0026-895X/08/7402-330–337$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 74:330–337, 2008

Vol. 74, No. 2
47621/3363541
Printed in U.S.A.

ACCELERATED COMMUNICATION

Simian Immunodeficiency Virus Is Susceptible to Inhibition by Carbohydrate-Binding Agents in a Manner Similar to That of HIV: Implications for Further Preclinical Drug Development

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Received March 31, 2008; accepted May 8, 2008

ABSTRACT

Carbohydrate-binding agents (CBAs), such as the plant lectins *Hippeastrum* hybrid agglutinin (HHA) and *Urtica dioica* agglutinin (UDA), but also the nonpeptidic antibiotic pradimicin A (PRM-A), inhibit entry of HIV into its target cells by binding to the glycans of gp120. Given the high sequence identity and similarity between the envelope gp120 glycoproteins of HIV and simian immunodeficiency virus (SIV), the inhibitory activity of a variety of CBAs were evaluated against HIV-1, HIV-2, and SIV. There seemed to be a close correlation for the inhibitory potential of CBAs against HIV-1, HIV-2, and SIV replication in cell culture and syncytia formation in cocultures of persistently SIV-infected HUT-78 cell cultures and uninfected CEM cells. CBAs also inhibit transmission of the SIV to T lymphocytes after capture of the virus by dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN)-expressing cells. A total of 8 different SIV strains were isolated after prolonged HHA, UDA, and PRM-A exposure in virus-infected cell cultures. Each virus isolate consistently contained at least 2 or 3 glycan deletions in its gp120 envelope and showed decreased sensitivity to the CBAs and cross-resistance toward all CBAs. Our data revealed that CBAs afford SIV and HIV-1 inhibition in a similar manner regarding prevention of virus infection, DC-SIGN-directed virus capture-related transmission, and selection of drug-resistant mutant virus strains. Therefore, SIV_{mac251}-infected monkeys might represent a relevant animal model to study the efficacy of CBAs in vivo.

The envelope glycoproteins of HIV and simian immunodeficiency virus (SIV) mediate attachment and entry into the host cells. The envelope proteins consist of a surface glycoprotein (in both cases designated gp120) and a transmembrane glycoprotein (designated gp41 for HIV and gp32 for SIV, respectively) (Ohgimoto et al., 1998). Infection with HIV and SIV occurs through the binding of gp120 with the CD4 receptor and a coreceptor (Berger et al., 1999; Pöhlmann and Doms, 2002). Although HIV can use both CCR5 and CXCR4 as coreceptor, most SIV strains can only efficiently infect its target cells using the CCR5 coreceptor (Chen et al., 1997; Edinger et al., 1997; Marcon et al., 1997), although it was reported that a few laboratory SIV strains can also use the CXCR4 coreceptor for entry (Marx and Chen, 1998). The C-type lectin DC-SIGN, present on dendritic cells of the innate immune system (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b), is also able to capture SIV and transmit the virus to $CD4^+$ T lymphocytes present in the lymph nodes (Pöhlmann et al., 2001). In contrast to HIV-1 infection of chimpanzees, infection of macaque species with SIV isolates results in a disease that share many similarities with HIV infection and AIDS in humans (Van Rompay, 2005).

The three dimensional crystal structures of HIV gp120 and SIV gp120 were recently elucidated (Kwong et al., 1998; Wyatt et al., 1998; Chen et al., 2005a,b). Comparison of the gp120 core of SIV_{mac32H} with $HIV\text{-}1_{HXBc2}$ shows a 35% sequence identity and more than 70% nucleotide sequence sim-

This work was supported by the European Commission (René Descartes Prize-2001, Krediet no. HPAW-2002-90001; and EMPRO 503558 of the 6th Frame Work Programme), the Fonds for Wetenschappelijk Onderzoek (FWO) Krediet nr. G-0485-08 and the Centers of Excellence of the Katholieke Universiteit Leuven (Krediet no. EF/05/015).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.047621.

ABBREVIATIONS: SIV, simian immunodeficiency virus; CBA, carbohydrate-binding agent; PRM-A, pradimicin A; HHA, *Hippeastrum* hybrid agglutinin; GNA, *Galanthus nivalis* agglutinin; UDA, *Urtica dioica* agglutinin; PVAS, polyvinyl alcohol sulfate; DC-SIGN, dendritic cell-specific ICAM3-grabbing nonintegrin; dNTP, deoxynucleotide triphosphate; PCR, polymerase chain reaction; FCS, fetal calf serum.

ilarity. Moreover, a considerable number of glycosylation sites on HIV-1 and SIV gp120 are highly conserved between both viruses or are shifted by no more than one or two amino acid residues (Chen et al., 2005b). Because of the high degree of envelope similarity, SIV infection of monkeys could represent a reliable model for the study of HIV entry inhibitors that involve glycan binding.

Despite the continuous search for new entry inhibitors, only one HIV fusion inhibitor, enfuvirtide (T20; Fuzeon), has been approved for HIV treatment (Kilby et al., 1998). An interesting new approach, however, is targeting the glycans of the HIV gp120 by chemotherapeutics (Balzarini, 2005, 2007). Several carbohydrate-binding agents (CBAs) derived from prokaryotic, plant, invertebrate, or vertebrate species with specificity for mannose, β-galactose, or *N*-acetylglucosamine have been reported to be endowed with pronounced antiviral activity in a variety of cell culture systems, in particular against HIV (Balzarini, 2006). Under escalating pressure by CBAs including plant lectins derived from *Hippeastrum* hybrid (HHA) and *Urtica dioica* (UDA), several mutant HIV strains could be isolated predominantly containing deletions of glycans in the gp120 envelope protein (Balzarini et al., 2004, 2005). In addition, the nonpeptidic low-molecular-weight antibiotic pradimicin A (PRM-A) has been shown to select for glycan deletions in HIV-1 gp120 (Balzarini et al., 2007b). PRM-A has a unique nonpeptidic structure that contains the amino acid D-Ala and the carbohydrate D-xylose and 4,6-dideoxy-4-methylamino-D-galactose attached to a substituted 5,6-dihydrobenzo[a]naphthacenequinone. It specifically binds to the terminal D-mannose pyranosides of α(1,2)-mannose oligomers. It requires the presence of Ca^{2+}, which is thought to bind two PRM-A molecules through the free carboxylic acid group present on each molecule (Ueki et al., 1993).

