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Polyanionic (i.e., Polysulfonate) Dendrimers Can Inhibit the Replication of Human Immunodeficiency Virus by Interfering with Both Virus Adsorption and Later Steps (Reverse Transcriptase/Integrase) in the Virus Replicative Cycle

MYRIAM WITVROUW, VALERY FIKKERT, WIM PLUYMERS, BARRY MATTHEWS, KAREN MARDEL, DOMINIQUE SCHOLS, JOHN RAFF, ZEGER DEBYSER, ERIK DE CLERCQ, GEORGE HOLAN, and CHRISTOPHE PANNECOUQUE

Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium (M.W., V.F., W.P., D.S., Z.D., E.D.C., C.P.); Biomolecular Research Institute, Clayton, Victoria, Australia (B.M., G.H.); and Starpharma, Ltd., Clayton, Victoria, Australia (K.M., J.R.)

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ABSTRACT

Polyanionic dendrimers were synthesized and evaluated for their antiviral effects. Phenyldicarboxylic acid (BRI6195) and naphthyldisulfonic acid (BRI2923) dendrimers were found to inhibit the replication of human immunodeficiency virus type 1 (HIV-1; strain III_B) in MT-4 cells at a EC_{50} of 0.1 and 0.3 μg/ml, respectively. The dendrimers were not toxic to MT-4 cells up to the highest concentrations tested (250 μg/ml). These compounds were also effective against various other HIV-1 strains, including clinical isolates, HIV-2 strains, simian immunodeficiency virus (SIV, strain MAC_{251}), and HIV-1 strains that were resistant to reverse transcriptase inhibitors. HIV strains containing mutations in the envelope glycoprotein gp120 (engendering resistance to known adsorption inhibitors) displayed reduced sensitivity to the dendrimers. The compounds inhibited the binding of wild-type virus and recombinant virus (containing wild-type gp120) to MT-4 cells at concentrations comparable to those that inhibited the replication of HIV-1(III_B) in these cells. Cellular uptake studies indicated that BRI2923, but not BRI6195, permeates into MT-4 and CEM cells. Accordingly, the naphtyldisulfonic acid dendrimer (BRI2923) proved able to inhibit later steps of the replication cycle of HIV, i.e., reverse transcriptase and integrase. NL4.3 strains resistant to BRI2923 were selected after passage of the virus in the presence of increasing concentrations of BRI2923. The virus mutants showed 15-fold reduced sensitivity to BRI2923 and cross-resistance to known adsorption inhibitors. However, these virus mutants were not cross-resistant to reverse transcriptase inhibitors or protease inhibitors. We identified several mutations in the envelope glycoprotein gp120 gene (i.e., V2, V3, and C3, V4, and C4 regions) of the BRI2923-resistant NL4.3 strains that were not present in the wild-type NL4.3 strain, whereas no mutations were found in the reverse transcriptase or integrase genes.

In the last decade intensive efforts have been undertaken to develop drugs against human immunodeficiency virus (HIV), the causative agent of AIDS. At present, 14 compounds have been approved by the U.S. Food and Drug Administration for the treatment of HIV infections: the dideoxynucleoside analogs [nucleoside reverse transcriptase inhibitors (NRTIs)] zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (1592U89); the non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) nevirapine (BI-RG587), delavirdine (PNU-90152T), and efavirenz (DMP266); and the protease inhibitors saquinavir (Ro31-8959), ritonavir, indinavir (L735524), nelfinavir, and amprenavir. Drug-resistant HIV-1 strains emerge after treatment with all of these drugs. The emergence of drug-resistant HIV strains has led to the search for agents with other mechanisms of action than those currently used in therapy.

