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Novel anti-HIV-1 activity produced by conjugating unsulfated dextran with polyL-lysine

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ABSTRACT

A conjugate of polyL-lysine (PLL) with unsulfated dextran produced by reductive amination was found to have remarkable anti-HIV-1 activity against both the macrophage-tropic R5 virus Ba-L and T-cell line tropic X4 virus IIIB strains, although neither PLL nor dextran has such activity. The conjugate is a pseudoproteoglycan (pseudoPG) that simulates the structure of a proteoglycan. Conjugation with dextran was found to produce an antiviral effect in three kinds of assay systems including a human $CD4^+$ T-cell line, and the pseudoPG synthesized using 10 kDa PLL and 10 kDa dextran showed EC_{50} 4–40 times lower than that of sulfated dextran or heparin against Ba-L and EC_{50} equal to that against IIIB, indicating that PLL–dextran (PLL–Dex) was more effective against R5 virus than sulfated polysaccharides. PLL–Dex significantly suppressed a clinically isolated R5 virus from primary peripheral blood mononuclear cells. PLL–Dex interacted with the virus during adsorption to the cell and also decreased virus entry into the cell, suggesting PLL–Dex has multiple preventive mechanisms against HIV-1.

1. Introduction

The currently approved drugs for clinical treatment of human immunodeficiency virus type 1 (HIV-1) target viral entry or inhibit HIV-1 reverse transcriptase, integrase, or protease (De Clercq, 2010; Nair and Chi, 2007). Despite major achievements in HIV-1 pharmacotherapy, an urgent need remains for more-potent and less toxic conceptually novel antiretroviral drugs because long-term administration of a combination therapy using various inhibitors quite often increases the selective pressure on viruses, and subsequently induces emergence of multiple drug-resistant HIV-1 variants (Baba et al., 1988b; Balzarini et al., 2010). It has been reported that HIV-1 utilizes the cell surface heparan sulfate proteoglycan syndecan as a *cis* receptor to infect macrophages and as a *trans* receptor to infect T lymphocytes. Accordingly, removal of the cell surface heparan sulfate or use of sulfated polysaccharides as competing agents reduced HIV-1 attachment and entry into several cell lines including CD4-positive HeLa cells, macrophages, and T-cell lines (Balzarini et al., 2010; Saphire et al., 2001; Zhang et al., 2002) because the V3 loop of gp120 and the CD4-induced chemokine receptor-binding region overlap with the sulfated glycan-binding region (Zhang et al., 2002). Inhibition of the negative charge of sulfated glycans is also credited with inhibiting the replication of HIV-1 (Mondor et al., 1998; Ohshiro et al., 1996; Pearce-Pratt and Phillips, 1996; Saphire et al., 2001; Vives et al., 2005; Weiler et al., 1990). Similarly, several acidic polysaccharides, i.e., dextran sulfate, carrageenan, mannan sulfate, curdlan sulfate, and pentosan polysulfate inhibit a wide variety of enveloped viruses (Aoki et al., 1991; Baba et al., 1988b; Ito et al., 1987; Mastromarino et al., 1997; Ueno and Kuno, 1987).

Our previous study demonstrated that a polyL-lysine (PLL)-linked heparin, a heparin pseudoproteoglycan (pseudoPG), enhanced binding to rat cyclophilin A and HSP90 due to its unique higher-order structure (Nakagawa et al., 2009). Cyclophilin A was

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reported to associate with the HIV-1 capsid protein and play important roles in viral assembly and disassembly (Lammers et al., 2010; Li et al., 2007) by counteracting host restriction factors to evade resistance to the retroviral infection (Towers et al., 2003). In this context, we prepared pseudoPGs by conjugating peptides with various glycans to develop a new anti-HIV-1 reagent with the bifunctional actions of inhibiting HIV-1 interaction with the cell surface PG and entry into the cell by incorporating cyclophilin A, and examined their anti-HIV-1 activities using various cells and viral strains. The pseudoPGs made of sulfated glycans did not efficiently suppress HIV-1 infection. However, although unsulfated glycans such as dextran are lacking in anti-HIV-1 activity, dextran unexpectedly acquired a novel activity when conjugated with PLL. Here, we report a series of pseudoPGs prepared by coupling inactive unsulfated materials to develop a novel anti-HIV-1 activity.

2. Materials and methods

2.1. Materials

Various sizes of α-PLL hydrobromides, dextran sulfate (DexS, 50 kDa), and azidothymidine (AZT) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dextran T10 (Dex, 10 kD) was obtained from GE Healthcare UK Ltd. (Buckinghamshire, England). TAK-779 was provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals used were of analytical grade. The monocytotropic R5 type HIV-1 Ba-L (Aoki et al., 1991; Baba et al., 1988a) and the T-cell line-tropic X4 type HIV-1 IIIB (Baba et al., 1988b; Balzarini et al., 2010; Couffin-Hoarau et al., 2009; De Clercq, 2010) strains were used for anti-HIV-1 assays. The macrophage-tropic Ba-L strain was kindly provided by Dr. Tatsuo Shioda (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), and the T-cell line-tropic IIIB strain was kindly provided by Dr. Naoki Yamamoto (Tokyo Medical and Dental University School of Medicine, Tokyo, Japan). The human CD4-positive T-cell lines C8166 (Salahuddin et al., 1983) and C8166/CCR5 (clone 8) (De Clercq, 2010; Soda et al., 1999) were maintained in RPMI 1640 medium (Nissui Co., Ltd., Tokyo, Japan) containing 10% FBS. C8166/CCR5 cells stably express human CCR5 (De Clercq, 2010; Soda et al., 1999). The culture supernatants of C8166 cells infected with the IIIB HIV strain were harvested as viral stocks when cytopathicity was observed microscopically. The Ba-L strain was propagated in C8166/CCR5 cells as previously described (Soda et al., 1999). HIV-1 clinical isolates (CXCR4-tropic KK-1 and CCR5-tropic KK-20) were obtained from HIV-1-infected Japanese male patients by co-cultivation of their peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA)-stimulated PBMCs from healthy, HIV-seronegative donors. The coreceptor tropism of primary isolates was determined by their ability to infect MT-2 cells (Berger et al., 1998). The viruses were recovered by filtering the culture supernatants through a 0.45-mm pore filter on days 7–14 after infection and stored at −80 °C until use.

