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Glycoside analogs of β-galactosylceramide, a novel class of small molecule antiviral agents that inhibit HIV-1 entry

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ABSTRACT

The interaction between HIV gp120 and galactose-containing cell surface glycolipids such as GalCer or Gb_3 is known to facilitate HIV binding to both $CD4^+$ as well as $CD4^-$ cells. In an effort to develop small molecule HIV-1 entry inhibitors with improved solubility and efficacy, we have synthesized a series of *C*-glycoside analogs of GalCer and tested their anti HIV-1 activity. The analogs were tested for gp120 binding using a HIV-1 (IIIB) V3-loop specific peptide. Two of the six analogs that interfered with gp120 binding also inhibited HIV Env-mediated cell-to-cell fusion and viral entry in the absence of any significant cytotoxicity. Analogs with two side chains did not show inhibition of fusion and/or infection under identical conditions. The inhibition of virus infection seen by these compounds was not coreceptor dependent, as they inhibited CXCR4, CCR5 as well as dual tropic viruses. These compounds showed inhibition of HIV entry at early steps in viral infection since the compounds were inactive if added post viral entry. Temperature-arrested state experiments showed that the compounds act at the level of virus attachment to the cells likely at a pre-CD4 engagement step. These compounds also showed inhibition of VSV glycoprotein-pseudotyped virus. The results presented here show that the glycoside derivatives of GalCer with simple side chains may serve as a novel class of small molecule HIV-1 entry inhibitors that would be active against a number of HIV isolates as well as other enveloped viruses.

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1. Introduction

HIV gains entry into cells via binding of Env glycoprotein to CD4 and a chemokine receptor (CXCR4/CCR5). Besides the use of these well-defined receptor and coreceptor other cofactors involved in HIV-1 entry have been suggested (Rawat et al., 2006). A variety of glycosphingolipids (GSL) have been shown to regulate Env-mediated fusion including galactosyl ceramide (GalCer) and a monoganglioside GM3 (Fantini et al., 2000; Hammache et al., 1998). More specifically GalCer has been shown to be a cofactor in HIV Env binding and infection of $CD4^+$ cells (Fantini et al., 1997).

The binding of HIV gp120 to cell surface expressed GalCer maps to the V3 loop region (Fantini et al., 1997; Bhat et al., 1993). The V3 loop of HIV, though highly variable, has a few conserved basic residues. This region has therefore been shown to bind a variety of anionic compounds such as polysulfonated albumin (Kuipers et al., 1996), suramin (Yahi et al., 1994) and heparin sulfate (Rider et al., 1994). A number of soluble analogs of GalCer as well as other sphingolipids have been shown to inhibit HIV infection (Mahfoud et al., 2002; Villard et al., 2002; Lund et al., 2006).

With a view towards new anti HIV agents we have previously examined the gp120 binding of both a *C*-glycoside (LAA-4) and an aza-*C*-glycoside (LAA-5), analogs of GalCer in which one or other of the acetal oxygens is replaced with a methylene or amino group and the ceramide residue substituted with a simple hydrocarbon chain (Augustin et al., 2006). The binding of both LAA-4 and LAA-5 was found to be similar to that of GalCer (Tanahashi et al., 1997). In the present study we have widened the range of structures to include analogs with *O*-glycoside residues and branched-chain ceramide substitutes and examined their binding to a synthetic peptide

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corresponding to the glycolipid binding domain of the V3 loop of HIV-1 gp120. The inhibition of HIV-1 Env glycoprotein-mediated fusion and infection was also investigated. Binding to the HIV-1 peptide was found to vary with both the structure of the glycoside or pseudoglycoside linkage and the ceramide replacement, and selected analogs showed a potent inhibition of both HIV Env glycoprotein-mediated cell-to-cell fusion as well as viral infection. Analysis of intermediate steps in the fusion process revealed that the inhibition is at the level of viral entry and is independent of coreceptor use by the virus.

2. Material and methods

2.1. C-glycoside analogs of GalCer

The syntheses of *O*-glycosides LAA-3 (Tanahashi et al., 1997) and LAA-6, *C*-glycoside LAA-4 and aza-*C*-glycoside LAA-5 (Augustin et al., 2006) have been previously described (Nehete et al., 1993). The new analogs LAA-1 and LAA-2 were prepared by modification of these procedures.

2.1.1. Physical data for LAA-1 and LAA-2

LAA-1. R_f = 0.6 (MeOH/$CHCl_3$ 15:85); ^{1}H NMR (6/1 $CDCl_3$/CD_3OD, 500 MHz) δ 3.95 (bs, 1H), 3.8 (J = 5.8, 11.6 Hz, 1H), 3.75 (J = 5.4, 11.6 Hz, 1H), 3.45 (m, 3H), 3.05 (t, J = 8.4 Hz, 1H), 2.2 (bs, 2H), 1.8 (m, 2H), 1.05–1.5 (m, 53H), 0.85 (t, J = 7.2 Hz, 6H). ^{13}C NMR (6/1 $CDCl_3$/CD_3OD, 125 MHz) δ 14.0, 22.6, 26.7, 26.8, 29.0, 29.3, 29.5, 29.6, 29.7, 30.0, 30.1, 30.2, 31.4, 31.9, 33.5, 33.6, 37.6, 62.2, 69.8, 71.9, 75.3, 77.6, 80.5. FABHRMS: calcd for $C_{37}H_74O_5Na$ 621.5434, found 621.5434.