Here we report a new class of polyanionic compound, i.e., polyanionic dendrimers. Dendritic molecules or dendrimers are highly branched macromolecules that are built up in layers (generations) from a reactive core group by the use of

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; CC_{50}, 50% cytotoxic concentration; DS, dextran sulfate; RT, reverse transcriptase; IN, integrase; PBMC, peripheral blood mononuclear cell; CPE, cytopathic effect; m.o.i., multiplicity of infection; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; AZT, zidovudine; ddI, didanosine; ddC, zalcitabine; d4T, stavudine; 3TC, lamivudine; PAMAM, polyamidoamine; BI-RG587, nevirapine; PNU-90152T, delavirdine; L735524, indinavir; Ro31-8959, saquinavir; BRI2923, naphthyldisulfonic acid; BRI6195, phenyldicarboxylic acid; PBS, phosphate-buffered saline.

branched building blocks. At the periphery, dendrimers can carry a high density of functional groups that determine the properties of the molecule. The means by which these molecules are put together results in materials in which such parameters as size, shape, and surface functionality can be precisely controlled. The synthesis of polyamidoamine (PAMAM) dendrimers proceeds by repeated cycles of Michael additions of methyl acrylate, followed by amidation with ethylene diamine from reactive cores such as ammonia [e.g., naphthyldisulfonic acid (BRI2923)] or ethylene diamine [e.g., phenyldicarboxylic acid (BRI6195)] to give spherical molecules. Dendrimers, as a result of their unique structure, show properties unlike most other available materials and this has led to research in a wide range of applications such as drug delivery, gene therapy, new catalysts, and energy harvesting and as agents for the stimulation of therapeutic antibodies (Newkome et al., 1996). When dendrimers were used as pharmaceutical agents, it was envisaged that the precise control over the size, shape, and functionality would allow these molecules to be designed to interact optimally with biological surfaces. Additionally, it was expected that the polyvalent functionality provided by the dendrimers would result in stronger binding to biological receptors. BRI2923 consists of a fourth generation PAMAM dendrimer scaffold built from an ammonia core, which is fully capped on the surface with 24 naphthyl disulfonic acids. BRI6195 is a fourth generation PAMAM dendrimer based on an ethylene diamine core, which is fully capped on the surface with 32 phenyl dicarboxylic acid groups. In both BRI2923 and BRI6195 the linkage group between the amino end groups of the dendrimers and the terminal capping groups are thioureas, and all the anionic groups are in the form of sodium salts. Both of these macromolecules represent single molecular structures, e.g., they are essentially monodisperse, in contrast with polydisperse polymers that are mixtures of different molecular weight species.

We have examined the antiviral activities of the polyanionic dendrimers BRI2923 and BRI6195 against different HIV-1 strains (including clinical isolates), HIV-2 strains, and SIV(MAC_{251}) and HIV-1 strains that are resistant to NRTIs, NNRTIs, or virus adsorption inhibitors. HIV-1 strains were selected after in vitro passage of NL4.3 in the presence of BRI2923, and the mode (i.e., target) of anti-HIV action of BRI2923 was elucidated.

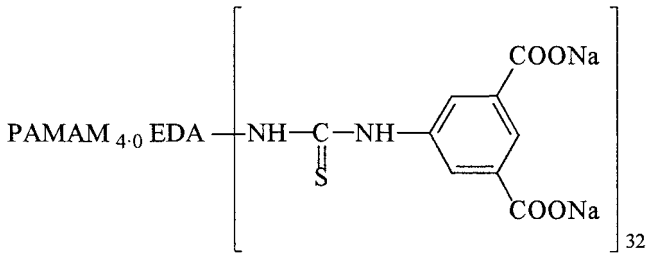
Materials and Methods

Compounds. AZT was synthesized as previously described (Horwitz et al., 1964). Dextran sulfate (DS; mol. wt. 5000), ddI, ddC, and d4T were purchased from Sigma (Bornem, Belgium). Abacavir (1592U89) and 3TC were a gift from GlaxoWellcome (Stevenage, UK). Adefovir and tenofovir were kindly provided by Gilead Sciences (Foster City, CA). BI-RG587 was obtained from Boehringer Ingelheim (Ridgefield, CT) PNU-90152T was kindly provided by Pharmacia and Upjohn (Kalamazoo, MI) by B. Bruce. DMP266 was obtained from Dr. Lee Bacheler (DuPont Pharmaceuticals, Wilmington, DE). L735524 was obtained from Dr. Huff (Merck Research Laboratories, West Point, PA), and Ro31-8959 was a gift from Dr. N. Roberts (Roche Products Limited, Welwyn Garden City, UK). The bicyclam AMD31000 was kindly provided by Geoffrey Henson, AnorMed (Langley, British Columbia, Canada).