2.2. Synthesis and purification of PLL–Dex pseudoPGs

PLLs and Dex were conjugated by reductive amination as reported previously (Nakagawa et al., 2009) with a slight modification. Briefly, the molar ratio of the glycan to the polypeptide was 20:1; for example, 50 mg of Dex and 5.7 mg of PLL (25 kD) was dissolved in 5 ml of 0.1 M $H_3BO_4/Na_2B_4O_7$ (pH 8) with 2 M NaCl. The solution was preincubated for 2 h at 45 °C, then a 10-time molar excess of $NaCNBH_3$ was added to the glycan and incubated at 45 °C for 2 weeks. Formation of the conjugate was monitored by size exclusion chromatography and multiangle laser light scattering (SEC–MALLS). The pseudoPG was purified by gel filtration chromatography on a Toyopearl HW50F column (φ20 × 450 mm, Tosoh Co., Tokyo, Japan) equilibrated with 0.067 M phosphate buffer (pH 7.4) containing 2 M NaCl. The reaction mixture (no more than 2 ml in volume) was applied to the column, eluted at a flow rate of 0.4 ml/min at room temperature, and collected in 2-ml tubes. The elution of pseudoPG, dextran, and PLL was monitored by absorbance at A_{220} nm, the carbohydrate concentration was measured by the phenol–sulfuric acid method (Saha and Brewer, 1994), and protein was assayed using Pierce BCA protein assay reagent (Pierce Biotechnology, Rockford, IL) with Dex and PLL as the standards, respectively. The peak fractions of pseudoPG were pooled and dialyzed against water, then lyophilized for storage at −20 °C until use.

2.3. SEC–MALLS analysis

SEC–MALLS was performed on tandemly connected columns of Ultrahydrogel 250 (7.8 × 300 mm) and Ultrahydrogel 500 (7.8 × 300 mm) (Japan Waters Ltd., Tokyo, Japan) equipped with a MALLS detector (MiniDawn, Wyatt Technology Co., Santa Barbara, CA) and a refractometer (Shodex, Model RI-101, Shoko Co., Ltd., Tokyo, Japan). The refractive index (RI) increment (dn/dc) was measured by an Optilab rEX refractometer (Wyatt Technology) at 658 nm. Samples were dissolved at a concentration of 2 mg/ml in 0.067 M phosphate buffer (pH 7.4)–2 M NaCl, injected in 100-μl aliquots, and separated by SEC using tandemly connected columns that had been equilibrated with the same buffer at a flow rate of 1 ml/min at 40 °C. The molecular mass was calculated using analysis software (Astra V, Wyatt Technology) assuming a specific refractive index increment of PLL–Dex dn/dc = 0.115 ml/g. Pullulan standards (Shodex; Mr 5.9 k–212 k) were used to characterize the performance of the SEC column.

2.4. Chemical composition analyses

The carbohydrate concentration was measured by the phenol–sulfuric acid method (Saha and Brewer, 1994) using underivatized Dex as a standard. The peptide concentration was measured by a BCA assay (Pierce Biotechnology) according to the manufacturer's instructions using PLL as a standard.

2.5. Anti-HIV-1 activity

Inhibition of HIV-1 infection was measured using three assay systems: GFP- and coreceptor-expressing human glioma NP-2 cells, MAGIC-5 cells, and C8166 human T-cells, which are non-fluorescent.

2.5.1. N4R5/iGFP and N4X4/iGFP assays

Because all HIV-1 viruses require expression of a coreceptor, either CCR5 or CXCR4 or both, on the cells for infection (Habasque et al., 2002), human glioma NP-2 cells were prepared to express GFP as a signal of HIV-1 infection by introducing a vector containing GFP cDNA that had been ligated with the nuclear-translocating signal downstream of the HIV-1 LTR sequence (Hoshino et al., to be published). Cells that stably express the coreceptors CD4 and CCR5 (N4R5/iGFP) for the CCR5-tropic virus or CD4 and CXCR4 (N4X4/iGFP) for the CXCR4-tropic virus but do not express GFP in an uninfected state were used to indicate when a cell was infected with HIV-1. The cells were suspended in Eagle's minimum essential medium containing 10% (v/v) fetal calf serum (FCS) at 5×10^3 cells/100 μL, seeded in wells of 96-well microtiter plates, and incubated overnight. The sample compound was serially diluted with the medium, a 10-μL aliquot was added to each well, and the cells were incubated at 37 °C for 1 h. One hundred microliter of HIV-1, the CCR5-tropic Ba-L strain for N4R5/iGFP cells, or the CXCR4-tropic IIIB strain at a

multiplicity of infection (MOI) of 0.01–0.04 for N4X4/iGFP cells, suspended in the medium was added so that 50–200 cells became positive in the absence of drugs. The medium without HIV-1 was used as a control. The GFP-positive polynucleated cells were counted under a fluorescent microscope after 2 d incubation in the presence or absence of the sample, and the EC_{50} was calculated. To determine the cytotoxicity, the cells were incubated with Tetracolor One (Seikagaku Co., Tokyo, Japan) for 2 h, and the percentage of living cells was measured at absorbance A_{450} nm.

2.5.2. MAGIC-5 assays

For MAGIC-5 assays, the anti-HIV-1 activities of the compounds were determined using the CCR5-expressing tumor cell line MAGIC-5 (kindly provided by Dr. Masashi Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan), which was developed from MAGI (HeLa-CD4-LTR-β-galactosidase) cells. MAGIC-5 cells express the β-galactosidase gene driven by the HIV-1 LTR, and infected cells are detected as blue foci after incubation with 5-bromo-4-chloro-3-indolyl-β−D-galactopyranoside (X-gal). MAGIC-5 cells were plated on 96-well plates at 7×10^3 cells/well in Dulbecco's modified Eagle medium containing 2.5% FCS and incubated overnight at 37 °C. After the culture medium was removed, fivefold serially diluted compounds and 100–150 blue foci per 50 μL of the HIV-1 suspension of the CCR5-tropic Ba-L strain, or of clinical isolates, CCR5-tropic KK-20 or CXCR4-tropic KK-1, were added and incubated at 37 °C. After 44–48 h incubation, the culture medium was removed and the cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature, then washed and incubated with the staining solution consisting of 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 400 μg/ml X-gal for 1 h at 37 °C. After the staining solution was removed and the cells were washed, blue foci were counted under an optical microscope, and the EC_{50} values of the compounds were calculated on the basis of the reduction in the number of blue foci. To determine cytotoxicity, the viability of MAGIC-5 cells was measured using Tetracolor One as described for N4R5/iGFP assays.

2.5.3. C8166 and C8166/CCR5 assays

The human $CD4^+$ T-cell line C8166, which expresses CXCR4 but not CCR5, is susceptible to the CXCR4-tropic HIV-1 IIIB strain, while CCR5-transduced C8166 cells, C8166/CCR5, are susceptible to both CXCR4-tropic HIV-1 IIIB and CCR5-tropic HIV-1 Ba-L strains. These cells were cultured in RPMI 1640 medium containing 10% FCS (10% RPMI), and 4×10^4/400 μl of C8166 (for IIIB strain) or C8166/CCR5 (for Ba-L strain) cells were seeded in each well of 48-well plates. After overnight incubation, 50 μl of the sample compounds (serially diluted with 10% RPMI) was added per well, incubated for 1 h at 37 °C, and then inoculated with HIV-1 at a concentration that infected more than 60% of cells within 2 d. After 2 d incubation, cells were washed with PBS, and infected cells were detected by an indirect immunofluorescence assay using pooled sera derived from HIV-1-infected humans, as described previously (Shimizu et al., 2008). The numbers of infected cells at different concentrations of the sample compounds were counted under a fluorescent microscope, and the EC_{50} was calculated. The cytotoxicity of the sample compounds was detected with Tetracolor One as described above.