LAA-2. R_f = 0.5 (MeOH/$CHCl_3$ 15:85); ^{1}H NMR (6/1 $CDCl_3$/CD_3OD, 500 MHz) δ 3.86 (bs, 1H), 3.6 (bd, 1H), 3.3 (m, 3H), 2.7 (t, J = 5.7 Hz, 1H), 2.4(dt, J = 7.9, 2.4 Hz, 1H), 1.8 (m, 1H), 1.05–1.3 (m, 56H), 0.9 (t, J = 7.0 Hz, 6H). ^{13}C NMR (6/1 $CDCl_3$/CD_3OD, 125 MHz) δ 13.9, 22.6, 26.6, 26.7, 29.0, 29.3, 29.6, 29.7, 30.1, 31.8, 33.4, 33.6, 37.8, 62.8, 58.9, 60.0, 69.9, 73.0, 76.2. ESI HRMS: calcd for $C_{37}H_{76}NO_4$ (M+H) 598.5774, found 598.5750.

2.2. Cells and reagent

HL2/3 is a HeLa cell derived cell line that expresses HIV-1 HXB2 Env Rev and Tat proteins. It is commonly used in fusion assays as Env expressing cells to induce fusion with receptor and coreceptor expressing cells. The presence of tat allows for gene reporter assays based on tat dependent luciferase expression in target cell lines. TZM cells are also HeLa derived cells that express HIV receptor CD4 and coreceptor CXCR4 and CCR5. The cells express luciferase in the presence of HIV tat. These cells are commonly used as target cells for HIV infection and cell-to-cell fusion assays with luciferase gene reporter as read out. HL2/3 and TZM cells (NIH AIDS research and reference reagent program) were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% FBS and penicillin and streptomycin (5000 U/ml). Fusion inhibitors C34 and AMD3100 were provided by NIH AIDS research and reference reagent program. Anti CD4 antibody Leu3A was obtained from BD Biosciences. The synthetic V3 loop peptide, RIQRGPGRAFVTIGK was obtained as previously described (Garmy et al., 2005).

2.3. Surface pressure measurements

The surface pressure was measured with a fully automated microtensiometer (μTROUGH SX; Kibron, Inc., Helsinki, Finland). The apparatus allowed the real-time recording of the kinetics of interaction of a soluble ligand with the monomolecular film using a set of specially designed Teflon troughs. All experiments were carried out in a controlled atmosphere at 20 ± 1 °C. Monomolecular films of LAA 1–6 were spread on pure water subphases (volume of 800 μl) from hexane–chloroform–ethanol solution as described previously (Augustin et al., 2006; Garmy et al., 2005). After spreading of the film, 5 min was allowed for solvent evaporation. To measure the interaction of the V3 peptide with GalCer and its analogs, the peptide was injected in the subphase with a 10 μl Hamilton syringe to a concentration of 10 μM, and pressure increases were recorded until reaching a stable value ($\Delta\pi$). The experiment was repeated at different values of the initial surface pressure (π_i) of the monolayer. The data were analyzed with the Filmware 2.5 program (Kibron, Inc.). The results are expressed as the variations of $\Delta\pi$ as a function of π_i for compounds LAA 1–6. The accuracy of the system under the experimental conditions was ±0.25 mN/m for surface pressure.

2.4. Determination of critical micelle concentrations

Stock solutions of glycolipids were prepared in hexane:chloroform:ethanol (11:5:4, v/v/v) and injected in water with a Hamilton microsyringe (dilution 1:1000). The surface tension was continuously recorded with the Kibron microtensiometer. Below the CMC, a drop in surface tension was recorded after each glycolipid injection. Increasing the concentration of the added glycolipid resulted in a linear decrease in surface tension. The CMC was determined as the concentration of glycolipid which did not induce any further decrease in surface tension.

2.5. Virus stock preparation

Plasmids containing infectious molecular clone of X4 tropic NL4-3 and dual tropic 89.6 were obtained from NIH AIDS research and reference reagent program. Molecular clone of R5 virus NLAD8 was a kind gift from Eric Freed (NCI Frederick). Virus stocks were prepared by 293T transfection using infectious molecular clones and Ex Gen 500 transfection reagent (Fermentas, Glen Burnie, MD) as per the manufacturer's protocol. Virus supernatant was collected 48 h post transfection, cleared of cellular components by centrifugation, aliquoted and stored at −70 °C. All stocks were titrated on TZM cells prior to use. VSV pseudotyped virus were prepared by co transfection of 293T cells with pNLLuc and VSVG. Supernatants were collected and titrated in TZM cells before use.

2.6. Cell-to-cell fusion assay

HL2-3 cells expressing HIV-1 Env glycoprotein as well as Tat protein from HXB2 strain were seeded in 96-well plates at 2×10^4 cells/well. TZM cells expressing CD4 and CXCR4/CCR5 as well as Tat dependent luciferase reporter gene were added at the same amount. The cells were cocultured for 6 h following which fusion was determined as luciferase activity measured by BriteLite luciferase substrate (PerkinElmer, Waltham, MS).