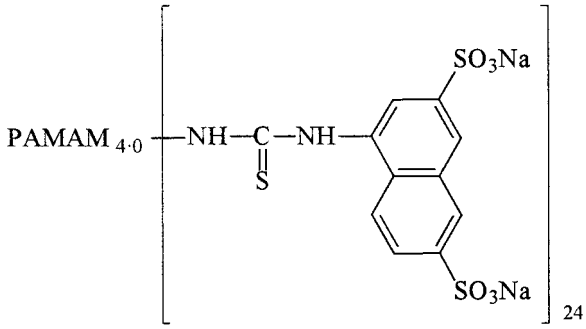
BRI2923 and BRI6195 (Fig. 1) were synthesized as described below. Microanalysis was performed for both samples. The observed deviations to the theoretical values were less than 0.4% taking into account the amount of associated water as determined by thermal gravimetry analysis. ^{1}H and ^{13}C NMR were recorded on a Varian Gemini 200 NMR spectrometer. Final products had slightly broadened ^{1}H and ^{13}C NMR signals. Starburst PAMAM 4.0 [EDA] refers to Polyamidoamine Starburst Polymer of generation 4.0 based on an ethylene diamine core and was purchased from Sigma-Aldrich (Castle Hill, Australia). Starburst PAMAM 4.0 refers to Polyamidoamine Starburst Polymer of generation 4.0 based on an ammonia core and was prepared as described in the literature (Tomalia et al., 1986; Smith et al., 1987).

Preparation of Sodium 3,6-Disulfonapthylthiourea Terminated PAMAM 4.0 Dendrimer (BRI2923). Solid sodium 3,6-disulfonapthylisothiocyanate (160 mg; 0.41 mmol) was added to a solution of PAMAM 4.0 (51 mg; 0.01 mmol) in water (3 ml), and the resulting solution was heated under nitrogen at 53°C for 2 h and then cooled. The solution was concentrated and the brown solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give the BRI2923 as a brownish solid (73 mg). ^{1}H NMR (D_2O): δ 2.30; 2.60; 2.74; 3.20; 3.57; 7.75; 7.86; 8.28. ^{13}C NMR (D_2O): δ 35.0; 39.9; 43.1; 48.1; 53.8; 56.1; 128.4; 128.6; 129.3; 131.0; 131.3; 136.0; 136.8; 138.2; 145.5; 146.0; 177.2; 177.8; 185.5.

Preparation of Sodium 3,6-Dicarboxyphenylthiourea Terminated PAMAM 4.0 [EDA] Dendrimer (BRI6195). Solid 3,5-



BRI6195



BRI2923

Fig. 1. Chemical structure of the sulfonated (BRI2923) and carboxylated (BRI6195) dendrimers.

dicarboxyphenylisothiocyanate (112 mg; 0.5 mmol) was added to a solution of PAMAM 4.0 [EDA] (69 mg; 0.01 mmol) in water (5 ml), and the pH of the solution was adjusted to 10 with 1 M sodium carbonate solution. The resulting solution was heated under nitrogen at 53ºC for 2 h and then cooled. The solution was concentrated and the solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give the BRI6195 as a white solid (112 mg). ^{1}H NMR (D_2O): δ 2.23; 2.48; 2.65; 3.12; 3.57; 7.70; 8.05. ^{13}C NMR (D_2O): δ 35.5; 40.2; 43.1; 48.1; 53.7; 56.1; 131.7; 132.6; 134.2; 141.4; 142.2; 144.3; 178.1; 184.5; 185.5.