To reveal whether SIV could serve as a reliable model for studying the anti-HIV activity of CBAs in the in vivo (monkey) setting, three relevant CBAs [the α(1,3)/α(1,6)-mannose specific HHA, the GlcNAc-specific UDA, and the α(1,2)-mannose-specific PRM-A] have been investigated against SIV for their antiviral potential in virus-infected cell cultures. In addition, mutant SIV_{mac251} strains were selected under escalating drug pressure of HHA, UDA, and PRM-A, the resulting drug-related mutations in SIV gp120 were determined, and (cross-)resistance of the mutant SIV strains against several other CBAs was examined. Finally, the potential of the CBAs to prevent capture of SIV by DC-SIGN was studied.

Materials and Methods

Test Compounds. PRM-A (molecular weight, 838) was isolated and purified from the fermentation broth of *Actinomyces* sp. TP-A0016 (Dairi et al., 1997). The mannose-specific plant lectins from *Galanthus nivalis* (GNA) and HHA and UDA were derived and purified from plants, as described previously (Van Damme et al., 1988a,b). Dextran sulfate-5000 was obtained from Sigma (St. Louis, MO). Polyvinyl alcohol sulfate (PVAS) was kindly provided by Dr. S. Görög (Budapest, Hungary).

Cells. Human T lymphocytic CEM, MT4, C8166, and HUT-78 cells were cultivated in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; Lonza Verviers SPRL, Verviers, Belgium), 2 mM L-glutamine, and 20 μg/ml gentamicin (Invitrogen).

The Raji/DC-SIGN cells were constructed by Geijtenbeek et al (2000a). Wild-type Raji/0 and DC-SIGN-expressing Raji/DC-SIGN cells were kindly provided by Dr. L. Burleigh (Institut Pasteur, Paris, France) and cultivated in RPMI-1640 culture medium as described above.

U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cells were established as described previously (Princen et al., 2004) and cultivated in DMEM containing 10% FCS supplemented with 0.4% G418 (Geneticin; Invitrogen) and 1% puromycin (Invitrogen).

Viruses. HIV_{IIIB} was kindly provided by R. C. Gallo (at that time at the National Cancer Institute, National Institutes of Health, Bethesda, MD) and $HIV\text{-}2_{ROD}$ by Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France). Simian immunodeficiency virus SIV_{mac251} was provided by Dr. H. Egberink (Utrecht, The Netherlands).

Antiretrovirus Assays. The methodology of the anti-HIV and anti-SIV assays has been described previously (Balzarini et al., 1991). In brief, CEM cells (4.5×10^5 cells/ml) were suspended in

TABLE 1
Primers for the amplification and sequencing of the envelope of SIV_{mac251}

Primer	Sequence	Description
KF92	5′-TCAACTATACCGCCCTCTTGAGGCGTGC-3′	Outer sense primer for amplification of *env*
KF93	5′-TTGCACTGTAATAAATCCCTTCCAGTCCCC-3′	Outer antisense primer for amplification of *env*
KF94	5′-TATGAGCAGTCACGAAAGAGAAGAAGAACT-3′	Inner sense primer for amplification of region 1
KF95	5′-CTTAGGGGAACTTTTGGCCTCACTGATACC-3′	Inner antisense primer for amplification of region 1
KF102	5′-AATTTGTCCACATGAAGGTAACTTCCG-3′	Inner sense primer for amplification of region 2
KF103	5′-AACCAATCAATGATAGGCCAAAGCAGGC-3′	Inner antisense primer for amplification of region 2
KF94	5′-TATGAGCAGTCACGAAAGAGAAGAAGAACT-3′	Sense primer for sequencing of region 1
KF67	5′-ACTTGGGGAACAACTCAGTGCC-3′	Sense primer for sequencing of region 1
KF68	5′-GAGACTAGTTCTTGTATAGC-3′	Sense primer for sequencing of region 1
KF69	5′-CAAGGATGATGGAGACACAGAC-3′	Sense primer for sequencing of region 1
KF70	5′-TAAATTGGGTAGARGATAGG-3′	Sense primer for sequencing of region 2
KF71	5′-TAGGGTTCTTGGGTTTTCTCG-3′	Sense primer for sequencing of region 2
KF72	5′-ATGTATGAATTACAAAAGTTG-3′	Sense primer for sequencing of region 2
KF73	5′-AGYATACCAGATCCTCCAACC-3′	Sense primer for sequencing of region 2
KF74	5′-TCCACCGTCTCRTTCTTTGCC-3′	Antisense primer for sequencing of region 2
KF75	5′-AACCTTTCGCTCCCACTCTTGCC-3′	Antisense primer for sequencing of region 2
KF76	5′-TGTACCTCTTCACATCTGTGGG-3′	Antisense primer for sequencing of region 2
KF77	5′-TTATCAGTATTGTTAGTTCC-3′	Antisense primer for sequencing of region 1
KF78	5′-AAAGCATAACCTGGAGGTGC-3′	Antisense primer for sequencing of region 1
KF79	5′-CCCCATCTATCTGTCTCACTT-3′	Antisense primer for sequencing of region 1
KF95	5′-CTTAGGGGAACTTTTGGCCTCACTGATACC-3′	Antisense primer for sequencing of region 2