2.7. Virus infection assay

TZM cells were seeded in 96-well plates at 2×10^4 cells per well and allowed to adhere overnight. The subsequent day cells were infected with different viruses in the presence of 20 μg/ml DEAE-dextran. Test compounds at various concentrations in DMSO were

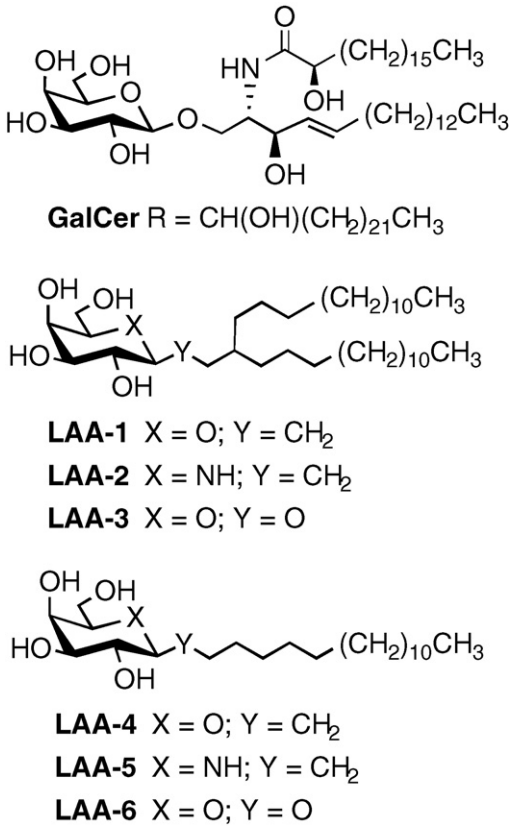

Fig. 1. Structures of GalCer and synthetic analogs LAA 1–6. LAA 1–6 were prepared following published procedures.

added at the time of virus addition. Twenty-four hours post infection the luciferase activity was measured using Brite Lite Luciferase substrate.

2.8. Temperature arrested state induction

TZM cells were incubated with NL4-3 virus in the presence of 20 μg/ml DEAE-dextran. Temperature-arrested state (TAS) was induced by incubation of cells at 23 °C for 2 h (Mkrtchyan et al., 2005). Various inhibitors were added either prior to or post 2 h TAS incubation. The plates were subsequently transferred to 37 °C and incubated for an additional 24 h. Finally the virus infection was determined as luciferase activity.

2.9. Cytotoxicity assay

Cytotoxicity was determined on TZM cells by incubating with serial dilutions of the test compounds for 24 h. Cell viability was determined using Cell titer Blue assay (Promega Corp. Madison, WI) as per the manufacturer's instructions.

3. Results

3.1. Structure and binding of glycoside analogs of GalCer

Our interest has been in GalCer analogs that are amenable to development as therapeutic agents. In this context we have synthesized hydrolytically stable analogs with simple hydrocarbon chains as replacements for the ceramide residue (Fig. 1). Accordingly, the gp120 binding of *C*-glycoside LAA-4 and aza-*C*-glycoside LAA-5 with a linear hydrocarbon chain as a ceramide substitute were previously examined in a monolayer assay. The relative binding was determined by measuring the change in surface pressure of the glycolipid monolayer, on exposure to an aqueous solution of recombinant gp120 (Augustin et al., 2006). The critical pressure of insertion was in the range of 24 mN/m for both GalCer and the *C*-glycoside LAA-4, and 28.5 mN/m for the aza-*C*-glycoside LAA-5. This data suggests that gp120 exhibits similar binding to monolayers of GalCer and LAA-4, and noticeably higher affinity for the monolayer formed from LAA-5 (Marsh, 1996). In order to develop a wider structure activity relationship, we extended this study to LAA-6, the *O*-glycoside corresponding to LAA-4 and LAA-5, and the series of *C*-, aza-*C*- and *O*-glycosides with a branched chain hydrocarbon as the ceramide substitute (Fig. 2).

A synthetic V3 loop peptide, RIQRGPGRAFVTIGK, corresponding to the R15K peptide, from gp120 of HIV-1 IIIb isolate was used, instead of the recombinant gp120 molecule, for binding studies. This synthetic peptide was originally designed as an inhibitor of HIV-1 infection in vitro (Nehete et al., 1993) and was later shown to bind to monomolecular films of GalCer, Gb3 and GM3, and therefore, could be used as a model for the glycolipid binding domain of gp120 (Nehete et al., 2002). To validate this hypothesis and also to allow for more accurate comparison of the data, the binding of LAA-4 and LAA-5 was re-evaluated against the synthetic peptide. Indeed, the values for the critical pressure of insertion for GalCer, LAA-4 and LAA-5 with respect to the synthetic peptide (ca. 22, 22, and 30 mN/m respectively), were very close to those determined for the binding with gp120. GlcCer showed no insertion at all, regardless of the initial pressure of the film, and was used as a negative control.