Cells. MT-4 (Miyoshi et al., 1982) and C8166 (Salahuddin et al., 1983) cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% sodium bicarbonate, and 20 μg of gentamicin per ml. Peripheral blood mononuclear cells (PBMCs) were isolated from HIV-seronegative donor buffy coats using Lymphoprep (Nycomed, Oslo, Norway), stimulated for 3 days in medium containing phytohemagglutinin (2 μg/ml; Sigma, Bornem, Belgium) and human interleukin-2 (5 U/ml; Boehringer Mannheim, Mannheim, Germany), washed, and resuspended in RPMI 1640, supplemented with 2 mM L-glutamine, gentamicin (50 μg/ml), 15% heat-inactivated fetal calf serum, and recombinant human interleukin-2 (10 U/ml).

Viruses. The origin of the HIV-1 virus stocks (III_B and RF) have been described (Popovic et al., 1984). The HIV-1(NL4.3) strain (Adachi et al., 1986) is a molecular clone obtained from the National Institutes of Health (Bethesda, MD). The strains NL4.3/DS5000, NL4.3/AMD3100, and NL4.3/AR177 were selected in our laboratory after serial passage of HIV-1(NL4.3) in MT-4 cells in the presence of increasing concentrations of DS, AMD3100 (De Vreese et al., 1996), or AR177 (zintevir) (Esté et al., 1998), respectively. The strain 13MB1 (L100I) was isolated in our laboratory after serial passage of HIV-1(III_B) in MT-4 cells in the presence of R82913 (TIBO). L1, L2, L4, and L6 are clinical isolates from the same seropositive patient before and after sequential treatment with the dideoxynucleoside analogs AZT, ddI, ddC, d4T, and 3TC and the HIV-1-specific NNRTI loviride (R89439) (L2, RT mutation: Q151M; L4, RT mutations: V75I, F77L, F116Y, Q151M; and L6, RT mutations: V75I, F77L, K103N, F116Y, Q151M, and M184V; Schmit et al., 1996). HIV-1(HE) represents a clinical isolate from a Belgian patient with AIDS [for HIV-1(HE), virus stocks were prepared from the supernatants of MT-4 cells that had been infected with the supernatants of the fifth passage of cocultures of the patient's PBMCs with cord blood lymphocytes (at a ratio of 1:1)]. HIV-1 and HIV-2(ROD) (Barré-Sinoussi et al., 1983) and HIV-2(EHO) (Rey et al., 1989) stocks were obtained from the culture supernatants of HIV-1- or HIV-2-infected cell lines (Pauwels et al., 1987; Schols et al., 1989). SIV(MAC_{251}) was originally isolated by Daniel et al. (1987) and was obtained from C. Bruck (Smith Kline-RIT, Rixensart, Belgium).

Antiviral Activity Assays. The inhibitory effects of the dendrimers on HIV-1, HIV-2, and SIV replication were monitored by the inhibition of virus-induced cytopathicity in MT-4 and C8166 cells 5 days after infection as described (Pauwels et al., 1988; Witvrouw et al., 1998). Cytotoxicity of the compounds was determined by measuring the viability of mock-infected cells on day 5 for MT-4 and C8166 cells. PBMCs (2×10^5/200 μl) were plated in the presence of serial dilutions of the test compound and were infected with HIV-1(III_B) at 1000 $CCID_{50}$ per ml. Four days postinfection, 125 μl of the supernatant of the infected cultures were removed and replaced with 150 μl of fresh medium containing the test compound at the appropriate concentration. Seven days after the cells were plated, p24 antigen was detected in the culture supernatant by an enzyme-linked immunosorbent assay (NEN, Dreieich, Germany).

Gp120-Binding Assays. The inhibitory effect of the test compounds on recombinant gp120 [HIV-1(III_B); Intracel, London, UK] binding was measured using an indirect immunofluorescence-laser flow cytometric method that had been specifically designed for this purpose (Schols et al., 1989). Briefly, MT-4 cells were exposed to gp120 (10 μg/ml) in the presence or absence of different concentrations of test compounds. The compounds were added 10 to 20 s before the virus was added. The cells were processed for gp120 binding using an anti-gp120 monoclonal antibody (9305, NEN, Dreieich, Germany) and analyzed for cell-bound gp120 by flow cytometry after 30 min of incubation at 37°C.