fresh culture medium and infected with HIV-1 or HIV-2 at 100 times the cell culture 50% infective dose ($CCID_{50}$) per ml of cell suspension in the presence of appropriate dilutions of the test compounds. After 4 to 5 days of incubation at 37°C, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC_{50}) corresponds to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures. MT4-cells (5×10^5 cells/ml) were suspended in fresh culture medium and infected with SIV at 100 times the $CCID_{50}$ per ml of cell suspension in the presence of appropriate dilutions of the test compounds. After 4 days' incubation at 37°C, virus-induced destruction of the MT4-cells was recorded by the trypan blue dye exclusion method.

Cross-resistance of mutant SIV strains to CBAs was determined using U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cells. In a 48-well plate, 1.8×10^4 U87/$CD4^+$/$CXCR4^+$/CCR5 cells/ml were seeded and incubated for 2 h at 37°C. Then, 2500 to 5000 pg/ml SIV_{mac251} was added to the cell cultures in the presence of appropriate dilutions of the test compounds. After 7 days of incubation at 37°C, giant cell formation was recorded microscopically. The 50% effective concentration (EC_{50}) corresponds to the compound concentrations required to prevent syncytium formation in the virus-infected U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cell cultures by 50%.

Generation of Persistently SIV_{mac251}-Infected HUT78 Cells, and Effect of CBAs on Giant Cell Formation in Cocultures of HUT-78/SIV and C8166 Cells. Persistently SIV_{mac251}-infected HUT-78 (designated HUT-78/SIV) were generated by infection of 1×10^5 HUT78 cells/ml with SIV_{mac251} (800,000 pg/ml). The cells were subcultured every 3 to 4 days, and infection was monitored using the SIV p27 antigen enzyme-linked immunosorbent assay kit (Gentaur, Brussels, Belgium). For the cocultivation assay, HUT78/SIV cells were washed to remove free virus form the culture medium, and 5×10^4 cells (50 μl) were transferred to 96-well microtiter plates. Then, a similar amount of C8166 cells (50 μl), along with an appropriate concentration of test compound (100 μl), was added to each well. After 2 days, the EC_{50} values were determined based on the appearance of syncytia in the cocultures.

Flow Cytometric Analyses. The persistently SIV_{mac251}-infected HUT78 cells were incubated with anti-gp120 mAb 9205 (PerkinElmer Life and Analytical Sciences, Zaventem, Belgium) and were processed for flow cytometry as described previously (Schols et al., 1990). The anti-gp120 mAb (9205) [raised against a synthetic peptide representing amino acids 308 to 322 of the HIV-1 IIIB strain (RIQRGPGRAFVTIGK)] was purchased from PerkinElmer Life and Analytical Sciences (Skinner et al., 1988). Although the anti-gp120 mAb does not react with SIV_{agm385}, it does cross-react with SIV_{mac251}. In brief, after washing with PBS containing 2% FCS, SIV_{mac251}-infected HUT78 cells were preincubated with the anti-gp120 mAb 9205 for 30 min at 4°C. Then the cells were washed and incubated with phycoerythrin-labeled goat-anti-mouse IgG (Invitrogen) for 30 min at 4°C. As a control for aspecific background staining, cells were stained in parallel with goat-anti-mouse IgG-phycoerythrin only.

Capture of SIV_{mac251} by Raji/DC-SIGN and Subsequent Cocultivation with C8166 Cells. Wild-type Raji/0 and Raji/DC-SIGN cells were suspended in cell culture medium at 1×10^6 cells/500 μl. SIV_{mac251} (100 μl) was added, in the presence of 400 μl of test compound or medium (mock-treated). After 60 min of incubation at 37°C, the cells were carefully washed three times to remove unbound virus particles and resuspended in 1 ml of cell culture medium. Two hundred microliters was added to uninfected 2×10^5 C8166 cells (800 μl) in a 48-well plate. The cocultures were incubated at 37°C, and giant cell formation was microscopically estimated the next day.

Selection and Isolation of CBA-Resistant SIV_{mac251} Strains. The procedure followed for the selection of drug-resistant SIV mutants was comparable with that described for HHA, UDA, and PRM-A against HIV-1 (Balzarini et al., 2004, 2005). In brief, SIV_{mac251} was added to MT4 cell cultures in 48-well plates in the presence of HHA, UDA, or PRM-A at a concentration equal to 1- to 2-fold the respective EC_{50} values. Subcultivations occurred after every 3 to 4 days by transferring 50 μl of the drug-exposed SIV-infected cells to 950 μl of uninfected freshly prepared cell cultures. For the generation of drug-resistant virus mutants, escalating 2-fold higher drug concentrations were administered when, in the previous cell culture, the virus afforded a full cytopathogenic effect. The drug resistance selection schedules for the individual experiments are depicted in Fig. 2, A–C.