2.5.4. PBMC assay with clinical isolates

For the drug susceptibility assay, PHA-stimulated donor PBMCs were incubated with HIV-1 clinical isolates at a MOI of 0.001 at 37 °C for 2 h. After being washed, the infected cells were resuspended in RPMI-1640 medium containing 20% FCS and 100 units/ml of IL-2 and cultured in 96-well plates at 2×10^5 cells/well with or without serially diluted sample compounds. On day 5, half of the medium was removed from each well and replaced with fresh drug-containing medium. On day 10, culture supernatants were collected and assayed for p24 antigen by ELISA (Retro-Tek, ZeptoMetrix Corp., Buffalo, NY). The EC_{50} values of the compounds were calculated on the basis of the reduction of the p24 antigen in the culture supernatants. The viability of PBMC was measured using Tetracolor One as described for N4R5/iGFP assays.

2.6. Effects of pseudoPG on virus adsorption and/or entry into cells

The effects of pseudoPG on virus adsorption, adsorption and entry, or entry into the cells after adsorption were measured separately using a combination of N4X4/iGFP assays and incubation at various temperatures. During the assay protocols described above, CXCR4-tropic virus was first mixed with various concentrations of PLL (10 k)–Dex (10 k) or heparin, which was used as a positive control, and then the mixture was incubated with N4X4/iGFPcells at 4 °C, a temperature at which the virus binds to but does not enter the cells, for 1 h to measure the effects of pseudoPGs on virus adsorption, or at 37 °C, a temperature at which the virus can both bind and enter the cells, for 1 h to measure the effects on viral adsorption and entry into the cells. The effects of pseudoPGs on virus entry into the cells was measured by first incubating N4X4/iGFP cells with X4 virus at 4 °C for 1 h, then PLL–Dex was added and incubated at 37 °C for 1 h. In each experiment, unbound viruses were removed by centrifuging the cells at 4 °C, and then the cells were lysed with PBS/0.75% NP-40 (Mckeating, 1995). The amounts of virus that were adsorbed by and/or entered the cell were compared by measuring the viral protein p24 by ELISA (Ed Harlow, 1988) in the cell lysates to show the infection efficiency. The wells of ELISA plates were coated with a monoclonal antibody against p24. After blocking with 3% (W/V) BSA in PBS, the cell lysate was added and the bound p24 was detected with HIV-1-positive human serum and HRP-anti-human IgG antibodies.

2.7. Effects of preincubation of pseudoPG with virus or cells

To elucidate whether pseudoPG acts on the virus or N4R5/iGFP and N4X4/iGFP cells to inhibit the infection process, PLL (10 k)–Dex (10 k) or heparin at various concentrations (1–100 μg/ml) was preincubated with either cells or virus in microtiter plates at 37 °C for 1 h. After preincubation of the cells with the compounds, the cells were washed by centrifugation to remove the compounds; the virus was added to the cells for incubation at 37 °C for 1 h, following the protocols of the N4R5/iGFP and N4X4/iGFP assays. Then the cells were washed, and the culture medium was added. After 2 d, the propagation of HIV-1 in the cells was estimated by measuring the amount of p24 using ELISA. To examine the effects of compounds on HIV-1, the cells were added to microtiter plates containing preincubated mixtures of HIV-1 and the test compounds, and the infection assay was performed in the same way.

2.8. In vivo toxicity test

Pathogen-free female BALB/c mice (8-weeks-old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The mice were handled daily for 1 week before the study to minimize the effects of stress occasioned by human handling. The mice were injected intraperitoneally with the test compounds dissolved in physiological saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Assessment of the toxicity of test compounds was initiated immediately after injection. The condition and body weights of the mice were recorded at 30 min, 1 h, 3 h, 6 h, 24 h, and 3 d after the injection. The care and use of the experimental animals in this study followed "The Ethical Guidelines of

Animal Care, Handling and Termination" described by the National Institute of Health Sciences.

3. Results

3.1. Preparation of PLL–Dex pseudoPGs

We first examined the optimal conditions for the reductive amination reaction of Dex with PLL (1, 6, 10, and 25 kDa) by changing the pH, time, and temperature, and the conjugation was monitored by subjecting aliquots of the reaction mixture to SEC–MALLS. After choosing the best condition, described in Section 2, the conjugation was performed for 2 weeks because the reaction reached a plateau within that period. As shown in Fig. 1A, each pseudoPG synthesized was eluted as the first peak in front of the major peak of Dex on gel-filtration chromatography on a Toyopearl HW55F column and separated from the starting materials. The high-molecular-weight fractions (fractions 58–67) on gel-filtration chromatography were subjected to SEC–MALLS. As shown in Fig. 1B, PLL (25 k)–Dex and PLL (10 k)–Dex had average molecular masses of 1.4×10^5 and 9.8×10^4 Da, respectively; based on the average molecular mass of each monomer, PLL and Dex, the conjugates were calculated to contain on average 10 and 8 mol, respectively, of Dex chains per mole of PLL chain, as illustrated in Fig. 1C. The average molecular masses of the pseudoPGs are summarized in Table 1 together with those of the components, PLLs and Dex. The molar ratios of PLL to Dex in each pseudoPG were calculated and are shown in Table 1.

3.2. Anti-HIV-1 activity measured by coreceptor-expressing GFP-reporter cells

Anti-HIV-1 activities of pseudoPG were demonstrated by using coreceptor-expressing N4R5/iGFP and N4X4/iGFP cells, which were constructed to express GFP in the nucleus on infection with the HIV-1 CCR5-tropic Ba-L or CXCR4-tropic IIIB virus strain, respectively. The relative infectivity with HIV-1 was measured by taking the number of GFP-positive polynucleated cells, instead of the absolute number of total cells, in the absence of the compound as 100%. Fig. 2A and B shows that the pseudoPG concentration-dependently suppressed the infection of N4R5/iGFP and N4X4/iGFP cells by Ba-L and IIIB strains, respectively, by presenting PLL (6 k)–Dex and PLL (10 k)–Dex as representative pseudoPGs. The anti-HIV-1 activity and cellular toxicity of the compounds tested using MAGIC-5, C8166, and C8166/CCR5 cells were concentration-dependent and were similar to those of N4R5/iGFP cells. Thus, the data of N4R5/iGFP and N4X4/iGFP cells (Fig. 2) are shown as representative. The EC_{50} of the compounds prepared in this study, which is defined as the drug concentration suppressing the HIV-1 infection by 50%, are summarized in Table 1, together with those of the components and the known anti-HIV-1 compounds AZT, DexS, and heparin. The pseudoPG prepared with PLL (1 k) did not exhibit significant anti-HIV-1 activity even at high concentrations, while those prepared with the longer PLLs inhibited HIV-1 infection with maximum activity using a PLL of 10 k (Fig. 2A and B, and Table 1). PLL (6 k)–Dex and PLL (10 k)–Dex had EC_{50} of 0.030 ± 0.004 and 0.053 ± 0.024 μM against the Ba-L strain and 0.033 ± 0.002 and 0.017 ± 0.008 μM against the IIIB strain, respectively. The EC_{50} of PLL (10 k)–Dex was 4.2 and 40 times lower against Ba-L than the EC_{50} of DexS and heparin, respectively, indicating marked activities against the Ba-L virus, which is tolerant of sulfated polysaccharides.