The trend that appears to emerge for the complete set of analogs LAA 1–6 with the V3 peptide is that *C*-glycosides exhibit similar binding as GalCer whereas the interaction of the aza-*C* and *O*-glycosides is significantly stronger (Fig. 2). Generally, independent of the structure of the glycoside linkage, the linear chain ceramide analogs showed a sharper increase in surface pressure with decreasing initial pressure, compared with the branched chain

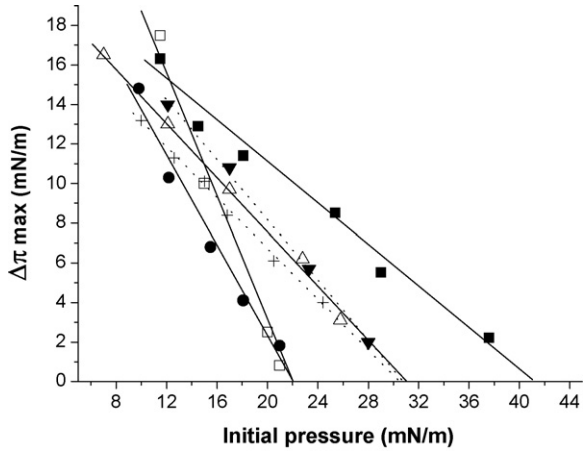

Fig. 2. Effect of HIV-1 gp120 synthetic peptide on the surface pressure of synthetic glycolipid monolayers. Each analog was spread on the surface of a water subphase at various values of the initial surface pressure (range 9.8–37.6 mN/m). A stable monomolecular film was formed after evaporation of the solvent. The synthetic HIV-1 gp120 peptide was then added in the subphase at a final concentration of 10 μM. The maximal surface pressure increase ($\Delta\pi_{max}$) was determined after 2–3 h of incubation, i.e. when the equilibrium was reached. A linear fit allowed to determine the critical pressure of insertion as the theortical value of the initial pressure giving a null $\Delta\pi_{max}$. The following symbols were used: LAA-1, solid line, ●; LAA-2, solid line, ■; LAA-3, solid line, △; LAA-4, solid line, □; LAA-5, dot line, +; LAA-6, dot line, ▼. The critical pressure of insertion of authentic GalCer was determined to be 22 mN/m (data not shown).

compounds. However, the effect of ceramide modification on the critical pressure of insertion varied with the structure of the glycoside linkage. For the *O*- and *C*-glycosides, the critical pressure of insertion was relatively unchanged, but LAA-2, the aza-*C*-glycoside with the branched chain ceramide mimic was markedly more active than its congener with the linear chain (ca. 41 vs 30 mN/m).

CMC measurements for the straight chain analogs LAA-4, LAA-5 and LAA-6 in water were performed in order to determine the physical state of the glycolipids at the concentrations used in the cellular assays (*vide infra*). The data obtained in three separate experiments were: LAA-4 (1.9, 1.8, and 1.9 μM); LAA-5 (6.7, 6.5, and 6.5 μM); LAA-6 (4.5, 5.2, and 5.0 μM). When these experiments were extended to the branched chain *O*-glycoside LAA-3, a regular decrease in surface tension from 72.8 mN/m (pure water) to 0 mN/m (glycolipid film collapse) in the concentration range of 0.25–5.8 μM, was observed. A similar observation was made when the CMC determination of natural GalCer was attempted. One interpretation of the results observed for LAA-3 and GalCer is that these analogs form precipitates rather than micelles above a critical concentration.

3.2. Glycoside analogs of GalCer inhibit cell-to-cell fusion mediated by HIV-1 Env glycoprotein

To determine whether the gp120 binding compounds from our initial screen had an effect on cell-to-cell fusion, a simple gene reporter based fusion assay was conducted. HL2/3 cells expressing HIV-1 HXB2 Env along with Tat protein were cocultured with TZM target cells expressing CD4 and coreceptors (CXCR4 and CCR5) as well as a Tat dependent luciferase reporter gene. Fusion was determined by measuring luciferase activity 6 h post coculture of HL2/3 and TZM cells at 37 °C. Our initial experiments showed that compounds LAA-4, LAA-5 and LAA-6 had significant inhibitory activity (Fig. 3A) at 100 μM concentration. Compounds LAA-1, LAA-2 and LAA-3 did not show any significant activity. Since the CMCs for LAA-4, LAA-5 and LAA-6 were found to be in the 5 μM range, these analogs are present as micelles at the active concentrations. As far as the data for LAA-3 is representative of the branched chain analogs, the latter apparently do not form micelles and exist as precipitates at the concentrations required for significant activity. This solubility behavior is not surprising given the expectedly greater hydrophobicity of the branched chain analogs. Further analysis using LAA-4, LAA-5 and LAA-6 showed a dose-dependent inhibition of HIV cell fusion (Fig. 3B).

3.3. Glycoside analogs of GalCer inhibit HIV infection in a coreceptor use-independent fashion

Next, we asked whether these glycoside analogs inhibit infection by HIV particles and whether this inhibition was coreceptor dependent. To address this issue, TZM cells that express both CXCR4 and CCR5 were infected with either an X4 tropic (NL4-3), R5 (NLAD8) or the dual tropic (89.6) virus. As seen in Fig. 4, LAA-4, -5 and -6 inhibited the virus infection in a dose-dependent manner, suggesting that the inhibition was not dependent on coreceptor use of the viral envelope. Although LAA-5 appeared to be the most potent to inhibit viral infection (Fig. 4), an *in vitro* toxicity assay showed LAA-5 to have significant toxicity with a TC_{50} of around 24 μM (Fig. 5, Table 1). Although the inhibitory activity of LAA-5 (IC_{50} 2–3 μM) was most likely not due to cytotoxicity (TC_{50} 24 μM), this compound was not used in subsequent experiments. Since, LAA-4 and LAA-6 showed inhibition of virus infection with an IC_{50} in the range of 10–40 μM with no significant toxicity seen up to 400 μM (Table 1), these analogs were further investigated.

