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A synthetic globotriaosylceramide analogue inhibits HIV-1 infection *in vitro* by two mechanisms

Amanda L. Harrison · Martin L. Olsson · R. Brad Jones · Stephanie Ramkumar · Darinka Sakac · Beth Binnington · Stephen Henry · Clifford A. Lingwood · Donald R. Branch

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Abstract Previously, it was shown that the cell-membrane-expressed glycosphingolipid, globotriaosylceramide (Gb_3/P^k/CD77), protects against HIV-1 infection and may be a newly described natural resistance factor against HIV infection. We have now investigated the potential of a novel, water soluble, non-toxic and completely synthetic analogue of Gb_3/P^k (FSL-Gb_3) to inhibit HIV-1 infection *in vitro*. A uniquely designed analogue, FSL-Gb_3, of the natural Gb_3/P^k molecule was synthesized. HIV-1_{IIIB} (X4 virus) and HIV-$1_{Ba\text{-}L}$ (R5 virus) infection of PHA/interleukin-2-activated, peripheral blood mononuclear cells (PBMCs) and Jurkat T cells *in vitro* was assessed, as well as infection of U87.CD4.CCR5 by various clinical R5 tropic viruses after treatment with FSL-Gb_3. We monitored Gb_3, CD4 and CXCR4 expression by fluorescent antibody cell sorting and viral replication by $p24^{gag}$ ELISA. Total cellular Gb_3 was examined by glycosphingolipid extraction and thin layer chromatography. *In vivo* toxicity was monitored in mice by histological assessment of vital organs and lymphoid tissue. FSL-Gb_3 blocked X4 and R5 of both lab and clinical viral strains in activated PBMCs or the U87.CD4.CCR5 cell line with a 50% inhibitory concentration (IC_{50}) of approximately 200–250 μM. FACS and TLC overlay showed that FSL-Gb_3 can insert itself into cellular plasma membranes and that cellular membrane-absorbed FSL-Gb_3 is able to inhibit subsequent HIV-1 infection. There was no effect of FSL-Gb_3 on cell surface levels of CD4 or CXCR4. Thus, FSL-Gb_3 can inhibit HIV-1 by two mechanisms: direct inhibition of virus and inhibition of viral entry. Infusion of FSL-Gb_3 into laboratory mice at doses well in excess of theoretical therapeutic doses was tolerated with no untoward reactions. Our results demonstrate the potential utility of using a completely synthetic, water soluble globotriaosylceramide analogue, FSL-Gb_3, having low toxicity, for possible future use as a novel therapeutic approach for the systemic treatment of HIV/AIDS.

Keywords Glycosphingolipids · Globotriaosylceramide · Gb_3 · P^k blood group antigen · HIV · HIV infection

A. L. Harrison · D. Sakac · D. R. Branch (✉)
Canadian Blood Services Research and Development/Toronto General Research Institute,
67 College St.,
Toronto, Ontario M5G 2M1, Canada
e-mail: don.branch@utoronto.ca

A. L. Harrison · S. Ramkumar · C. A. Lingwood · D. R. Branch
Department of Laboratory Medicine and Pathobiology,
University of Toronto,
Toronto, Canada

A. L. Harrison · S. Ramkumar · B. Binnington · C. A. Lingwood
Hospital for Sick Children Research Institute,
Toronto, Canada

M. L. Olsson
Division of Hematology and Transfusion Medicine,
Department of Laboratory Medicine, Lund University,
Lund, Sweden

R. B. Jones
Department of Immunology, University of Toronto,
Toronto, Canada

S. Henry
Biotechnology Research Institute, AUT University,
Auckland, New Zealand

Introduction

Previously, studies have associated the histo-blood groups with pathogen-host interactions [1]. In particular, the

P/P1/P^k blood group antigens have defined pathogen interactions [1, 2]. Studies by our group using different experimental approaches have implicated the P^k blood group antigen (Fig. 1), also defined as the glycosphingolipid (GSL), globotriaosylceramide (Gb_3) and the CD77 marker for germinal centre B lymphocytes [3], as a resistance factor in HIV infection [4]. GSLs are an important part of the lipid raft microdomains of cellular membranes, which are a key component in the HIV infection cycle [5–7]. Indeed, HIV binds to several GSLs *in vitro*, including Gb_3 [8–10].

We have shown previously that a soluble Gb_3 mimic modified from natural globotriaosylceramide isolated from human kidney by our group, called adamantyl-Gb_3 [11], binds to the HIV envelope glycoprotein gp120 [10], and is a fusion inhibitor of HIV regardless of viral strain or resistance status in both HIV target cell lines and primary cells [12]. PBMCs from patients with Fabry disease, who accumulate Gb_3 cellularly due to a defective α-galactosidase A required for Gb_3 degradation are resistant to R5 HIV-1 infection [13]. Additionally, we have analyzed differences in Gb_3 expression caused by genetic polymorphisms in the genes governing the P^k and P blood group antigens [4]. Individuals with the p phenotype express no Gb_3 due to a defective 4-α-galactosyltransferase (Gb_3/P^k synthase) caused by mutation in the *A4GALT* gene [14–17]. In contrast, $P_1{}^k$ individuals accumulate Gb_3 due to a defective 3-β-*N*-acetylgalactosaminyltransferase (Gb_4/P synthase) caused by mutation in the *B3GALNT1* gene [17, 18]. We have shown that PBMCs from p persons are highly susceptible to both X4 and R5 HIV infection, while $P_1{}^k$ individuals, who accumulate Gb_3, resisted infection [4]. Also, Gb_3 synthase gene silencing using siRNAs results in increased HIV infection while introduction and overexpression of Gb_3 synthase in cells cause resistance to HIV infection [4]. In addition we examined the effect of pharmacological manipulation of Gb_3 levels on infection status. The chemical induction of Gb_3 accumulation in HIV-1 infectable cell lines results in resistance to infection, while pharmacological blocking of the Gb_3 synthetic pathways increases cellular susceptibility to HIV-1 infection [19]. These findings taken together strongly argue that globotriaosylceramide can play a major inhibitory role in HIV infection.