Amplification of the SIV_{mac251} *env* Region. Proviral DNA was extracted from cell pellets using the DNeasy Tissue Kits (QIAGEN, Hilden, Germany). The primers used for the amplification and sequencing of the envelope of SIV_{mac251} are depicted in Table 1. A 3038-bp nucleotide fragment, encompassing the entire *env* region, was amplified in an outer PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) under the following conditions: 1× Expand HF Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primer KF92, 0.4 μM primer KF93, and 2.625 units of the Expand High Fidelity PCR System enzyme mix. Cycling conditions

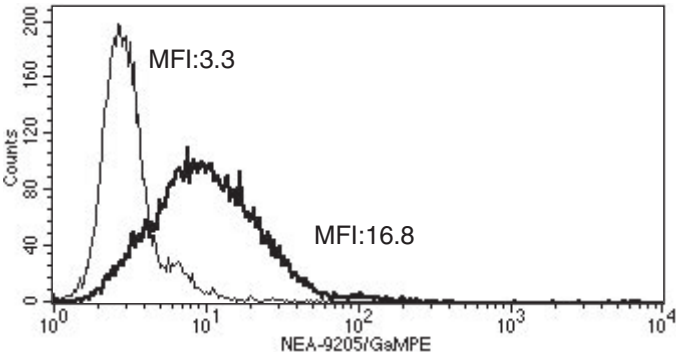

Fig. 1. Staining of anti-gp120 mAb 9205 on persistently SIV_{mac251}-infected HUT78 cells. The MFI of the background fluorescence (thin histogram) is 3.3 and the MFI (bold histogram) for the mAb 9205 specific binding is 16.8.

TABLE 2
Antiviral activity of HHA, GNA, UDA, and PRM-A in cell culture
Values are presented as mean ± S.D. of two to three independent experiments. Molecular weights of the CBAs: HHA, 50,000; GNA, 50,000; UDA, 8,700; PRM-A, 838.

CBA[c]	EC_{50}		
	HIV-1_{IIIB} (CEM)	HIV-2_{ROD} (CEM)	SIV_{mac251} (MT-4)
	μM		
HHA	0.006 ± 0.003	0.016 ± 0.000	0.010 ± 0.0008
GNA	0.018 ± 0.0	0.011 ± 0.007	0.042 ± 0.012
UDA	0.140 ± 0.040	0.330 ± 0.090	0.162 ± 0.034
PRM-A	3.4 ± 1.2	1.8 ± 0.0	5.0 ± 0.30

TABLE 3
Inhibitory activity of CBAs against giant cell formation in co-cultures of SIV- or HIV-1-exposed Raji/DC-SIGN cells and uninfected C8166 cells
Values are presented as mean ± S.D. of two to three independent experiments.

CBA	IC_{50} (Giant Cell Formation)	
	Raji/DC-SIGN/HIV-1 and C8166 Cocultures	Raji/DC-SIGN/SIV and C8166 Cocultures
	μM	
HHA	0.64 ± 0.15[a]	0.032 ± 0.017
GNA	0.84 ± 0.05[a]	0.046 ± 0.040
UDA	3.4 ± 1.1[a]	0.38 ± 0.33
PRM-A	23	19 ± 8.9

[a] Data taken from Balzarini et al. (2007a).

were: a denaturation step of 2 min at 95°C followed by 10 cycles of 15 s at 95°C, 30 s at 55°C, and 4 min at 68°C followed by 30 cycles of 15 s at 95°C, 30 s at 55°C, 4 min at 68°C + cycle elongation of 5 s for each cycle and a final extension step of 10 min at 72°C. A 1239-bp nucleotide fragment (region 1) was amplified in an inner PCR using the Expand High-Fidelity PCR System under the following conditions: 1× Expand HF Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primer KF94, 0.4 μM primer KF102, and 2.625 U of the Expand High Fidelity PCR system enzyme mix. Cycling conditions were as follows: a denaturation step of 2 min at 95°C followed by 10 cycles of 15 s at 95°C, 30 s at 56°C, 3 min at 68°C followed by 20 cycles of 15 s at 95°C, 30 s at 56°C, 3 min at 68°C + cycle elongation of 5 s for each cycle and a final extension step of 10 min at 72°C. A 1772-base pair nucleotide fragment (region 2) was amplified in an inner PCR using Expand High Fidelity PCR System under the following conditions: 1× Expand HF Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primer KF95, 0.4 μM primer KF103, and 2.625 U of the Expand High Fidelity PCR system enzyme mix. Cycling conditions were as follows: a denaturation step of 2 min at 95°C followed by 10 cycles of 15 s at 95°C, 30 s at 56°C, and 3 min at 68°C followed by 20 cycles of 15 s at 95°C, 30 s at 56°C, 3 min at 68°C + cycle elongation of 5 s for each cycle and a final extension step of 10 min at 72°C.

Genotyping of the SIV_{mac251} *env* Region. PCR products were purified with the Qiaquick PCR Purification kit (QIAGEN). Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit, and the reactions were run on an ABI3100 Genetic Analyzer (Applera, Nieuwekerk a/d Issel, The Netherlands).

Results

Antiviral Activity of CBAs against $HIV\text{-}1_{IIIB}$, $HIV\text{-}2_{ROD}$, and SIV_{mac251} Infection. The α(1,3)/α(1,6)-mannose-specific HHA, the α(1,3)-mannose-specific GNA, the GlcNAc-specific UDA, and the α(1,2)-mannose-specific nonpeptidic PRM-A antibiotic were examined for their potential to inhibit HIV-1-, HIV-2- and SIV-induced cytopathicity in cell culture (Table 2). The antiviral activity of HHA, GNA, UDA, and PRM-A against HIV-1 and HIV-2 was analyzed in CEM cell cultures, whereas for SIV, MT4 cell cultures were used. Each individual CBA inhibited the three different viruses to a similar extent. HHA and GNA were the most active compounds against HIV-1, HIV-2, and SIV; EC_{50} values ranged between 0.006 and 0.042 μM. UDA was less active against the three different viruses; EC_{50} values ranged between 0.140 and 0.330 μM. PRM-A was the least active compound with EC_{50} values in the lower micromolar range (1.8–5.0 μM) (Table 2).