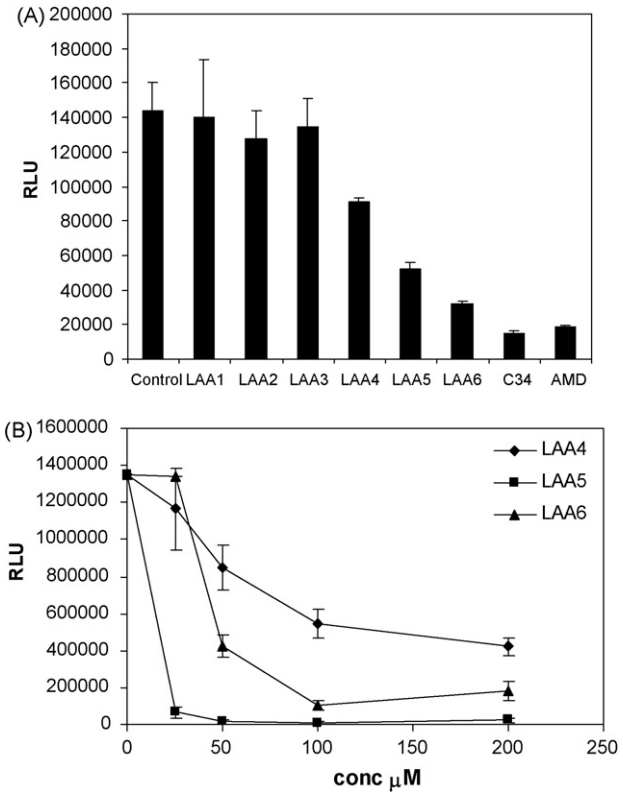

Fig. 3. GalCer analogs inhibit cell-to-cell fusion mediated by HIV-1 Env. (A) HL2/3 cells expressing HIV-1 Env were cocultured with TZM cells in the presence or absence of GalCer analogs at 100 μM. Fusion was determined 6 h post coculture by luciferase gene reporter activity. (B) Cell-to-cell fusion was conducted in the presence of varying concentrations of analogs LAA-4, -5 and -6. Fusion was determined as above. Data are mean ± S.D. of triplicate observations.

3.4. Glycoside analogs of GalCer inhibit infection at and early entry stage of infection

To determine whether the inhibition of HIV infection by the glycoside analogs was at the level of viral entry, we performed a wash-out experiment whereby the compounds were added either prior to incubation of virus (pre infection) or after washing the virus post incubation for 4 h (post infection) with target cells at 37 °C. As seen in Fig. 6, addition of compounds prior to infection followed by washing still showed significant activity. However addition of compounds 4 h post infection failed to inhibit the virus infection. Similar results were obtained with gp41 fusion inhibitor C34, CXCR4 antagonist AMD3100 and anti-CD4 antibody Leu3A, all known to inhibit HIV entry. This suggests that the inhibitory activity of the compounds was at the level of HIV Env-mediated fusion. This is consistent with the binding activity of the compounds with gp120 and inhibition of cell-to-cell fusion.

3.5. Glycoside analogs of GalCer inhibit infection at a pre-CD4 binding step

To further characterize the mechanism of action for these compounds, a temperature-arrested state experiment was conducted. HIV Env-mediated fusion is quite complex and involves several intermediate steps and conformational changes before the fusion reaction is completed (Gallo et al., 2003). Binding of HIV gp120 to CD4 induces conformational changes in gp120 that result in exposure of the coreceptor (CXCR4/CCR5) binding site. Once gp120 binds to the coreceptor, the HIV-1 gp41 six helix bundle forms resulting