Here, we have analyzed a novel, totally synthetic, form of Gb_3, (Fig. 1) termed **F**unctional head-**S**pacer-**L**ipid tail (FSL)-Gb_3. This synthetic analogue of natural Gb_3 is uniquely formulated, having the same Gb_3/P^k trisaccharide Galα4Galβ4Glcβ-R recognition elements but with a unique dioleoyl-phosphatidyethanolamine lipid tail [20]. This

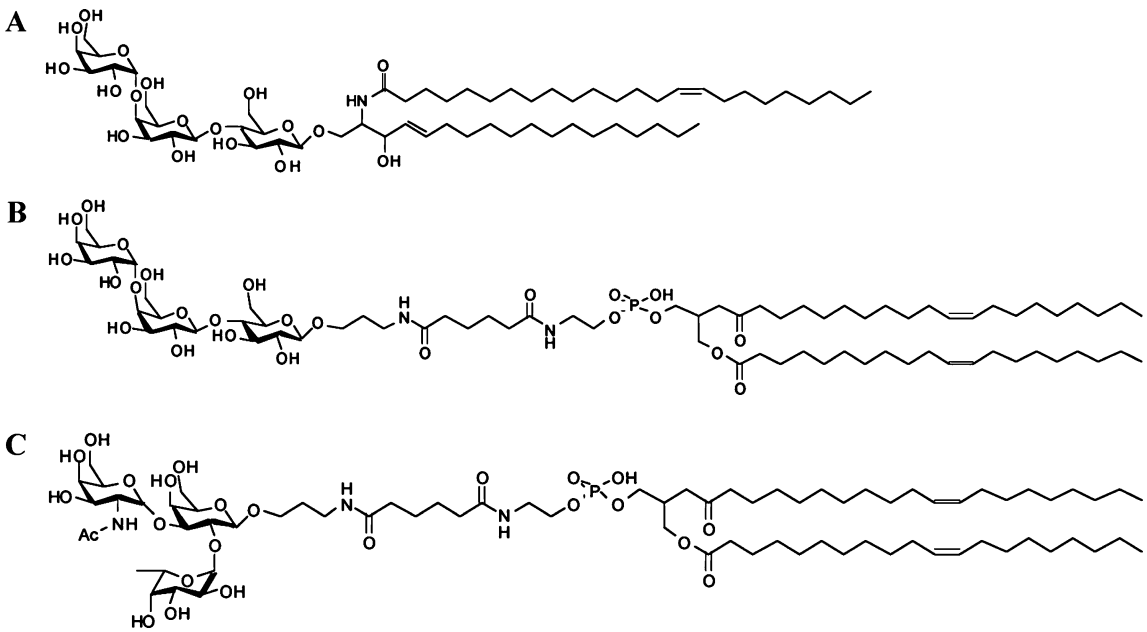

Fig. 1 Glycosphingolipid globotriaosylceramide vs FSL-Gb_3. Schematic diagram contrasting: (**a**) a natural Gb_3 glycosphinogolipid with the Galα4Galβ4Glcβ glycotope and a nervoic acid C24 nonsaturated cis fatty acid ceramine with (**b**) FSL-Gb_3 (Cat #352439) also with the Galα4Galβ4Glcβ epitope but conjugated via an $O(CH_2)_3NH$ spacer to an activated adipate derivative of dioleylphosphatidylethanolamine; and the control construct (**c**) FSL-A (Cat # 421604) which has the same spacer-lipid contruction as FSL-Gb_3 but instead has the blood group A GalNAcα3(Fucα2)Galβ trisaccharide glycotope as its functional head

synthetic Gb_3 analogue is completely water dispersible, has low toxicity, shows potent HIV-1 inhibitory properties *in vitro*, and great promise for its potential to inhibit systemic HIV-1 infection *in vivo*.

Materials and methods

Cells and chemicals

FSL-Gb_3 (Blood group P^k; GB3(GALa4GALb4GLCb)-SA1-L1, Cat No. 352439, MW 1438) and FSL-A (Blood group A; A(GALNa3[Fa2]GALb)-SA1-L1, Cat No. 421604, MW 1463) were supplied by KODE Biotech Materials Ltd (Auckland, New Zealand; kodebiotech.com). Adamantyl-Gb_3 was produced as described [11] from human kidney extracted Gb_3. Whole blood was obtained from healthy volunteers after informed consent under an approved protocol from the Canadian Blood Services Research Ethics Board (REB) committee. PBMCs were isolated using Ficoll-Paque (Amersham/Pharmacia, Baied 'Urfe, Quebec, Canada) density gradients from whole blood. PBMCs were activated for 3 days in 5 μg/ml phytohemagglutinin (Sigma-Aldrich, Oakville, Ontario, Canada) and IL-2 (Invitrogen, Burlington, Ontario, Canada, 100 U/ml). The Jurkat C non-adherent T-cell line was maintained in complete RPMI (RPMI-1640, 10% FBS, 10 μM gentamycin antibiotics (Invitrogen, Burlington, Ontario, Canada). The U87.CD4.CCR5 cell line (NIH/AIDS Research and Reference Reagent Programme (NIH/AIDS)) was maintained in complete DMEM supplemented with 15% FBS, 1 μg/mL puromycin, 300 μg/mL G418, and pen/strep (Invitrogen).

Viruses and *in vitro* infections

X4 HIV-1_{IIIB} and R5 HIV-$1_{Ba\text{-}L}$ were from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: $HTLV_{IIIB}$ = HIV-1_{IIIB} from Dr. Robert Gallo, HIV-$1_{Ba\text{-}L}$ from Dr. Suzanne Gartner. The R5 HIV-1 clinical strains 90SE_364, KNH1207, and KSM4030 were also obtained from NIH/AIDS as described [26]. Multiplicity of infection was determined as described [21], for HIV-1_{IIIB}, or calculated based on total $p24^{gag}$ levels measured by enzyme-linked immunosorbent assay (ELISA, ZeptoMetrix, Buffalo, NY) for HIV-$1_{Ba\text{-}L}$. For pre-incubation experiments, HIV-1_{IIIB} or HIV-$1_{Ba\text{-}L}$ or clinical virus was incubated for 1 h at 37°C with FSL-Gb_3 or FSL-A control over the concentration range 50–1000 μM prior to 1 h infection of 1×10^6 PBMCs or 5×10^5 U87.CD4.CCR5 adherent cells plated 24 h previously. Culture supernatants were taken 3 days after infection and every other day thereafter, up to day 12 for measurement of $p24^{gag}$ antigen production by ELISA. For cell pre-incubation experiments, 1×10^6 Jurkat C cells were incubated for 1 hr at 37°C with varying concentrations of FSL-Gb_3 or RPMI media as control and then infected for 1 hr with HIV-1_{IIIB} (m.o.i. 0.1). Cells were washed and cultured for up to 5 days.