Inhibitory Activity of CBAs on Giant Cell Formation in a Cocultivation Assay Between Persistently SIV-Infected HUT-78/SIV and Uninfected CEM Cells. The HUT-78 cells persistently infected with SIV_{mac251} were selected by exposing uninfected HUT-78 cells to SIV and culturing the infected cell cultures for several weeks (subcultivations twice a week). The HUT-78/SIV cells derived after 4 weeks stably expressed SIV gp120 in the cell membrane, as assessed by flow cytometric analysis (Fig. 1).

The inhibitory activity of HHA and PRM-A was then determined in a cocultivation assay between the persistently SIV-infected HUT-78/SIV cells and uninfected T-lymphocytic CEM cells. After 24 h, the IC_{50} values were determined microscopically based on the quantification of giant cell formation. The CBAs efficiently prevented syncytia formation at an IC_{50} (50% inhibitory concentration) of 0.22 ± 0.15 μM (for HHA) and 5.8 ± 0.42 μM (for PRM-A), respectively.

Inhibitory Effect of the CBAs on the Ability of Raji/DC-SIGN to Capture and Transmit SIV_{mac251} to Uninfected C8166. It was previously shown that the CBAs such as HHA, UDA, and PRM-A were able to efficiently prevent capture of HIV-1 by DC-SIGN (Balzarini et al., 2007a). In

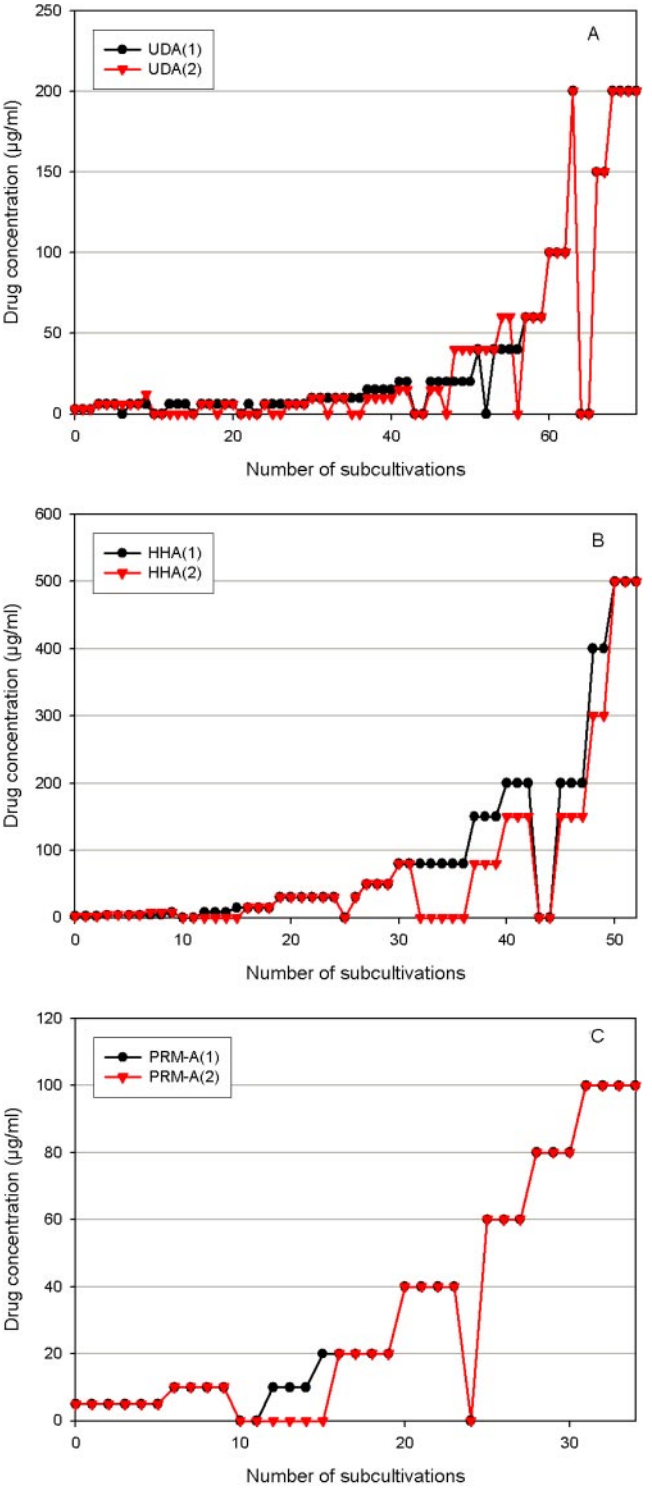

Fig. 2. Selection pathway for SIV_{mac251} strains resistant to UDA (A), HHA (B), and PRM-A (C). The drug concentrations were increased when abundant viral cytopathicity was observed in the cell cultures. Each subcultivation was performed every 4 to 5 days. Fifty microliters of the virus-infected, drug-exposed MT4 cell cultures were added to 950 μl of fresh uninfected MT-4 cell culture at each passage.

this study, we wanted to investigate whether the CBAs were also able to prevent SIV_{mac251} capture by DC-SIGN-expressing cells followed by transmission of SIV to uninfected T-lymphocyte cells. Capture of SIV by Raji/DC-SIGN cells followed by subsequent transmission of SIV-captured virus to uninfected C8166 cells was markedly inhibited by the CBAs. HHA and GNA were most pronounced in their inhibitory activity (EC_{50}, 0.032–0.046 μM), whereas PRM-A inhibited virus capture by DC-SIGN in the lower micromolar range (EC_{50}, 19 μM) (Table 3). Raji/0 cells that do not express DC-SIGN do not capture SIV (data not shown). There was a good correlation for the inhibitory activity of the CBAs against HIV-1 and SIV-capture by DC-SIGN and subsequent virus transmission to T lymphocytes ($r^2 = 0.99$) (Table 3).