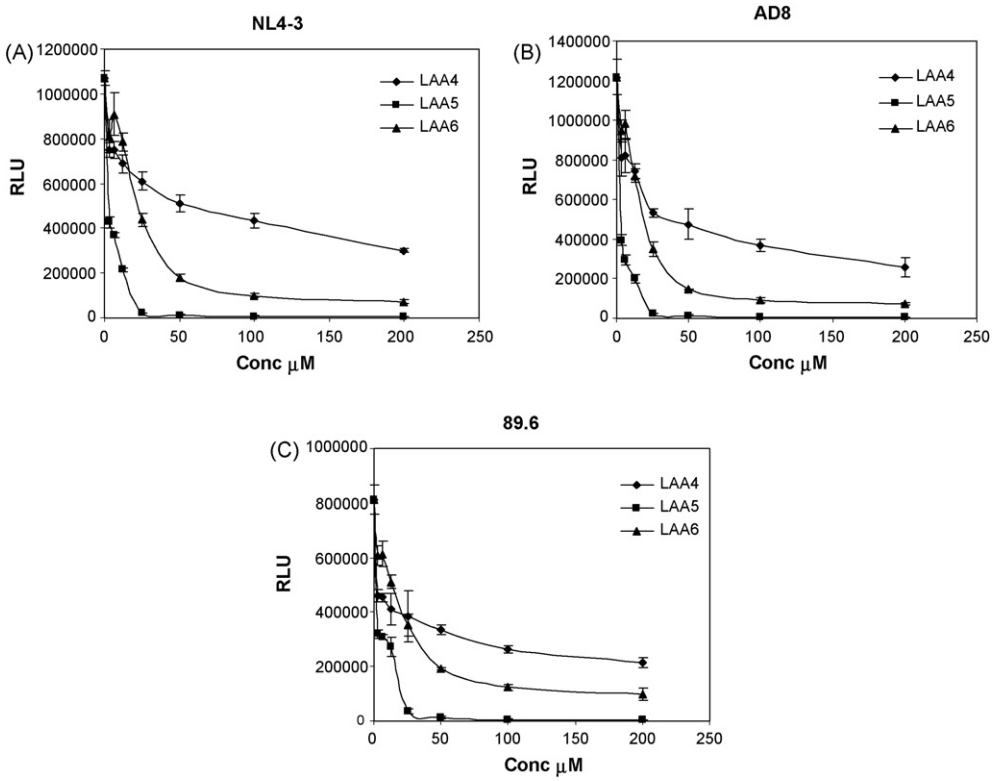

Fig. 4. Glycoside analogs of GalCer inhibit virus infection in a coreceptor independent manner. TZM cells were infected with either X4 tropic NL4-3 (A) or R5 tropic NLAD8 (B) or dual tropic 89.6 (C) virus in the presence of varying concentrations of the compounds. Virus infection was monitored 24 h post infection by luciferase gene reporter activity. Data are mean ± S.D. of triplicate observations.

in the fusion of viral and cellular membranes. Incubating the virus with cells at lower than optimal temperature for fusion can arrest the fusion process at the TAS step that involves binding of gp120 to CD4 and to variable levels the coreceptor (CXCR4/CCR5) in the absence of fusion (Mkrtchyan et al., 2005). We used the TAS model to study the step at which the glycoside analogs inhibit HIV fusion. As seen in Fig. 7, the compounds LAA-4 and -6 lost significant activity during the 2 h TAS. This was followed by loss of Leu3A activity, indicating CD4 engagement and to some degree loss of AMD3100 activity, suggesting CXCR4 recruitment. However, C34 did not loose any activity, suggesting that gp41 structural changes do not occur during TAS, which is consistent with previous reports (Mkrtchyan et al., 2005). The loss of activity was more significant for LAA-4 and LAA-6 than for Leu3A suggesting that the compounds inhibit a pre-CD4 step likely involving HIV gp120 binding to cell surface glycolipids or glycoproteins.

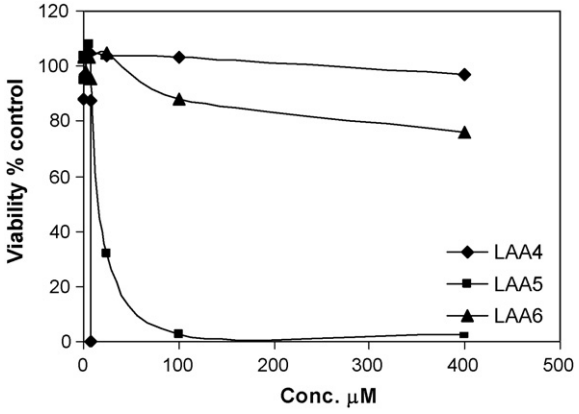

Fig. 5. Toxicity of glycoside analogs of GalCer. TZM cells were incubated with various concentrations of the compounds for 24 h. Viability was determined by Cell Titer Blue assay and expressed as % control (no treatment).

3.6. Glycoside analogs of GalCer inhibit infection mediated by VSVG protein pseudotyped virus

The inhibition of HIV-1 viral entry by the glycoside analogs at a pre-CD4 step suggested that the compounds may not be specific to HIV and also inhibit other viruses. We tested this hypothesis by utilizing HIV particles pseudotyped with vesicular stomatitis virus G (VSVG) protein. Infection of TZM cells with VSVG pseudotyped HIV showed inhibition of infection by LAA-4 and LAA-6 similar to HIV Env containing particles (Fig. 8A). Interestingly, while HIV specific inhibitors Leu3A, C34 and AMD failed to inhibit VSV pseudotype

Table 1
IC_{50} (μM) and toxicity of *C*-glycoside analogs

	Coreceptor	LAA-4	LAA-5	LAA-6
NL4-3	X4	42.18 ± 3.78	3.0 ± 0.70	32.43 ± 12.78
NLAD8	R5	29.69 ± 13.62	2.82 ± 1.71	17.62 ± 2.89
89.6	X4/R5	9.72 ± 3.02	3.69 ± 1.44	12.79 ± 8.76
Toxicity (TC_{50})		> 400	24.0	> 400

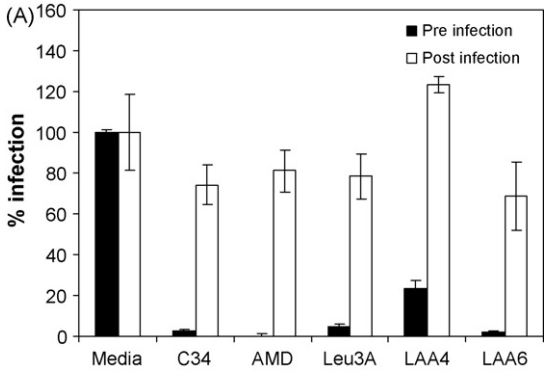

Fig. 6. Glycoside analogs of GalCer inhibit early step in virus infection. TZM cells were infected with NL4-3 virus for 4 h followed by washing twice with PBS. The compound at 100 μM was either added before incubation with the virus (pre infection) or after 4 h and washing (post infection). Control inhibitors C34 and AMD3100 were used at 2 μM and anti-CD4 antibody Leu3A at 1 μg/ml. The cells were cultured for 24 h following which infection was monitored by luciferase assay.