Flow cytometry

To determine the effects of pre-incubation of FSL-Gb_3 on cell surface Gb_3 expression and/or cell surface receptor expression, 5×10^5 Jurkat C cells were incubated with varying concentrations of FSL-Gb_3 for 1 h at 37°C. Cells were then washed with PBS and pelleted in Eppendorf tubes for 5 min by centrifugation at 4000 rpm. Cells were resuspended in 100 μL 10% human serum plus FACS buffer (PBS, 2% FBS, 0.1% sodium azide, 5 mM EDTA) and incubated on ice for 20 min, then centrifuged again and resuspended in 100 μL FACS buffer. For Gb_3 staining, 1.5 μL of mouse anti-Gb_3 monoclonal antibody (clone BGR23, SeikagakuCorp., Tokyo, Japan) was added per tube, then incubated on ice for 30 min, washed and resuspended in 100 μL FACS buffer. Then 10 μL of the secondary antibody APC-labelled goat anti-mouse (10% solution; Invitrogen Molecular Probes) was added, followed by a 30 min incubation on ice and washing as above. If another conjugated antibody was used after this, the cells were blocked with 20 μL 10% mouse serum in FACS buffer for 20 min on ice. After washing and resuspending in 100 μL FACS buffer, 5 μL of the following conjugated antibodies were added: CXCR4-PE (Serotec Ltd., Oxford, UK), CD4-FITC (BD Biosciences, Mississauga, Ontario, Canada) and incubated on ice for 30 min. The cells were then washed, resuspended in 500 μL FACS buffer and transferred to FACS tubes through filter tops. Data was collected on a FACSCalibur cell cytometer (BD Biosciences, Mississauga, Ontario, Canada) and analyses were conducted using CellQuest software.

Verotoxin1 TLC overlay

At least 2×10^6 Jurkat C cells per treatment group were centrifuged and washed in PBS lacking $MgCl_2/CaCl_2$ and stored at −20°C to disrupt the cell pellet. GSLs were then extracted from Jurkat C cells by vigorously shaking cells overnight in chloroform/methanol (C:M, 2:1 v/v). The mixture was filtered through glass wool and the lipids collected were dried under nitrogen gas. Extracts were saponifed to isolate the glycolipid fraction by resuspending in 1 M NaOH in methanol for 1 h at 37°C. The mixture was

then neutralized with 1 M acetic acid to achieve a slightly basic pH. Volumes were adjusted to form a Folch partition in chloroform/methanol/water (C:M:W, 8:4:3 v/v) and centrifuged to separate the upper and lower phases. The upper phase was removed and discarded and then replaced with an equal volume of C:M:W, 1:47:48. After another round of centrifugation, the upper phase was discarded and the lower phase was dried as described. The dried extract was re-suspended in C:M, 2:1 to approximately 10^5 cells extract equivalent/20 µL and the aliquots were applied to the TLC plate and then pre-cleared by C:M, 98:2. GSLs were then separated by TLC (C:M:W, 65:25:4) and the GSL species were detected by verotoxin-1 (VT1) binding using TLC overlay [22]. For the VT1 overlay, the TLC plate was run in the appropriate solvent (C:M:W, 65:25:4) and then dried in the fume hood. The plate was blocked with 1% bovine gelatin and after incubation at 37°C, the plate was washed with 50 mM TBS at pH 7.4. The plate was incubated for 45 min at room temperature with purified VT1, diluted to a concentration of 1 µg/10 mM in TBS. After washing, the plate was incubated for 45 min with a monoclonal anti-verotoxin B subunit [22, 23] diluted 1/2000 and then with HRP-conjugated goat anti-mouse IgG (diluted 1/2000; Bio-Rad). The plate was developed for 10 min with 3 mg/mL solution of 4-chloro-1-naphthol (4-CN) in methanol freshly mixed with 5 volumes of TBS and 1/2000 dilution of 30% H_2O_2.

In vivo toxicity

FSL-Gb_3 was prepared in sterile saline (21.2 mg/mL or 15 mM). Following a protocol approved by the University of Auckland Animal Ethics Committee (approval #R629), 15 C57BL/6 mice were weighed and anesthetized with isoflurane gas and electronically tagged. FSL-Gb_3 suspension, 200 µl (3 mM) or 110 µl (1.5 mM) per mouse, was then injected into the subclavian vein of 6 female and 4 male mice. The plasma volume of these mice was estimated to be 0.9–1.0 ml. Two females were 1 week pregnant at the time of infusion and their litters were also monitored. Three female and 2 male mice were kept unused as controls. Mice and their health and behavior were monitored and recorded by a veterinarian at the following intervals: immediately post infusion, 24 h, 48 h, 1 week, one month, 2 months and 3 months. Mice were autopsied at a 3 month time-point, which was considered a reasonable time to observe lesions or complications in mice by our Veterinary experts, and histological investigations were conducted on their organs (liver, spleen, hepatic lymph nodes, lung, kidney). Serum was analyzed for the presence of antibody to Gb_3 by thin layer chromatography antibody overlay analysis against FSL-Gb_3 construct.

Statistics

Data is represented as mean±SEM, where the Student's *t* test was used to determine statistical significance. Data was considered statistically significant if $p<0.05$.

Results

FSL-Gb_3 inhibits X4 and R5 HIV-1 infection of Jurkat T-cells and PBMCs

First, we assessed the ability of FSL-Gb_3 to prevent infection of Jurkat C with HIV-1_{IIIB} compared to the adamantyl-Gb_3. FSL-Gb_3 inhibited HIV-1_{IIIB} infection (m.o.i. 0.1) reaching complete inhibition by 400–600 µM and an IC_{50} of approximately 200 µM, (Fig. 2b), which was nearly identical to the results shown for adamantyl-Gb_3 (ada-Gb_3)(Fig. 2a).

Next, we investigated the ability of FSL-Gb_3 to prevent infection of PBMCs by viral pre-incubation. For both X4 and R5 HIV-1 infection, PBMCs were activated for 3 days by treatment with PHA/IL-2. FSL-Gb_3 inhibited X4 HIV-1_{IIIB} (m.o.i., 0.3) reaching complete inhibition by 200–600 µM with an IC_{50} of approximately 100 µM (Fig. 2c). Treatment of R5 HIV-1_{Ba-L} with FSL-Gb_3 resulted in increasing inhibition of infection over the concentration range with near complete inhibition at 400 µM with an IC_{50} of approximately 300 µM (Fig. 2d).