Selection of HHA-, UDA-, and PRM-A-resistant SIV_{mac251} strains. SIV_{mac251}-infected MT4 cell cultures in 48-well plates were exposed to a CBA concentration that corresponds to 1- to 2-fold the determined EC_{50} value of HHA, UDA and PRM-A. Every 3 to 4 days, the drug-exposed cultures were subcultured by transferring 50 μl of the drug-exposed virus-infected cell suspension to freshly prepared (950 μl) MT4 cell cultures. Giant cell formation was recorded microscopically, and once full cytopathic effect was observed, the drug concentration was increased. Besides microscopic determination of virus breakthrough, p27 levels were also regularly determined using the SIV p27 antigen enzyme-linked immunosorbent assay kit. At least 15 to 20 subcultures were required for SIV_{mac251} to replicate in the presence of PRM-A at concentrations that were at least >10-fold the EC_{50}, and approximately 25 to 50 SIV-infected MT4 cell subcultures were required to grow in the presence of more than 10-fold higher EC_{50} values for HHA and UDA (Fig. 2).

Mutational Analysis of the Envelope of SIV_{mac251} Strains Isolated upon Escalating HHA, UDA and PRM-A Drug Pressure. After extraction of the viral DNA from the MT4 cell cultures, the envelope gene of SIV_{mac251}, which consists of *gp120* and *gp32*, was amplified and sequenced. We compared the mutational patterns, obtained from eight independently selected SIV strains with the genetic *env* background in the wild-type SIV_{mac251}that had been subcultured for a similar number of passages in the absence of CBAs. In all drug-exposed virus strains, two to three *N*-glycosylation sites were deleted (Table 4, Fig. 3). Although the mutant virus strains are polyclonal, in most cases, only the mutant nucleotide sequence was detected in the sequencing assays. In only a few cases, a mixture of both mutant and wild-type nucleotide sequence was observed, as indicated in Table 4. Glycosylation motifs were most often deleted by mutating the asparagine residues (13 of 19 mutations). Only 1 virus strain selected under PRM-A drug pressure [designated SIV/PRM-A(4)] contained a glycosylation site mutation in the gp32 gene (636NDT638). All the selected SIV strains invariably contained a mutation at position Asn462, resulting in a deletion of the 462NST464 *N*-glycosylation motif. Moreover, five of the eight mutant SIV strains showed also an additional mutation at the Thr248 position, thus removing the 246NDT248 glycosylation motif in the SIV gp120 envelope. The threonine was mostly (four of five SIV strains) replaced by an isoleucine, but in one strain it was replaced by a proline. In three virus strains, the glycosylation motif 200NST202 was mutated, whereas the glycosylation sites at Asn70 and Asn373 were found to be mutated in only one of the isolated virus strains (Table 4).

Sensitivity of Mutant SIV_{mac251} Isolates against CBAs. The mutant SIV/HHA(1), SIV/UDA(1), and SIV/PRM-A(1) strains were evaluated for their sensitivity against the CBAs

TABLE 4

Mutations at the amino acid positions of the gp120 envelope of SIV_{mac251}

In most cases, solely the mutated nucleotide was detected. In a few cases, a mixture of mutant and wild-type nucleotide (roughly estimated to be at a ratio around 50/50) was observed and indicated by the designation of wild-type/mutant amino acid.

Glycosylated Asparagine Sites in SIV Env	Mutations in the Envelope That Emerged underEscalating Drug Exposure in MT4 Cell Cultures							
	PRM-A-1	PRM-A-2	PRM-A-3	PRM-A-4	HHA-1	HHA-2	UDA-1	UDA-2
37NAT39								
70NVT72						70Asn/Asp		
114NKS116								
148NET150								
158NCT160								
173NMT175[a]								
186NET188								
200NST202			200Asn/Ser		200Asn/Asp			
204NES206							204Asp	
214NTS216								
246NDT248	248Ile	248Ile	248Pro		248Ile	248Ile		
249NYS251								
280NGT282								
286NRT288								
297NRT299								
308NLT310								
318NKT320								
373NNT375								375Ile
379NLT381								
462NST464	462Ser	462Ser	462Asp	462Asp	462Thr	462Thr	462Ser	462Ser
478NQT480								
627NAS629								
636NDT638				636Asn/Lys				
652NIT654								

[a] In the SIV_{mac251} strain used in the selection experiments, an extra glycosylation site was created as a result of a T173N mutation.

GNA, HHA, UDA, and PRM-A, and the polyanions dextran sulfate (M_r 5000) and PVAS were included as controls (Table 5). SIV/HHA(1) proved approximately 12 times more phenotypically resistant to HHA, compared with wild-type SIV_{mac251}. SIV/UDA(1) and SIV/PRM-A(1) were 7 and 5 times less sensitive for UDA and PRM-A, respectively. The mutant SIV_{mac251} strains showed cross-resistance to the other CBAs evaluated, irrespective of the carbohydrate specificity of the particular CBA. In contrast, the antiviral activity of the polyanionic entry inhibitors dextran sulfate (M_r 5000) and PVAS remained essentially unchanged in all the mutant SIV strains (Table 5).