infection; other non-specific inhibitors of HIV like cyanovirin and suramin also inhibited infection of both VSVG pseudotypes and HIV-1. Suramin has been shown to inhibit HIV infection via binding to V3 loop similar to GalCer analogs, while cyanovirin is a high mannose binding protein that inhibits HIV as well as a variety of viruses including influenza and hepatitis-C, etc. These findings underscore the general antiviral activity of these compounds and underscore the common mechanism involved in infection by enveloped viruses.

4. Discussion

The need for new therapeutic agents to fight the HIV pandemic is still actual. With the approval and successful use of enfuvirtide, the first fusion inhibitor targeting HIV entry, the field of HIV entry inhibitors is gaining increased attention with several new compounds targeting HIV coreceptor CCR5 in clinical trials (Esté and Telenti, 2007). Other inhibitors of HIV entry that bind carbohydrate moieties on env glycoprotein such as cyanovirin (Dey et al., 2000) and retrocyclin (Wang et al., 2003) are also being investigated.

The HIV fusion reaction proceeds via binding of the gp120 subunit to receptor (CD4) and coreceptor (CXCR4/CCR5). This is

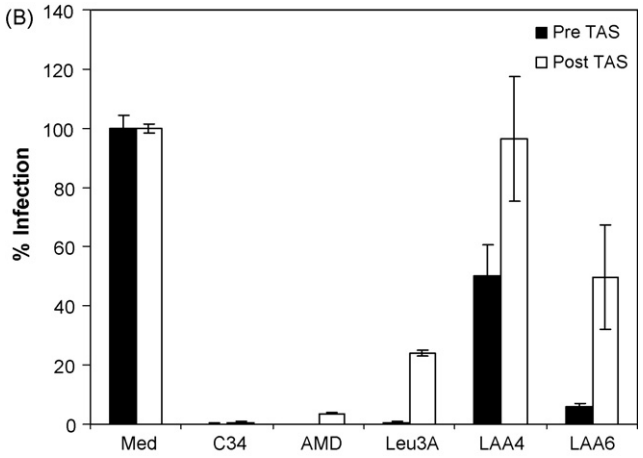

Fig. 7. Glycoside analogs of GalCer loose activity after 2 h at TAS. TZM cells were infected with NL4-3 virus at 23 °C to induce TAS. Different compounds were added either at the time of virus addition or 2 h post TAS induction. The cells were incubated for another 24 h before virus infection was monitored by luciferase activity.

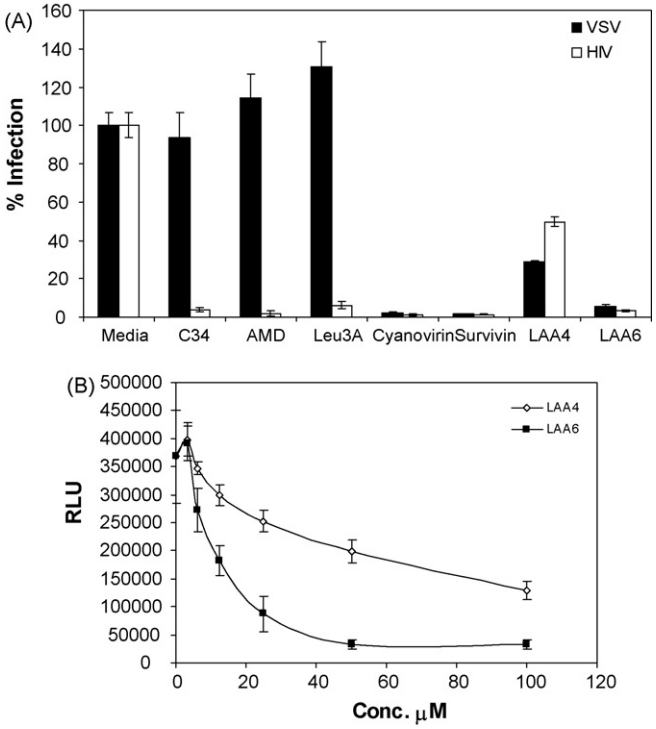

Fig. 8. Glycoside analogs of GalCer inhibit infection of VSVG pseudotyped virus: (A) TZM cells were infected with either VSVG-pseudotyped virus or HIV-1 NL4-3 in the presence or glycoside analogs LAA-4 and LLA-6 at 100 μM. Control inhibitors cyanovirin (10 μg/ml), suramin (40 μM), C34 (2 μM), AMD3100 (2 μM), and Leu3A (1 μg/ml) were added prior to infection with the virus. (B) VSVG-pseudotyped virus infection was inhibited by LAA-4 and LAA-6 at different concentrations.