When comparing the inhibitory effect of FSL-Gb_3 to the control molecule FSL-A at 200 µM using HIV-1_{Ba-L}, there was no difference between the FSL-A group and control, whereas a significant effect was observed between the control and FSL-Gb_3 treated group (Fig. 2e; $p<0.05$). PBMCs and Jurkat C were maintained in culture up to 7 days post-infection where inhibition at this time-point was still maintained as shown by $p24^{gag}$ ELISA.

FSL-Gb_3 inhibits HIV-1 R5 clinical isolate infection of U87.CD4.CCR5 cells

To further confirm the inhibitory activity of FSL-Gb_3, we investigated the ability of FSL-Gb_3 to prevent infection of various R5 tropic HIV-1 clinical isolates. FSL-Gb_3 used at a concentration of 500 µM was compared to FSL-A control. After 3 days of infection of susceptible U87 target cells, $p24^{gag}$ ELISA results indicated near complete inhibition of infection of the following HIV-1 isolates compared to control: 90SE_645 (Fig. 3a; $p=0.000102$), KNH1207 (Fig. 3b; $p=0.0265$) and KSM4030 (Fig. 3c; $p=0.161$ with variable control values of 740, 433 and 83 pg/mL $p24^{gag}$). Variability in the control group values resulted in a non-significant *p*-value for the KNH1207 virus; the low value of 83 pg/mL

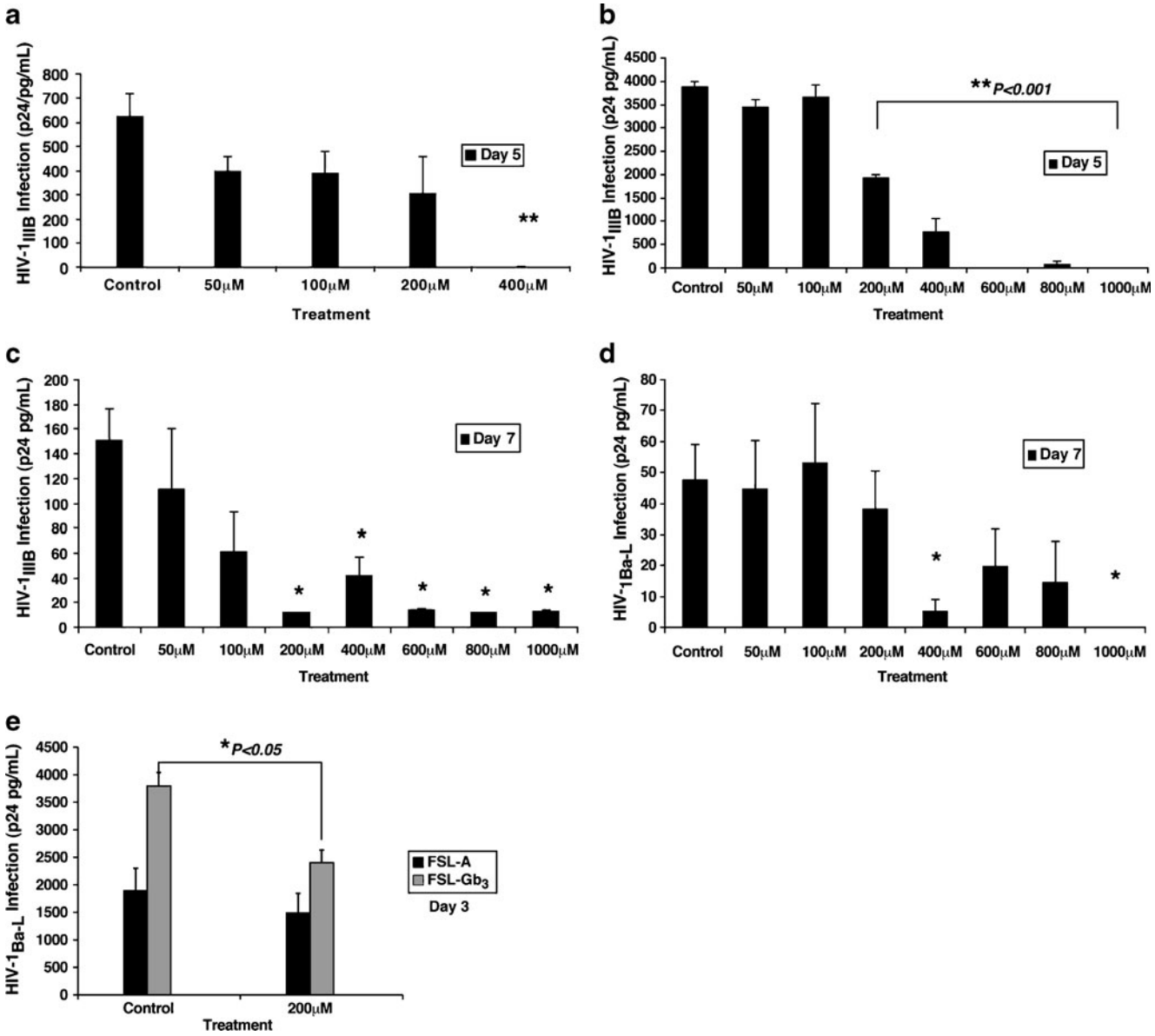

Fig. 2 FSL-Gb_3 inhibition of X4 HIV-1_{IIIB} and R5 HIV-$1_{Ba\text{-}L}$ infection of activated human peripheral blood mononuclear cells and Jurkat T-cells by viral pre-incubation. For Jurkat C T-cells, HIV-1_{IIIB} virus was pre-incubated for 1 h at 37°C with increasing doses of adamantly-Gb_3 (**a**) or FSL-Gb_3 (**b**) as compared to media control. PBMCs were isolated from the blood of healthy donors after informed consent and isolated via Ficoll-Paque density gradients. PBMCs were activated for 3 days prior to infection by (**c**) HIV-1_{IIIB} (m.o.i., 0.3) or (**d**) HIV-$1_{Ba\text{-}L}$ with PHA and IL-2 and were maintained in media containing IL-2 after infection. HIV-$1_{Ba\text{-}L}$ infection of PBMCs was compared after viral incubation with the control FSL-A compound and FSL-Gb_3 (**e**). $p24^{gag}$ ELISA was used to measure infection between days 5–7 post infection. One of three similar experiments were completed in triplicate or quadruplicate [$*P<0.05$ in comparison to media control]

is clearly an outlier but even with this value included, FSL-Gb_3 shows an inhibitory effect on the infection.