Similar concentration-dependent cellular toxicity was observed in both N4R5/iGFP and N4X4/iGFP cells for PLLs of more than 6 kD, as seen in Fig. 2C in which N4R5/iGFP cells are shown as representative. The CC_{50}, the drug concentration at which 50% of N4R5/iGFP cells died, was $>6.7 \pm 0.6$ μM for PLL (6 k) and 2.5 ± 0.5 μM for PLL (10 k). The relative infectivity could not be measured at drug concentrations over CC_{50} because whether cell death was caused by HIV-1 infection or the toxicity of the drug could not be determined. In contrast, PLL–Dex did not show significant cytotoxicity at concentrations up to 100 μM for PLL (6 k)–Dex and 200 μM for PLL (10 k)–Dex (data not shown), indicating that the cytotoxicity of PLL was decreased by conjugation with Dex. When PLL and Dex were simply mixed together without covalent conjugation, they did not show a comparable of elevation of anti-HIV-1 activity or decrease of cytotoxicity (data not shown). The results indicate that the conjugation of PLL and Dex produced an anti-HIV-1 activity that PLL or Dex alone does not possess and that simultaneously reduced the cytotoxicity of PLL at molecular masses higher than 6 k.

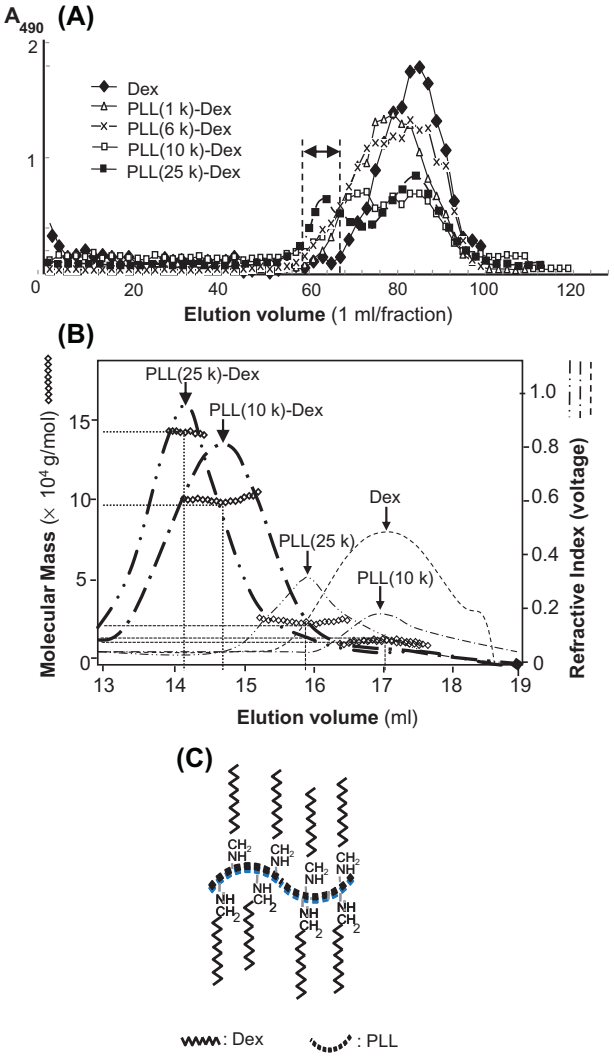

Fig. 1. Purification and measurement of molecular mass of PLL–Dex conjugates. Elution patterns of PLL–Dex and Dex on a Toyopearl-HW55F column (φ20 × 450 mm) (A) and SEC–MALLS (B). The structure of PLL–Dex (C). (A) PLLs of various sizes were coupled with Dex by reductive amination as described in the text, and the products were separated by gel-filtration chromatography and detected by the phenol–sulfuric acid method. The fractions indicated by a double-headed arrow were pooled and further analyzed by SEC–MALLS. (B) Size-exclusion HPLC on tandem KW802.5 and KW803 columns in connection with a MiniDAWN system. Chromatograms were detected by the refractive index (right of the *y*-axis); dots indicate the molecular mass (left of the *y*-axis), which was calculated from the MALLS signals using the Astra V program. (C) The deduced structure of PLL (10 k)–Dex, which was calculated from the average molecular mass of each monomer PLL and Dex, and PLL–Dex as measured by SEC–MALLS.

Table 1
Average molecular mass, composition, anti-HIV-1 activity, and cellular toxicity of PLLs, Dex, and PLL–Dex pseudoPGs in N4X4/iGFP and N4R5/iGFP, MAGIC-5, C8166, and C8166/CCR5 cells.