Discussion

Recent evidence has shown that CBAs bind to the glycans on the gp120 envelope of HIV, thereby preventing viral entry. CBAs could also force the virus to delete glycan moieties in its envelope to escape drug pressure [for an overview, see Balzarini (2007) and references therein]. It has been hypothesized that these glycan deletions may create "holes" in the protective glycan shield of the envelope, which may result in exposed immunogenic epitopes that can be targeted by the immune system of the host (Balzarini, 2005). However, the latter effect can only be investigated in a relevant animal model (i.e., SIV-infected monkeys). So far, no experiments have been conducted to investigate the impact of CBAs on SIV. In this study, HHA, GNA, UDA, and PRM-A were found to show strikingly comparable inhibitory activities against SIV, HIV-1, and HIV-2 in cell culture. In addition, syncytia formation between persistently SIV-infected HUT-78 cells and uninfected T lymphocytes, SIV capture by DC-SIGN-expressing cells, and subsequent transmission of the virus to uninfected T lymphocytes are comparably inhibited by the investigated CBAs irrespective of the nature of the lentivirus. Thus, our studies have provided the first evidence that CBAs could have a similar mode of antiviral action against SIV and HIV.

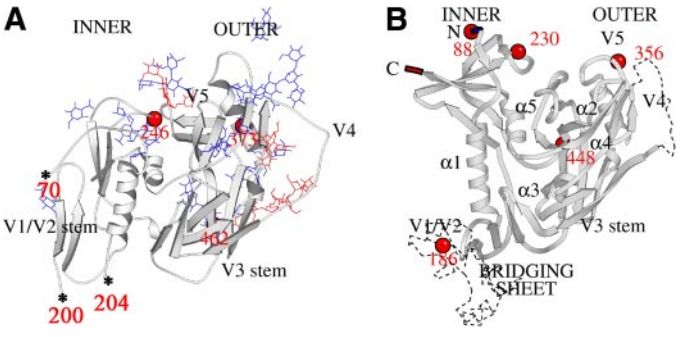

Fig. 3. Mapping of the deleted glycosylation sites in gp120 of SIV_{mac251} strains selected under escalating HHA, UDA, and PRM-A concentrations in MT4 cell culture and comparison of the SIV_{mac251} core gp120 with the HIV-1 core gp120. A, core structure of gp120 of SIV_{mac251}, as determined by Chen et al. (2005a,b). The mutated glycosylation sites are marked in red on the structure or with an asterisk in the N terminus and V1/V2 loop of SIV gp120 that were not resolved in the crystal structure. B, core structure of gp120 of HIV-1 (Kwong et al., 1998; Wyatt et al., 1998). The corresponding deleted glycans in HIV-1 gp120 are marked in red. Position Asn88 in HIV-1 corresponds to position Asn70 in SIV_{mac251}, position Asn186 to Asn200, position Asn230 to Asn246, position Asn356 to Asn373, and position Asn448 to Asn462. The *N*-glycan at position 204 in SIV does not have a corresponding glycan in HIV-1.

SIV and HIV contain a comparable number of glycans on their envelope. Of the 24 potential *N*-glycosylation motifs in HIV-1 gp120, 11 have been determined to be high-mannose type or hybrid type, and the remaining 13 were complex-type glycans (Leonard et al., 1990). The SIV strain used in this study also contains approximately 24 *N*-glycosylation motifs, but the nature of the glycans is currently not known. Chen et al (2005a) were able to crystallize the SIV gp120 core of SIV_{mac32H} without removal of the glycans, but the nature of the *N*-linked glycans could not be resolved in this study. However, of the 13 glycans present on the SIV gp120 core that could be resolved in the crystal structures, seven were fucosylated, which points to complex-type glycans. These glycans were located at the following amino acid positions in SIV_{mac251}: 246NDT248, 249NYS251, 280NGT282, 286NRT288, 297NRT299, 308NLT310, and 379NLT381. Their corresponding *N*-glycans in $HIV\text{-}1_{IIIB}$ are mentioned in the legend of Fig. 3. More investigations on the determination of the nature of the glycans of SIV gp120 will be necessary to be able to conclude whether a similar distribution between high-mannose and complex-type glycans exists between SIV and HIV-1. It is noteworthy that the selected SIV_{mac251} strains under CBA pressure invariably contained two to three *N*-glycan deletions. Studies involving selection of HIV-1-resistant strains in the presence of escalating CBA concentrations revealed virus strains containing up to six to nine glycan deletions in the gp120 envelope. Whereas a rather broad variety of different gp120 glycosylation sites could be affected upon CBA exposure against HIV (Balzarini, 2007), a more limited number of different glycosylation sites were mutated in SIV gp120 in our studies. This observation may point to a more targeted interaction of the CBAs to the glycans of SIV gp120. Indeed, there seems to be a clear and consistent preference for deleting the glycan moieties present on Asn246 and Asn462 of SIV gp120, because these glycosylation motifs were annihilated in virtually all (independently) selected virus strains under CBA pressure. The corresponding *N*-glycans in HIV-1 gp120 were high-mannose type, and the corresponding SIV gp120 Asn462 glycan in HIV-1 gp120 is highly conserved among a wide variety of HIV-1 clades (Balzarini et al., 2005).