Direct application of FSL-Gb_3 to Jurkat T-cells results in 100% Gb_3 expression on cell surface and inhibition of HIV-1_{IIIB} infection

To further explore the inhibitory effect of FSL-Gb_3 and because of the specific chemistry of FSL-Gb_3 which should allow this molecule to insert into the plasma membrane of cells, we next investigated whether Jurkat C T-cells pre-incubated with 1 mM or 3 mM FSL-Gb_3 showed increased Gb_3 expression via FACS analysis, since cell incubation would most closely mimic the scenario in treatment of HIV. Untreated Jurkat C cells revealed no Gb_3 expression (Fig. 4a), which after 1 h exposure to FSL-Gb_3 increased to 100% expression with 1 mM FSL-Gb_3 (Fig. 4b). MFI increased by nearly half log intensity from 1 to 3 mM (Fig. 4c) treatment

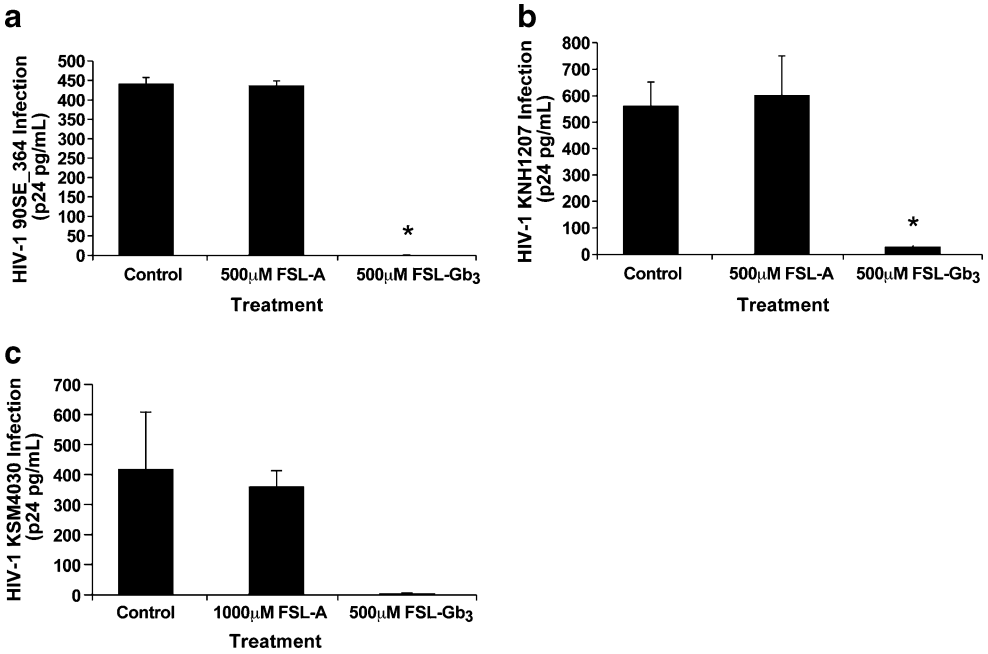

Fig. 3 FSL-Gb_3 inhibits R5 clinical HIV-1 isolate infection of U87.CD4.CCR5 cells. The R5 clinical viruses 90SE_645, KNH1207, and KSM4030 were incubated for 1 h with 500 μM FSL-Gb_3 or 500 or 1000 μM FSL-A compared to media control prior to 1 h infection of U87.CD4.CCR5 cells. $p24^{gag}$ ELISA was used to measure infection levels after 3 days of culture. **a** 90SE_645 infection, where $p=0.000102$. **b** KNH1207 infection where $p=0.0265$. **c** KSM4030 infection where $p=0.161$ with variable control values of 740, 433 and 83 pg/mL $p24^{gag}$

with FSL-Gb_3. These Gb_3-expressing Jurkat C T-cells were then infected with HIV-1_{IIIB} and HIV-$1_{Ba\text{-}L}$ for 1 h infection was measured by p24 expression 3 to 6 days post-infection. Cells pre-treated with FSL-Gb_3 inhibited HIV-1_{IIIB} infection (Fig. 4d) which showed a dose response over the concentration range tested and was significant at 3 mM treatment [$*P=0.016$ in comparison to untreated control]. Infection levels of HIV-$1_{Ba\text{-}L}$ revealed an increased initial infection at 1 mM which was not significant [$P=0.09$] and an overall slight decrease in infection at 3 mM, but this decrease was not statistically significant [$P=0.33$] (Fig. 4e).

Kinetics of absorption and retention of Gb_3 following Jurkat T-cell treatment with FSL-Gb_3

To determine the kinetics of retention of different amounts of FSL-Gb_3 absorbed onto HIV target cells, Jurkat C T-cells were treated for 1 h with FSL-Gb_3 over the concentration range 100–1000 μM and Gb_3 expression was followed for a period of up to 2 days by FACS and VT1 overlay. By FACS analysis, at 100 μM there was no difference between control and treated groups for MFI (Fig. 5b) however, percent Gb_3 expressing cells increased from 0 to 18% (Fig. 5a). By 200 μM, MFI increased significantly to 1765 and nearly 100% of cells were expressing Gb_3 (Fig. 5b), and by 1000 μM, MFI had reached 3028 (Fig. 5b). Retention of Gb_3 however, was reduced to background levels by both MFI and Percent Expression 24 h after treatment, for all concentrations except 1000 μM which retained 92% Gb_3 expressing cells, but MFI was near background levels (Fig. 5a and b). Additionally, total cellular GSLs were extracted from Jurkat cells treated with 400 μM FSL-Gb_3 before and 1 h after treatment, and at 24 and 48 h post treatment. We found Gb_3 to be cell-associated after initial 1 h incubation with FSL-Gb_3 but 24 h later was no longer detected in the cell population (Fig. 5c).

FSL-Gb_3 effects on cell surface receptor expression of Jurkat T-cells

Jurkat C T-cells exposed to FSL-Gb_3 for 1 h showed no difference in cell surface CD4 expression over time by MFI or percent expression (not shown). CXCR4 expression increased moderately at 24 h post FSL-Gb_3 treatment for the 100 μM group by MFI, however, this was not detected at 48 h. No other significant differences in CXCR4 expression were noted (not shown). CCR5 expression was not detectable in this cell line both prior to and after FSL-Gb_3 treatment (not shown).