Compound	Average molecular mass[a] (molar ratio = PLL:Dex)	N4R5/iGFP	N4X4/iGFP	N4R5/iGFP	MAGIC-5	MAGIC-5	C8166/CCR5	C8166	C8166
		EC50 (μM)[b]		CC50 (μM)[c]	EC50 (μM)[b]	CC50 (μM)[b]	EC50 (μM)[b]		CC50 (μM)[d]
		Ba-L	IIIB		Ba-L		Ba-L	IIIB	
PLL (1 k)	1.1×10^3	>91	>91	>91	NT	NT	NT	NT	NT
PLL (6 k)	5.8×10^3	2.1 ± 1.2	2.5 ± 0.05	6.7 ± 0.6	1.6 ± 0.7	15.6 ± 0.1	NE	NE	4.2 ± 1.8
PLL (10 k)	0.98×10^4	1.2 ± 0.7	1.8 ± 0.3	2.50 ± 0.5	0.9 ± 0.7	2.5 ± 0.7	0.3 ± 0.1	NE	2.8 ± 1.1
PLL (25 k)	2.5×10^4	0.48 ± 0.40	0.49 ± 0.40	2.5 ± 0.5	0.3 ± 0.1	0.75 ± 0.04	0.70 ± 0.04	0.14 ± 0.01	1.4
Dex (10 k)	1.1×10^4	NE	NE	>4	NE	>909	NE	NE	NT
PLL (1 k)–Dex	4.5×10^4 (1:4)	>1.3	>1.3	>100	NT	NT	NT	NT	NT
PLL (6 k)–Dex	9.3×10^4 (1:8)	0.030 ± 0.004	0.033 ± 0.002	>100.0 ± 0.6	NE	>10.8	0.019 ± 0.001	0.037 ± 0.003	>10.8[e]
PLL (10 k)–Dex	9.8×10^4 (1:8)	0.053 ± 0.024	0.017 ± 0.008	>200.0 ± 0.5	5.7 ± 1.6	>102	0.017 ± 0.008	0.026 ± 0.011	>102[e]
PLL (25 k)–Dex	14×10^4 (1:10)	0.032 ± 0.01	0.042 ± 0.028	>100.0 ± 0.6	0.61 ± 0.02	>7.1	0.017 ± 0.009	0.016 ± 0.006	>0.71[e]
AZT	267	0.0081 ± 0.0010	0.0067 ± 0.0014	>100	0.017 ± 0.006	>1	0.17 ± 0.07	0.19 ± 0.06	>100[e]
TAK-779	531	NT	NT	NT	0.018 ± 0.010	>1	NT	NT	NT
DexS	5.0×10^4	0.22 ± 0.16	0.0039 ± 0.0013	>2	6.2 ± 2.3	>652	NT	NT	NT
Heparin	3.0×10^4	2.1 ± 0.2	0.010 ± 0.000	>3	NT	NT	NT	NT	NT

[a] Average molecular mass was calculated using SEC–MALLS system as described in the text.
[b] Data are averages of three independent experiments ± S.D. NE, not effective. NT, not tested.
[c] CC50 was determined using N4R5/iGFP cells.
[d] CC50 for C8166 and C8166/CCR5 cells were similar. CC50 for C8166/CCR5 cells are shown.
[e] No cytotoxicity was observed at this concentration of drug.

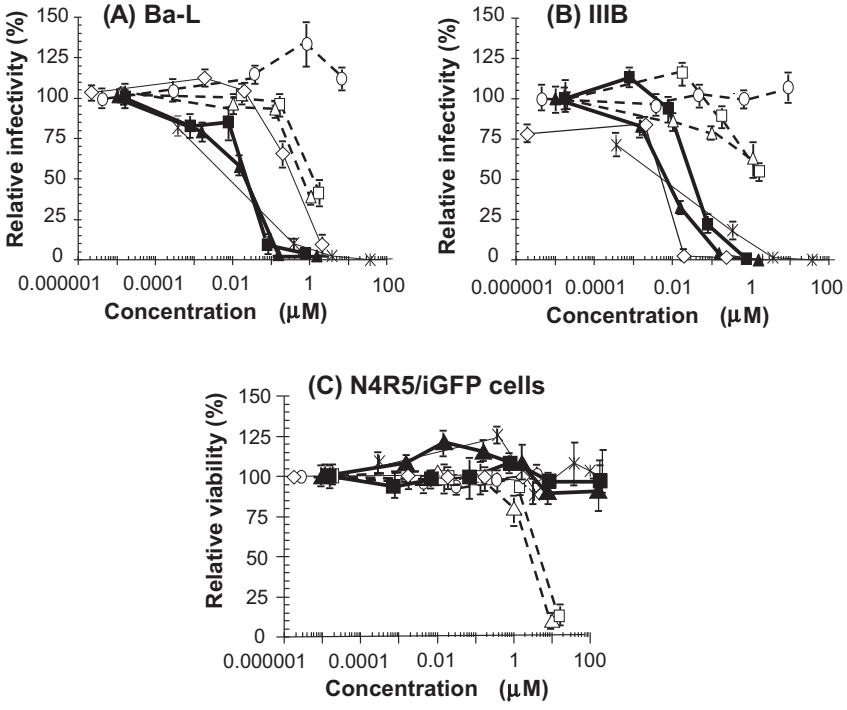

Fig. 2. Concentration dependence of anti-HIV-1 activity and cellular toxicity of PLL, Dex, and PLL–Dex against Ba-L and IIIB using N4R5/iGFP or N4X4/iGFP cells. The anti-HIV-1 activity (A and B) of the compounds against the Ba-L strain at various concentrations was measured using N4R5/iGFP cells (A) and the IIIB strain using N4X4/iGFP cells (B). The *X*-axis indicates the concentrations of the sample compounds. The *Y*-axis indicates the relative infectivity with HIV-1 expressed as % by taking the GFP-positive cells in the absence of the compound as 100% (A and B). Cellular toxicity is expressed as relative cell viability, which was measured in N4R5/iGFP cells using Tetracolor One, as described in the text, and is expressed as % by taking the viable cells in the absence of the compound as 100% (C). The data represent the mean values ± S.D. ($n = 3$, meaning three separate experiments), and three counts were taken at each concentration. Symbols used are; –○–: Dex, –□–: PLL (6 k), –△–: PLL (10 k), –■–: PLL (6 k)–Dex, –▲–: PLL (10 k)–Dex, –◇–: Dex sulfate (50 k), and –×–: AZT.

3.3. Anti-HIV-1 activity measured by MAGIC-5 assays

A separate study using MAGIC-5 cells confirmed the anti-HIV-1 activities of PLL (10 k)–Dex and PLL (25 k)–Dex. Both inhibited Ba-L strain infection of MAGIC-5 cells with EC_{50} of 5.7 ± 1.6 and 0.61 ± 0.02 μM, respectively, as summarized in Table 1. Although EC_{50} of PLL were obtained by MAGIC-5 assays (0.9 ± 0.7 and 0.3 ± 0.1 μM for PLL of 10 and 25 k, respectively, which are lower than CC_{50} of those measured using Tetracolor 1), significant cytopathic effects were observed microscopically in MAGIC-5 cells at these EC_{50} concentrations. In contrast, cytotoxic effects of PLL (10 k)–Dex and PLL (25 k)–Dex on MAGIC-5 cells were not observed either microscopically or by Tetracolor one assay at the concentrations examined (Table 1). The results may suggest that the

PLL moiety possesses both HIV-1-suppressing activity and cytotoxicity for MAGIC-5 cells but that conjugation with Dex significantly decreases the cytotoxic effect of PLL while retaining the anti-HIV-1 activity. Although the EC_{50} in the N4R5/iGFP and MAGIC-5 assays differed by more than one order of magnitude, probably because of differences in the assay systems and virus concentrations used for each assay, the results strongly indicate that anti-HIV-1 activity was formed by conjugating PLL and Dex.