followed by conformational changes in the fusion inducing gp41 subunit that mediates the fusion reaction (Gallo et al., 2003). The involvement of these steps is well documented and targeted by drugs like the gp41 peptide enfuvirtide and coreceptor antagonists like AMD3100 and TAK779. However, other cofactors like membrane glycosphingolipids (GSLs) are known to pay a supporting role in HIV env-mediated fusion reaction (Rawat et al., 2005). Indeed, specific glycolipids such as Gb3 and GM3 have been reported to act as cofactors for entry into $CD4^+$ T cells (Fantini et al., 2000). The binding of the V3 loop of gp120 to all of these glycolipids involves a common biochemical mechanism, i.e. a stacking interaction between the Phe residue in the GPGRAF core motif and the hydrophobic face of a terminal galactosyl ring in the glycolipid (Fantini, 2003). This explains why synthetic analogs of GalCer could inhibit HIV-1 infection in $CD4^+$ lymphocytes expressing Gb3 (Fantini et al., 2000). Based on this premise, we had earlier evaluated the gp120 binding of analogs of GalCer with simple ceramide replacements. In the current study we have examined the interaction of these and related structures with a synthetic peptide corresponding to the glycolipid binding domain of the V3 loop of HIV-1 gp120, and tested their efficiency to inhibit HIV Env-mediated fusion and viral entry.

The very similar binding profiles of LAA-4 and LAA-5 with respect to whole gp120 (previous study) (Augustin et al., 2006), and the synthetic V3 peptide (current study), is consistent with the notion that these glycolipids interact specifically with the consensus sequence on gp120. As far as the relative binding of the different analogs to the V3 peptide is concerned, the generally higher binding of the *O*- and aza-*C*-glycosides compared to the *C*-glycosides suggests that the heteroatoms of the acetal or pseudo-acetal moiety might be involved in polar interactions with the receptor, or

may be biasing a favored orientation of the sugar in relation to the hydrophobic chain (Taïeb et al., 2004). The sharper increase in surface pressure with decreasing initial pressure that was observed for the linear chain *O*- and *C*-glycoside analogs could be an indication that the branched chain congeners arrange into more densely packed glycolipid monolayers such that insertion of the peptide into the corresponding monolayers occurs less readily compared to insertion into the monolayers formed from the linear analogs. The markedly higher binding of the aza-*C*- compared with the *C*-glycosides might be connected to changes in monolayer packing and/or additional receptor interactions that result from the replacement of the ring oxygen by an amino group (Augustin et al., 2006).

While it appears likely that the difference in activity of the branched chain analogs in the peptide binding vs. cellular assays, is due to the poor water solubility of these compounds, any attempt to connect the activity of the linear chain analogs LAA-4, -5 and -6 to a similar molecular recognition process should be approached with caution. Such an analysis is complicated because of the absence of any clear structural information on the glycolipid receptor involved in the two assays, i.e. a monolayer vs. a monomer, micelle or a micelle aggregate. In addition, the possibility that receptors other than the V3 loop of gp120 may have an impact on the cellular effects should be considered. Not withstanding the molecular basis for interaction with gp120, the positive correlation between binding to the V3 peptide and inhibition of both cell-to-cell fusion and virus infection exhibited for LAA-4, -5 and -6 is consistent with the idea that antagonists to the glycolipid domain of the V3 loop may disrupt the attachment of HIV-1 to host cells.

The inhibition of both cell-to-cell fusion and virus infection suggests an inhibition of env function. We have also shown that the inhibition of viral entry was coreceptor-independent. The coreceptor-independent inhibition of viral entry mediated by LAA-4 and LAA-6 suggests that it is an early event common to a variety of HIV strains. This is further supported by the fact that inhibitory activity of the compounds is lost if added post viral entry. Our experiments using the TAS showed that these compounds rapidly loose activity within 2 h of TAS induction. The loss of activity was faster than that for CD4 engagement suggesting a pre-CD4 binding step may be involved. Hence, we speculate that these glycoside analogs of GalCer bind to the virus and inhibit its adsorption on the target membrane at a pre-CD4 binding step. Although one of the compounds (LAA-5) was significantly toxic to the cells, LAA-4 and LAA-6 showed inhibition at non-toxic concentrations. This holds promise for further development of these compounds into potential antiviral agents.

Not surprising was the fact that these compounds also inhibited VSVG-pseudotyped virus infection. The mechanism of action of these compounds is likely to be similar to other antiviral agents like suramin and cyanovirin which we have shown here for the first time also inhibit VSVG pseudotype infection. This not only supports the broad antiviral activity of these compounds but also underscores the notion that interaction with cell surface carbohydrates is a common feature of a variety of enveloped viruses.

Although the compounds may not have HIV specific activity, the general antiviral activity makes them neverthelss useful. Compound LAA-5 which had the highest activity was also significantly toxic to the cells; however LAA-4 and LAA-6 showed inhibition at non toxic concentrations holding promise for further development of these compounds into potential antiviral agents. Similar antiviral agents like cyanovirin (Chang and Bewley, 2002; Barrientos et al., 2003) retrocyclin (Wang et al., 2003), and scytovirin (Xiong et al., 2006) are proteins; inhibitors used in this study are small molecules that are not subject to the problems of immunogenicity that plague protein therapeutics. Other soluble analogs of GalCer have similarly been shown to inhibit HIV infection (Fantini et al., 1997); however, our compounds are simpler versions of these early analogs with smaller side chains, better solubility and significantly enhanced activity. We provide a mechanistic approach to the antiviral activity of these compounds and hope to provide important leads for the further development of small-molecule inhibitors of HIV as well as other viruses.

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