Daily administration of FSL-Gb_3 is effective at maintaining inhibition of HIV-1_{IIIB} infection

In order to assess the most effective administration schedule for FSL-Gb_3, we compared infection levels of Jurkat C T-cells treated once before infection to those treated initially and then once-daily for a 5 day infection period with HIV-1_{IIIB}. We chose a treatment concentration of 200 μM, since this was the minimum dose required to absorb FSL-Gb_3 resulting in 100% Gb_3 expressing cells by FACS analysis. Additionally, this was the lowest concentration to produce significant inhibition of HIV-1_{IIIB} infection by viral pre-incubation (Fig. 6a). Cells were incubated with FSL-Gb_3 for 1 h at 37°C and then infected with HIV-1_{IIIB} (m.o.i., 0.1). For cells in the daily treatment group, 200 μM FSL-Gb_3 was added daily based on a total culture volume of

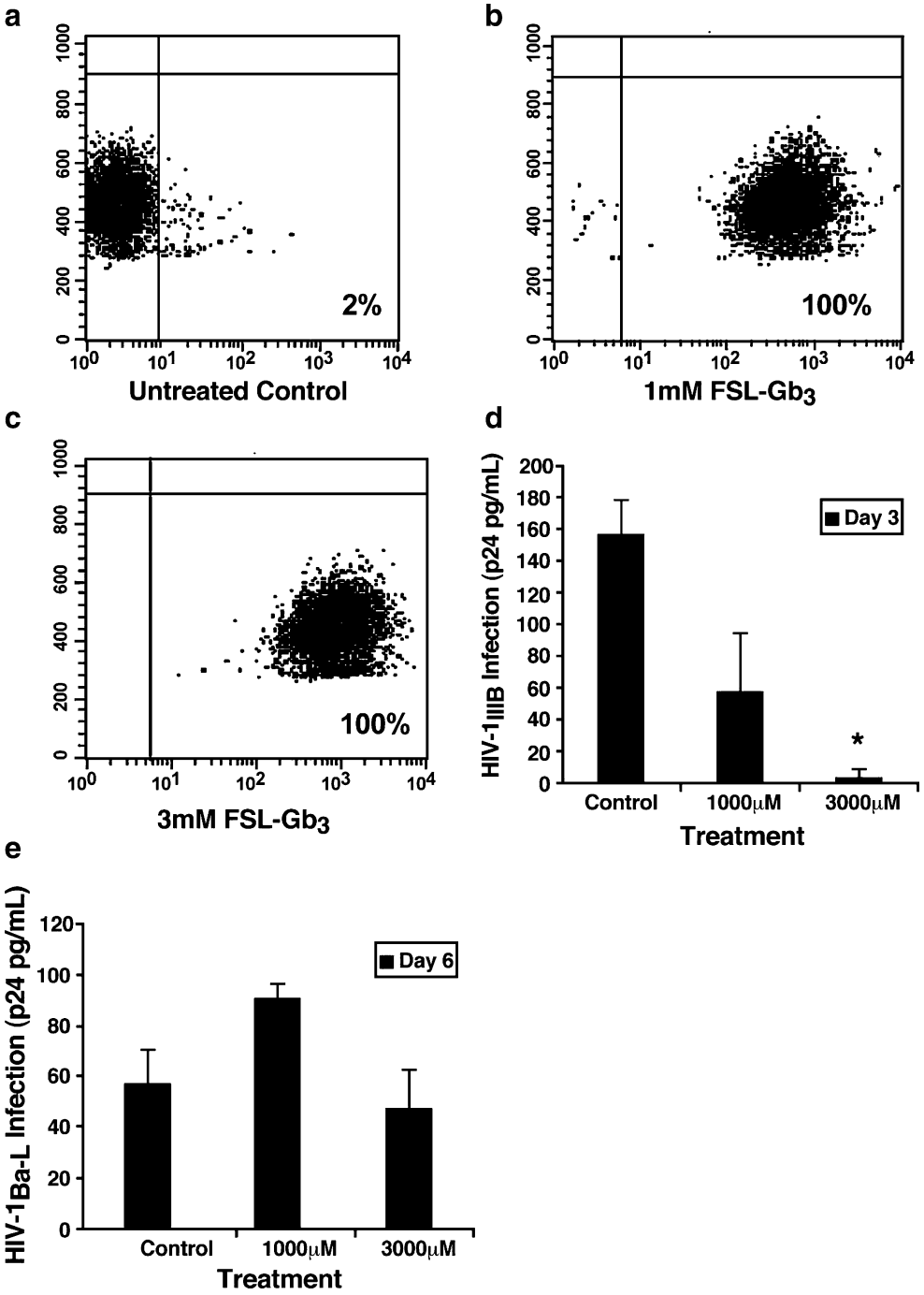

Fig. 4 Jurkat T-cell treatment with FSL-Gb_3 results in 100% Gb_3-expressing cells and a subsequent inhibition of HIV-1 infection. Jurkat C T-cells were incubated for 1 h with FSL-Gb_3 and then FACS analysis was performed to detect surface Gb_3 expression using anti-Gb_3 antibodies. **a** Percentage of untreated Jurkat cells expressing Gb_3. **b** Percentage of 1 mM treated Jurkat C expressing Gb_3. **c** Percentage of 3 mM treated Jurkat C expressing Gb_3. Treated and untreated cells were then infected with HIV-1_{IIIB} (m.o.i., 0.1) or sufficient HIV-1_{Ba-L} and monitored for p24 expression 3 to 6 days following infection. **d** HIV-1_{IIIB} infection in triplicate of Jurkat cells following cell pre-incubation with FSL-Gb_3 [*p=0.016 in comparison to untreated control]. **e** HIV-1_{Ba-L} infection in triplicate of Jurkat cells following cell pre-incubation with FSL-Gb_3

1 mL. Infection was monitored 5 days post infection by p24 ELISA. We found that compared to cells treated once only with FSL-Gb_3 (Fig. 6a), those receiving a daily treatment, showed significant inhibition of HIV-1_{IIIB} infection (Fig. 6b).

In vivo toxicity of FSL-Gb_3

All mice receiving either the large 200 μL or smaller 100 μL dose of FSL-Gb_3 did not show any adverse reaction to the subclavian vessel intravascular infusion, which was given under anesthetic. Mice evaluated at various time points throughout the study showed no overt clinical signs of toxicity. Litters born to pregnant female mice showed no signs of toxicity and thrived. Histological examination of spleen, liver, hepatic lymph nodes, kidney and lung at three months showed no evidence of toxic effects from the FSL-Gb_3 infusion. There was no evidence of antibody stimulation to Gb_3.