3.4. Anti-HIV-1 activity by C8166 and C8166/CCR5 assays

PLL (10 k)–Dex and PLL (25 k)–Dex inhibited Ba-L and IIIB strain infections of a human $CD4^+$ T-cell line, C8166/CCR5 and C8166 cells, with EC_{50} of 0.017 and 0.026–0.016 μM, respectively, as shown in Table 1. Cytotoxic effects of PLL (10 k)–Dex and PLL (25 k)–Dex on C8166 cells were not observed at 102 and 0.7 μM, respectively. PLL (6 k)–Dex did not inhibit HIV-1 infection of C8166 cells, indicating that the PLL chain length is important. The EC_{50} of PLL–Dex in the C8166 assay and those in the N4R5/iGFP assays corresponded except for this, indicating that PLL and Dex conjugates are also effective on lymphoid cell lines.

3.5. Anti-HIV-1 activity by PBMC assays with clinical isolates

As shown in Fig. 3 and Table 1, PLL (10 k)–Dex showed weak but significant suppression of infection of PBMC by KK-20 virus (EC_{50} = 6.9 μM) but not by KK-1 (EC_{50} > 102 μM). PLL (10 k)–Dex did not have cytotoxicity against PBMCs (CC_{50} > 102 μM). Thus, PLL–Dex was demonstrated to be effective against infection of PBMCs by clinically isolated CCR5-tropic HIV-1. In contrast, AZT showed high inhibition efficacy against both clinical isolates in PBMC, suggesting that PLL–Dex has a different action mechanism from that of AZT.

Although KK-1 was not suppressed by PLL–Dex in PBMC, the infection of MAGIC-5 cells, which express both the CCR5 and CXCR4 coreceptors, by KK-1 was inhibited by PLL–Dex as well as infection by KK-20, as shown in Table 2. It indicates that the effect of PLL–Dex on the KK-1 varies by cell.

3.6. Inhibition of virus adsorption and cell entry by PLL–Dex

As shown in Fig. 4A and B, PLL (10 k)–Dex at 100 μg/ml was found to inhibit IIIB virus adsorption by 70% and subsequent virus entry into the cells by 95%, while heparin at the same concentration suppressed adsorption and entry by 70% and 90%, respectively. After viral adsorption to the cells, PLL–Dex was shown to inhibit the entry of IIIB by 70% of the control (Fig. 4C), indicating that PLL–Dex acts on both the viral adsorption and cell entry processes to suppress the infection.

Fig. 5 shows whether the pseudoPG acts on cells or viruses. As shown in Fig. 5A, when N4R5/iGFP or N4X4/iGFP cells were first incubated with PLL–Dex, 100 μg/ml PLL–Dex concentration-dependently suppressed the infectivity of the cells to 40% of the control for Ba-L and to 45% for IIIB. In contrast, heparin only weakly suppressed the infectivity of the cells to 70% and 60%, respectively. On the other hand, as shown in Fig. 5B, when the virus was preincubated with PLL–Dex, the infection was almost completely inhibited, indicating that PLL–Dex acts both on cells and virus and that the effects are additive. Heparin at 1 μg/ml inhibited the IIIB virus better than PLL–Dex, but it did not inhibit Ba-L virus at all, indicating that PLL–Dex can suppress infection with both types of viruses while heparin preferentially inhibits IIIB and is less effective against Ba-L virus (Fig. 5B).

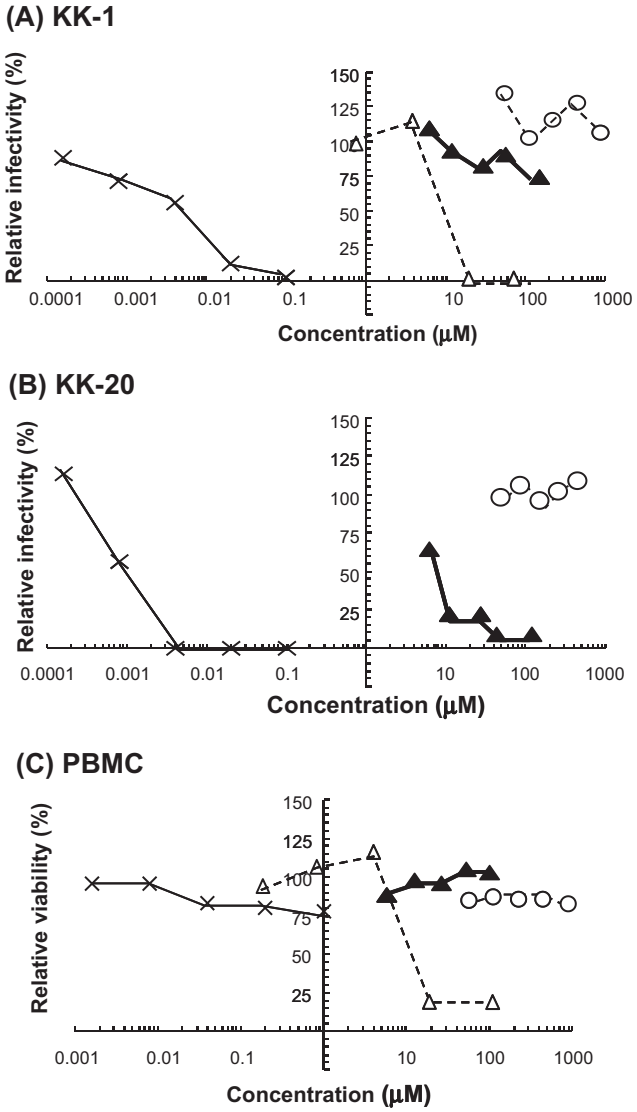

Fig. 3. Concentration dependence of anti-HIV-1 activity and cellular toxicity of PLL, Dex, and PLL–Dex on clinical HIV-1 isolates in primary PBMC. The anti-HIV-1 activity of the compounds was measured against the X4 virus, KK-1 strain (A), and the R5 virus, KK-20 strain (B) at various concentrations using PBMC. The EC_{50} values of the compounds were calculated on the basis of the reduction of the p24 antigen in the culture supernatants, as described in the text. The *X*-axis indicates the concentration of the sample compounds. The *Y*-axis indicates the relative infectivity with HIV-1 expressed as % by taking the PBMC in the absence of the compound as 100% (A and B). Cellular toxicity is expressed as relative cell viability, which was measured in PBMC using Tetracolor One as described in the text, and is expressed as % by taking the viable cells in the absence of compound as 100% (C). The data represent the mean values (n = 3, meaning three separate experiments), and three counts were taken at each concentration. Symbols used are; --○--: Dex, ⌙△⌙: PLL (10 k), ⌙▲⌙: PLL (10 k)–Dex, and x: AZT.

3.7. Toxicity of pseudoproteoglycan in vivo

When mice were injected intraperitoneally with 1, 10, or 50 mg/kg PLL (10 k), dose-dependent toxicity was observed in the PLL (10 k) experimental groups. At 1, 10, and 50 mg/kg, injected mice did not move for 30 min, 1 h, and 24 h, respectively. At a concentration of 100 mg/kg PLL (10 k), mice lost daily approximately 10% of their body weight and died on day 4. No toxic symptom or loss of body weight was observed after injection of PLL (10 k)–Dex or unconjugated Dex at the concentration of 50 mg/

Table 2
Anti-HIV-1 activity and cellular toxicity of PLL, Dex, and PLL–Dex for PBMC and MAGIC-5 cells using clinical strains KK-1 and KK-20.