TABLE 5
Antiviral activity of CBAs and polyanions against wild-type and mutant HHA-resistant, UDA-resistant, and PRM-A-resistant SIV_{mac251} strains

Virus Strain	EC_{50}					
	HHA	GNA	UDA	PRM-A	DS5000	PVAS
			μM			
SIV_{mac251}	0.002 ± 0.000	0.006 ± 0.002	0.036 ± 0.004	2.3 ± 0.06	0.38 ± 0.22	0.020 ± 0.011
SIV/UDA(1)	0.013 ± 0.008	0.013 ± 0.005	0.25 ± 0.13	9.7 ± 2.4	0.25 ± 0.13	0.017 ± 0.012
SIV/PRM-A(1)	0.034 ± 0.010	0.027 ± 0.012	0.32 ± 0.08	11 ± 0.4	0.35 ± 0.06	0.017 ± 0.003
SIV/HHA(1)	0.024 ± 0.012	0.030 ± 0.003	0.21 ± 0.04	12 ± 1.5	0.36 ± 0.04	0.017 ± 0.002

Seven years after the determination of the crystal structure of HIV-1 gp120 bound to CD4 in the absence of the glycan moieties on the envelope (Kwong et al., 1998; Wyatt et al., 1998), Chen et al (2005a,b) succeeded in crystallizing the gp120 core of SIV in an unliganded form, without removal of the *N*-linked glycans. These structures are of major importance for a better understanding of HIV entry and the conformational changes in gp120 needed for receptor binding and entry. Although the gp120 core of SIV and HIV only have 35% sequence identity, they have 70% sequence similarity (Chen et al., 2005b), which allows the assumption that the three-dimensional structure of HIV gp120 before CD4 binding might be very similar to the unliganded SIV gp120 structure, and vice versa. Moreover, all the *N*-glycans of SIV_{mac251} gp120 seem to have an equivalent in HIV-1 gp120, except for the 204NES206 glycan. When the mutations that occurred under CBA pressure in SIV_{mac251} gp120 were modeled on the three-dimensional structure of SIV gp120 and compared with the glycosylation site mutations that occurred under CBA pressure in HIV-1 gp120, the *N*-linked glycan 356NKT358 in HIV-1 has not been found to be mutated under CBA pressure (Balzarini, 2007), whereas its equivalent in SIV gp120, 373NNT375, was found to be mutated under UDA pressure. This difference could be due to a slightly different position of the *N*-glycan in SIV_{mac251} gp120 compared with HIV-1 gp120, which may affect the CBA binding (Fig. 2). The observed 636NDT638 mutation that appeared in the SIV/PRM-A(4) envelope is located in the gp32 region of the SIV gp160 and therefore could not be precisely mapped onto the SIV gp120 structure. It is rare to find a glycan mutation in the transmembrane region of SIV_{mac251} under CBA pressure. However, these findings are in agreement with similar observations made for CBA-exposed HIV-1 strains in which *N*-glycan deletions in HIV-1 gp41 also rarely occur.

Reitter et al. (1998) performed an interesting experiment in which several N-linked glycans were removed from the SIV gp120 envelope, after which the mutated virus was administered to monkeys. After 24 weeks, there was a marked increase in antibody binding to the previously hidden envelope epitopes that became uncovered upon deletion of the glycosylation sites, and a marked increase in neutralizing activity. In addition, mutant virus titers were substantially lower than wild-type virus in the infected monkeys. It is noteworthy that there seemed to be a tendency to reverse the mutated sites or to create new glycans near the mutated sites (Reitter et al., 1998). This study indicates that removal of as little as a few glycan moieties may trigger an antibody response and also provides evidence for the necessity of an intact glycan shield on the viral envelope to protect the virus against an efficient immunological attack. The importance of an intact glycan shield was also highlighted by Wei et al. (2003), who provided evidence of a continuously evolving glycan shield in HIV-1 gp120, to prevent and/or escape neutralizing antibody binding. In this respect, the CBAs may represent promising new antiviral drug leads with a dual mode of action: 1) they inhibit viral entry, by binding to the glycans on gp120, and 2) they may force the virus to remove glycan moieties in its gp120 to escape drug pressure, thus exposing the virus to the immune system triggering immunological recognition of previously hidden epitopes (Balzarini, 2005, 2007). Such additional mechanism of action can only be investigated in vivo; therefore, our findings on the similar behavior of HIV and SIV with respect to CBA pressure are important in this respect.

Besides the antiviral potential of the CBAs for systemic use, CBAs represent also interesting new agents that may qualify as microbicide drugs. Several classes of compounds were already studied for microbicidal use, such as detergents, anionic substances, and soluble CD4 (for review, see Veazey et al., 2005; Balzarini and Van Damme, 2007). It has been previously shown that macaques can be protected against chimeric pathogenic SIV/HIV-1 by vaginally delivered entry inhibitors such as the α(1,2)-mannose-specific cyanovirin N, a lectin derived from cyanobacteria. These findings provide already proof-of-concept that CBAs may show activity in the in vivo setting (Tsai et al., 2004; Veazey et al., 2005). Given the relevance of SIV-infected nonhuman primates as a valid animal model for anti-HIV drug efficacy and pathogenesis studies (Van Rompay, 2005), examining CBAs in such a monkey model would therefore be the next crucial step in the preclinical investigation of CBAs as potential new antiviral drugs, both from a systemic treatment and a microbicidal viewpoint.

In conclusion, CBAs behave markedly similarly for SIV and HIV with regard to prevention of virus infection of the target cells, virus transmission from infected cells to uninfected T lymphocytes, virus capture by DC-SIGN and subsequent transmission to T lymphocytes, and selection of virus strains containing deletions in the *N*-glycosylation motifs of their gp120 envelope. This study suggests that the impact of CBAs as potential therapeutics may be reliably investigated in SIV-infected monkeys in terms of prevention of virus (SIV) infection, virus spread and evolution, and potential involvement of the immune system upon mutation of SIV gp120 under CBA pressure.

Acknowledgments

We are grateful to Ann Absillis, Leen Ingels, and Rebecca Provinciael for excellent technical assistance, and Christiane Callebaut for dedicated editorial help.

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