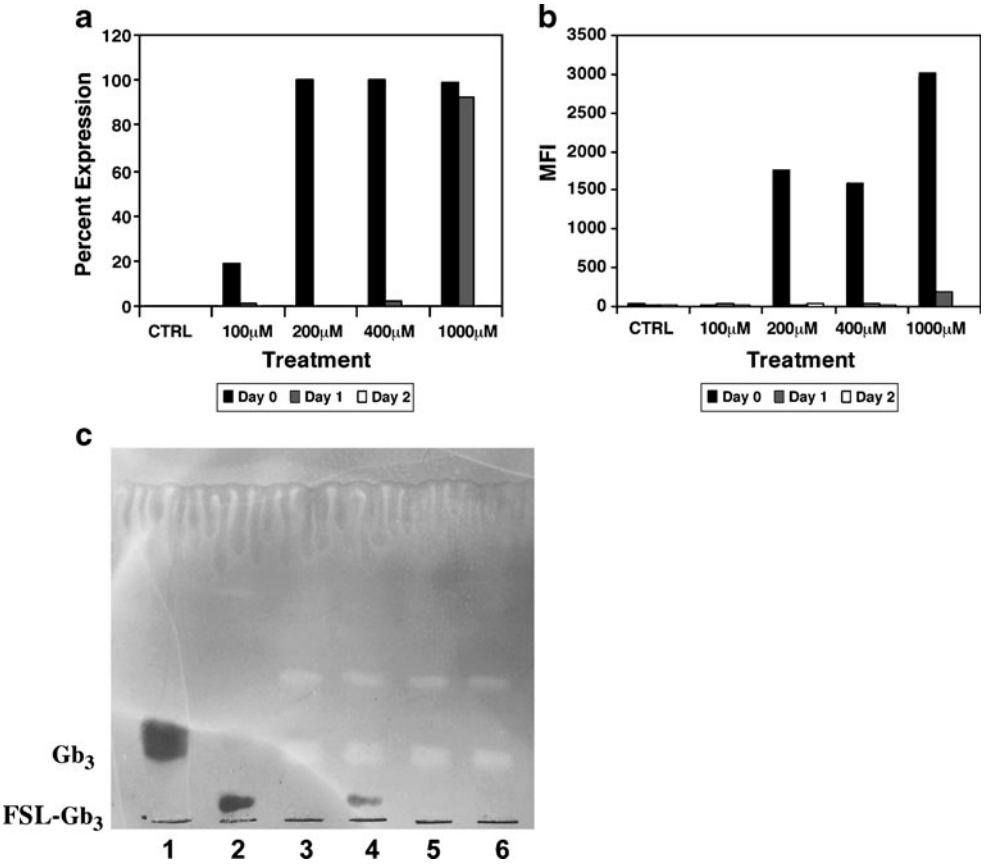

Fig. 5 Globotriaosylceramide expression over time following FSL-Gb_3 treatment of Jurkat T-cells. Jurkat C T-cells were incubated for 1 h at 37°C with 100–1000 μM FSL-Gb_3. Aliquots of cells were taken before treatment, after treatment and at 24 and 48 h for GSL extraction and Verotoxin/TLC overlay (400 μM for GSL extraction) and FACS analysis and washed and pelleted prior to analysis. FACS analysis of Gb_3 expression using anti-Gb_3 antibody was conducted 1 h, 24 and 48 h following treatment. **a** Percent expression of Jurkat C Gb_3 expression over time. **b** MFI of Jurkat C expressing Gb_3 following treatment over time. **c** Verotoxin1 TLC overlay, Lane 1, Gb_3 standard; lane 2, FSL-Gb_3 standard; lane 3 GSL from Jurkat C before treatment; lane 4, GSL from Jurkat C after 1 h incubation with FSL-Gb_3; lane 5, GSL from Jurkat C 24 h after treatment; lane 6, GSL after 48 h treatment

Discussion

The work conducted here supports previous studies by our group implicating globotriaosylceramide (Gb_3/P^k) as a resistance factor in HIV infection [4, 12, 13, 19], despite previous reports that suggested Gb_3 was a facilitator of membrane fusion [5]. However, as Gb_3 is either lacking or expressed at very low levels on resting or activated peripheral blood T-cells, a role for Gb_3 as facilitating the HIV infection, as has been previously proposed, [5], is unlikely. Our model proposes the following: Initially, the HIV envelope glycoprotein gp-120-C2 loop that binds CD4 on HIV target cells causes the V3 loop of gp120 to undergo a conformational change which exposes both the GSL (Gb_3) and chemokine co-receptor binding motifs. The binding motif XXXGPGRAFXXX [24], within the V3 loop, which binds Gb_3 overlaps with the chemokine consensus binding sequence S/GXXXGPGXXXXXXXE/D [25], also found in the V3 loop. We suggest that Gb_3 binds to its motif within the V3 loop and thus prevents subsequent interaction of the V3 loop with a chemokine co-receptor, which then prevents the conformational change necessary for gp41-mediated viral fusion [4]. Thus, if soluble globotriaosylceramide can be introduced into the blood stream where it can interact with circulating HIV-1 directly or be adsorbed onto the Gb_3-negative T-cell targets of HIV-1, effective inhibition or prevention of HIV-1 infection may be attained.

Herein, our aim was to evaluate a novel soluble, completely synthetic, Gb3 analogue, FSL-Gb_3, obtained from KODE Biotech Materials Ltd, for its efficacy in the inhibition of HIV infection as we had previously shown using a semi-synthetic Gb_3 analogue, adamantyl-Gb_3 [12]. FSL-Gb_3 is structured so that the carbohydrate moiety of Gb_3 is coupled to dioleoylphosphatidylethanolamine with help of an adipate linker [20]. We found that pre-incubation of HIV with FSL-Gb_3 inhibits both HIV-1_{IIIB} and HIV-$1_{Ba\text{-}L}$ infection of PBMCs and HIV-1_{IIIB} of Jurkat C similarly to adamantyl-Gb_3 (Fig. 2a–d) [12]. Although the IC_{50} for inhibition of HIV-1 infection using either FSL-Gb_3 or adamantyl-Gb_3 are similar, there are significant advantages to using FSL-Gb_3. First, unlike adamantyl-Gb_3 which is semi-synthesized starting from Gb_3 isolated from human kidney, FSL-Gb_3 is a completely synthetic molecule. Second, adamantyl-Gb_3 shows substantial *in vitro* toxicity [12], which has, thus far, precluded any *in vivo* toxicity studies and could prevent its use in humans. In contrast, FSL-Gb_3 shows no *in vitro* toxicities and also no significant toxicity in mice used at serum concentrations that would be expected to be of therapeutic benefit in humans. Indeed, *in vivo*, FSL compounds infused into laboratory animals (FSL-Gb_3 and FSL-A (blood group A)) have been shown to