Compound	EC50 (μM)[a]				CC50 (μM)[a]	
	PBMC		MAGIC-5		PBMC	MAGIC-5
	KK-1 (X4)	KK-20 (R5)	KK-1 (X4)	KK-20 (R5)		
PLL (10 k)	NE	NT	NE	NE	11.7 ± 0.9	2.52 ± 0.70
Dex	NE	NE	NE	NE	>909.1	>9.09
PLL (10 k)–Dex	NE	6.9 ± 2.4	4.05 ± 0.98	6.87 ± 1.12	>102	>10.2
AZT	0.0063 ± 0.0018	0.0015 ± 0.0001	0.0020 ± 0.0003	0.0010 ± 0.0001	>1	>1

NE, not effective. NT, not tested.
[a] Data are averages of three independent experiments ± S.D.

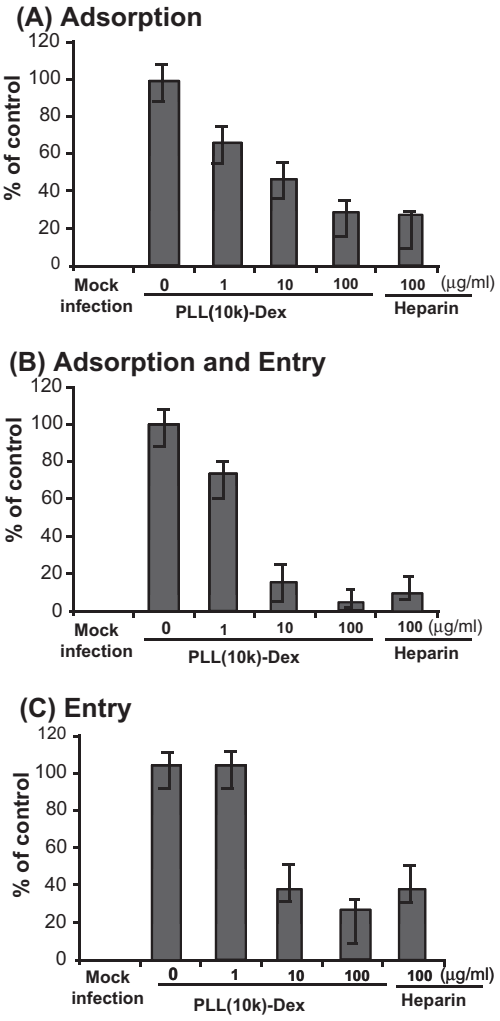

Fig. 4. Effects of PLL–Dex on absorption (A), adsorption and entry (B), and entry of HIV-1 (IIIB) to the N4X4/iGFP cells (C), detected as p24 by ELISA. (A) Effects of pseudoPG on the adsorption of HIV-1 were measured using N4X4/iGFP cells inoculated with IIIB by incubation in the presence of various amounts of PLL–Dex or heparin at 4 °C for 1 h. Then the cells were lysed, and HIV-1 p24 in the lysate was quantified by ELISA. (B) Effects on the adsorption and entry of HIV-1 were measured by the same procedure as shown in (A) but performed at 37 °C for 1 h. (C) Effect on the entry of HIV-1 was measured by incubating at 4 °C for 1 h in the presence of IIIB, then PLL–Dex was added and incubated at 37 °C for 1 h. Then the cells were lysed, and p24 was quantified by ELISA as described in the text. The data represent the mean ± S.D. (n = 3, meaning three separate experiments), and three counts were taken at each concentration.

kg. No mice in these experimental groups died before the end of the experiment.

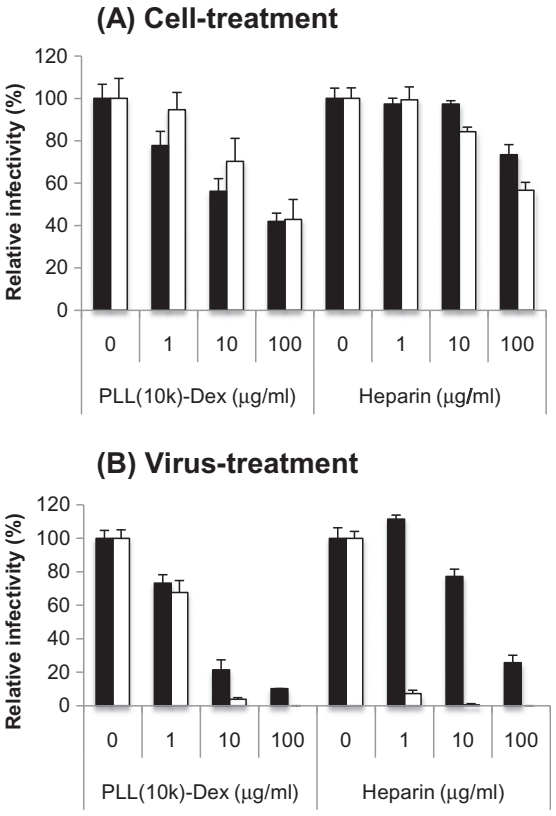

Fig. 5. Effect of incubation of PLL–Dex and cells (A) or viruses (B) on viral infectivity. PLL (10 k)–Dex (10 k) or heparin at various concentrations (1–100 μg/ml) were preincubated with either cells (A) or virus (B) in microtiter plates at 37 °C for 30 min. (A) After preincubation, the cells were centrifuged to remove the compounds, and the virus was added to the cells, followed by infection as described in the text. The propagated HIV-1 in the cells was measured as the amount of p24 by ELISA after cell lysis. (B) After preincubation of HIV-1 with one of the compounds, the cells were added to the microtiter plates containing the preincubated mixture of HIV-1 and the compounds, and the infection process and p24 were measured in the same way. Black bar: infectivity of N4R5/iGFP cells with Ba-L. White bar: infectivity of N4X4/iGFP cells with IIIB. The data represent the mean ± S.D. (n = 3, meaning three separate experiments), and three counts were taken at each concentration.

