNA. Viral p24 transcripts (692 bps) were amplified from 5 μL of cDNA in a 35-cycle PCR reaction using the forward primer CTATAGTGCAGAATCTC-CAAGG and the reverse primer CAACACTCTTGCTTTGTG-GCTAGGTC with the following parameters: denaturation, 94 °C for 1 min; annealing, 55 °C for 1 min; synthesis, 72 °C for 1 min and a final extension at 72 °C for 10 min. GAPDH (78 bps) was amplified using the GCCACATCGCTAAGACACCATGGG forward primer and the CCTGGTGACCAGGCGCCCAAT reverse primer, using similar PCR conditions except the annealing temperature of 60 °C.

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