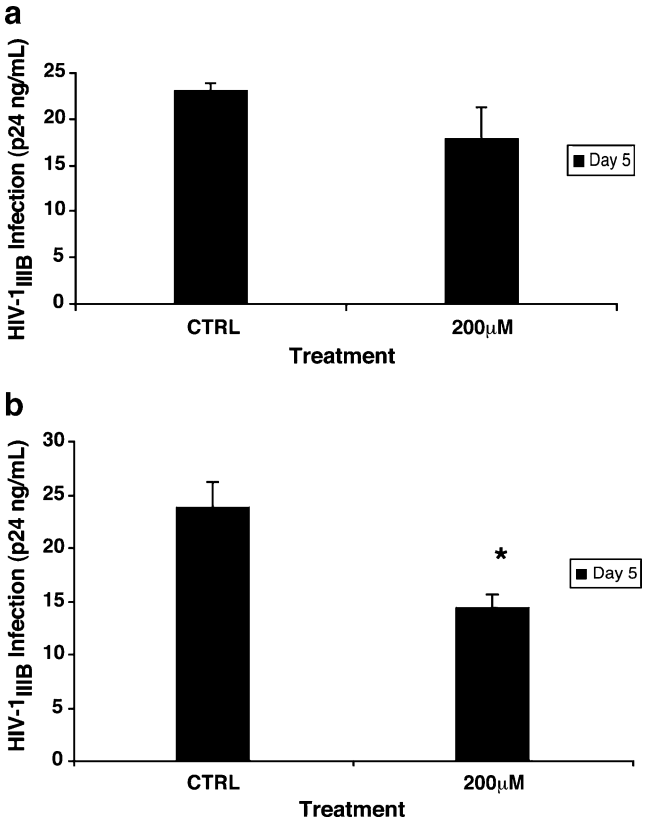

Fig. 6 Daily culture treatment with FSL-Gb_3 is more effective in inhibiting long-term HIV-1_{IIIB} infection when compared to one cellular treatment. Jurkat C T-cells were pre-incubated for 1 h at 37°C with 200 μM FSL-Gb_3 prior to infection with HIV-1_{IIIB} (m.o.i., 0.1). Infections were carried out in triplicate. HIV-1 infection was determined by $p24^{gag}$ ELISA 5 days post-infection. **a** Jurkat C treated once with FSL-Gb_3 compared to untreated control. **b** Jurkat C treated once initially as in (**a**) and then a daily dose of 200 μM FSL-Gb_3 based on total media volume was added to the cell culture every day sequentially up to 5 days post-infection, compared to untreated control [*P=0.04]

have no maximum tolerated dose up to 200 mg/kg body weight. Here, when used in millimolar concentrations (equivalent to 212 mg/kg) FSL-Gb_3 had no observable toxicity.

In unpublished work with blood group A FSL constructs (FSL-A), it can be shown that the constructs have strongly inserted into circulating cells within 10 min of infusion. Low toxicity would be critical if the compound were to be further explored for human use as a systemic and/or local potential treatment. Our *in vitro* observation could be qualified further by evaluation for any increase in apoptotic and necrotic cells after treatment with FSL-Gb_3 using FACS and Annexin V-FITC/PI staining. Also, more extensive *in vivo* toxicity testing is warranted. We have found that pre-incubation of the Jurkat C T-cell line with FSL-Gb_3 render these Gb_3-negative cells 100% Gb_3-positive by FACS analysis. Coating of the cells with FSL-Gb_3 caused them to resist HIV infection, at least at 3 days post infection. With HIV-1_{IIIB}, we show a significant inhibition of infection, and while infection with Ba-L was not significant, a trend towards overall inhibition was noted. However, Jurkat cells are T cells and not ideal CCR5 expressing cells for studies using R5 viruses. Indeed, when using primary PBMCs, FSL-Gb_3 was shown to significantly inhibit either X4 or R5 HIV-1 infection (Fig. 2).

The finding that FSL-Gb_3 can convert Gb_3-negative cells into cell surface-expressing Gb_3-positive cells offers potential ways to utilize the compound as a therapeutic, both through systemic inhibition and through the coating of mucosal target cells in a microbicide formulation. Furthermore, the finding that high surface Gb_3 expression on membranes can inhibit HIV infection parallels previous results with P_1^k PBMCs, where Gb_3 is highly expressed, and the cells resist both X4 and R5 HIV infection [4].

Cellular GSL analysis via VT1 TLC overlay revealed that FSL-Gb_3 was cell-associated initially after treatment but was lost from the cell within 24 h. These results were also consistent with our findings via FACS analysis. Additionally, treatment with FSL-Gb_3 had no effect on the HIV receptors CXCR4 and CD4. Consistent with our results that FSL-Gb_3 was lost from the cell membrane within 24 h, we found that when Jurkat cells were treated initially and then once daily for a 5 day period, that addition of FSL-Gb_3 to the culture medium was required to maintain a significant inhibition of long-term HIV infection in culture compared to cells treated once only. At this time, we are unsure of the mechanism of FSL-Gb_3 loss from membranes, but hypothesize that the molecule is lost into the culture medium or is internalized, and possibly associated with microparticles or LDL, or becomes degraded through cellular catabolic pathways. Surrogate unpublished data with FSL-A indicates the molecule is present in murine blood for several days. Although the finding that FSL-Gb_3 is lost within a 24 h period is disappointing, this is not at all unusual for pharmaceutical agents where daily dosing or even multidoses taken daily are required for therapeutic benefit. However, further studies exploring the kinetics of FSL-Gb_3 loss from cells and the mechanism involved would be valuable in determining its suitability as a therapeutic.

In summary, we report herein a novel, soluble, completely synthetic analogue of a natural HIV-1 resistance factor, globotriaosylceramide, which we call FSL-Gb_3, that has no *in vivo* toxicity in mice and which we have shown *in vitro* to have dual inhibitory properties. Further studies of this molecule may prove it to be a novel therapeutic for the treatment of HIV-1 infection by acting to both inhibit viremia by interacting as a soluble inhibitor with viruses present in the blood stream as well as able to insert itself into plasma membranes of Gb_3-negative HIV-1 targets, converting these cells to Gb_3-expressing cells capable of resisting HIV-1 infection.

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