4. Discussion

4.1. Anti-HIV activity of PLL–Dex

In this study, we demonstrated that pseudoPGs prepared by conjugating inactive Dex with PLL potently inhibited HIV-1 infection with both R5 and X4 viral strains. Assays using N4R5/iGFP, N4X4/iGFP, MAGIC-5, and C8166 or C8166/CCR5 cells indicated that the remarkable anti-HIV-1 activity occurred only when PLL

and Dex were covalently linked in a compound. PLL (10 k)–Dex was effective against not only Ba-L and IIIB viruses but also clinically isolated CCR5-tropic KK-20 virus in primary cells, PBMC, on which PLL (10 k)–Dex had practically no cytotoxicity. Although KK-1 was tolerant of PLL (10 k)–Dex in PBMC, KK-1, as well as KK-20, was inhibited by PLL–Dex in MAGIC-5 cells (Table 2). Furthermore, CXCR4-tropic IIIB virus was inhibited by PLL–Dex in a human T-cell line, C8166 cells, and N4X4/iGFP cells (Table 1). This indicates that inhibition of KK-1 by PLL–Dex is not simply dependent on the known coreceptor tropism of viral adhesion. It is considered that KK-1 may utilize an unknown coreceptor other than CXCR4 and CCR5, which MAGIC-5 does not have and PLL–Dex cannot inhibit, to infect PBMC, while the possibility remains that the lack of some other host restriction factor for KK-1 in PBMC is necessary for the suppression mechanism by PLL–Dex. Based on this observation, it is necessary to study more diverse HIV-1 strains to determine the HIV-1-suppressing mechanism of PLL–Dex. Because a pseudoPG made of PLL (1 k) with Dex (10 k) did not exhibit anti-HIV-1 activity and the anti-HIV-1 activity of pseudoPGs showed dependency on the size of the PLL (Table 1), the antiviral effects of PLL–Dex may largely depend on the PLL moiety. However, the Dex moiety was indispensable to the unexpectedly high anti-HIV-1 activity of the pseudoPGs, which reduced the EC_{50} by more than one order of magnitude and overcame the cytotoxicity of PLL (Table 1 and Table 2). Our preliminary experiments indicated that *N*-acetylation or partial biotinylation of amino groups in PLL reduced the cytotoxicity but completely destroyed the anti-HIV-1 activity (data not shown), suggesting that the modification of Dex with most free amino groups remaining on the PLL was necessary for the suppression of HIV-1 infection.

4.2. Comparison with sulfated glycans

Inhibition of HIV-1 adsorption on MT-4 cells by sulfated glycans, dextran sulfate, and heparin has been observed, but sulfated glycans were much less effective against the R5 virus (Aoki et al., 1991; Baba et al., 1988a,b). PLL–Dex showed a contrasting anti-HIV-1 efficacy, suppressing infection by the sulfated glycan-resistant R5 virus as well as infection by the X4 virus (Table 1). Supportingly, pseudoPGs that were made of sulfated glycans were inactive against HIV-1 infection, and PLL–Dex does not bind to the envelope gp120 of HIV-1 (our unpublished results). An inhibition mechanism for PLL–Dex would be novel among polysaccharide drugs on these points and is unique from that speculated for other conjugates. Therefore, the PLL–Dexs are expected to exhibit anti-HIV-1 activity by a different mechanism from that of the sulfated glycans.

4.3. Significance of pseudoPG structure

It is considered that PLL–Dex possesses a higher order structure that is essential for the anti-HIV-1 activity against both R5 and X4 viral strains without causing severe cellular toxicity. When the in vivo toxicity of each sample was tested by the intraperitoneal injection of PLL–Dex or Dex into mice at the concentration of 50 mg/kg, no toxic symptoms or loss of body weight were observed, although PLL alone caused significant acute toxicity at the same dose. A similarly low toxicity was reported for a compound made of peptides covalently linked to a ε-poly(L-lysine citramide) carrier (Couffin-Hoarau et al., 2009). Both conjugates have a similar higher order proteoglycan-like structure (Fig. 1C), but the backbone of pseudoPG in this study is α-PLL, retaining mostly unsubstituted amino groups except the substitution of 4–10 neutral Dex chains. In comparison, the peptide-poly(L-lysine citramide) conjugates do not possess free amino groups but have carboxyl groups on both the backbone carrier and hydrophobic peptide side chains (Couffin-Hoarau et al., 2009). Considering the markedly different surface charge distributions of the pseudoPG and peptide-poly(L-lysine citramide) conjugates, the action mechanisms of these two macromolecules would differ considerably, but involvement of the PG-like structure in the HIV-inhibition mechanism is still possible. In this context, this study proposes the use of a neoglycoconjugate, pseudoPG, to develop a novel concept of anti-HIV-1 activity.

The multivalent carbohydrate (MVC)-linked dendrimers (Rosa Borges et al., 2010) contain globotriose (average of 46 sugars/64mer) or 3′-sialyllactose (average of 28 sugars/64mer) covalently attached to a dendrimer core, which specifically inhibits HIV-1 adhesion to the membrane surface of carbohydrates. On the other hand, the synthesized PLL (25 k)-maltotriose pseudoproteoglycan containing on average 10 linked maltotriose chains per PLL chain completely lacked anti-HIV-1 activity (our unpublished data). This suggests that the length of the dextran chain in PLL–Dex is important, while the specific triose ligands on MVC-linked dendrimers also play an essential role in the respective anti-HIV-1 activities. Because the dextran chain is a linear polymer containing $\geqslant$50 glucose residues, PLL–Dex is quite different from the MVC-derivatized dendrimer, not only in oligosaccharide structure but also in steric structure. In solution, dextrans form helical chains that extend from the polyL-lysine backbone helix, and as a whole, they resemble a proteoglycan structure (Fig. 1C), in contrast to the spherical structure of a triose-coupled dendrimer. Thus, the anti-HIV-1 mechanisms of the PLL–Dex and the MVC dendrimer may be different.

Because of its rather high molecular weight, PLL–Dex would be suitable to prevent viral infection through use as an ointment or spray to prevent the attachment of HIV-1 to mucous membranes of healthy humans. In addition, PLL–Dex could be used as a coating material on medical instruments to inactivate the virus and inhibit infection of medical staff and patients' families. Although the current HAART therapy includes mainly a combination of approved antiretroviral drugs such as inhibitors of reverse transcriptases or proteases, the FDA recently approved Maraviroc, a CCR5 antagonist, for treatment of HIV-1 infection of chronically infected patients. Maraviroc prevents adsorption and entry of HIV-1 into the cell and is used most commonly for the treatment of R5 virus infection in patients who have failed to respond to other antiretroviral regimens (Wilkin and Gulick, 2011). Therefore, we consider that PLL–Dex may potentially serve as an anti-HIV-1 reagent like Maraviroc by suppressing adsorption and entry of HIV-1 into the cells. If the absorbability, effect on coagulation, and long-term toxicity in vivo of PLL–Dex were established, it might provide a new alternative for HIV-1 therapy. In the experiments on the preventive mechanisms of pseudoPGs against HIV-1, PLL–Dex pseudoPG was observed to almost completely inhibit the cellular entry at both the pre- and post-adsorption stages (Fig. 4B and C). PLL–Dex was found to act both on the virus and cells to suppress the infection (Fig. 5). The cell surface receptors for the pseudoPG, as well as the receptor on the virus, are yet unknown, and further studies are required to elucidate the mechanism of PLL–Dex.

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