Virus Adsorption Assays. In this assay the inhibitory effects of BRI2923 and DS on NL4.3 virus adsorption to MT-4 cells were measured as previously described (Witvrouw et al., 1994). Therefore, MT-4 cells (5×10^5 cells/tube) were incubated with the respective HIV strains (corresponding to 100 ng of p24) in the absence or presence of serial dilutions of the test compounds. After 2 h of incubation at 37°C, the cells were extensively washed with phosphate-buffered saline (PBS) to remove the unadsorbed virus particles. Then the cells were lysed with PBS containing 0.5% tergitol Nonidet P-40 (Sigma, St. Louis, MO). The amount of p24 antigen was determined by the p24 antigen enzyme-linked immunosorbent assay (NEN, Dreieich, Germany).

Time-of-Addition Experiments. MT-4 cells were infected with HIV-1(III_B) at a multiplicity of infection (m.o.i.) of 0.5, and the compounds were added at different times (0, 1, 2, 3, . . . 8, 25, and 26 h) after infection. Viral p24 antigen production was determined 31 h postinfection by the p24 antigen enzyme-linked immunosorbent assay (NEN). The reference compounds were added at a standardized concentration, i.e., 100 times their EC_{50} required to reduce by 50% the cytopathicity of HIV-1(III_B) (m.o.i., 0.01) in MT-4 cells (De Clercq et al., 1992).

Cellular Uptake Assays. MT-4 cells (2×10^6) were incubated for 24 h at 37°C in the presence of 10,000-fold the respective EC_{50} of BRI2923, BRI6195, or DS against the replication of HIV-1(III_B) (m.o.i., 0.01). Cells were washed five times to remove the compounds. Cells were lysed by adding milli-Q water followed by three cycles of freezing at −80°C and thawing at 37°C. The cellular debris was precipitated at 18,000 rpm for 4 h. Supernatant was carefully removed and cellular debris was lyophilized. The residue was dissolved in 400 μl of milli-Q water and was evaluated for anti-HIV-1(III_B) activity in MT-4 cells.

Confocal Microscopy. CEM cells were washed twice with cold PBS and resuspended at 10^7 cells/ml in cold PBS. Fluorescein-labeled analogs of BRI2923 or BRI6195 (10 μl) were added to 90 μl of cell suspension (final compound concentration, 15 μg/ml), incubated for 1 h at 4°C, and washed twice. The cells were examined under the confocal microscope Optiscan TM (Optiscan Imaging, Ltd., Clayton, Victoria, Australia) using fluorescein settings with ×60 oil immersion lens.

RT Assay. The RT reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, and 0.05% (w/v) Triton X-100. The template/primer [poly(C)/oligo$(dG)_{12-18}$] (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used at a concentration of 65 μg/ml. Tritium-labeled dGTP (Amersham, Pharmacia Biotech) was used at a final concentration of 2.5 μM. Specific activity was 11 Ci/mmol (1 Ci = 37 GBq). After various concentrations of the inhibitors and 10 μl of the different enzyme preparations were added, the reaction mixture was incubated for 1 h at 37°C. The incorporation rate was determined by a standard trichloracetic acid precipitation procedure using Whatman GF/C glass fiber filters (Whatman, Maidstone, England) and liquid scintillation counting (Ready-Protein; Beckman, Fullerton, CA).

HIV-1 Integrase (IN) Assays. The recombinant enzyme preparation of wild-type IN and the substrate and target DNA were as previously described (Cherepanov et al., 1997; Debyser et al., 2000). The 3′-processing and overall integration assays were slightly modified from published procedures. The final reaction mixture for the 3′-processing assays contained 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, 10 mM $MgCl_2$, 75 mM NaCl, 5% (v/v) polyethylene glycol 8000, 15% dimethyl sulfoxide, 30 nM oligonucleotide substrate, and 230 nM His-tag IN in a volume of 10 μl. Reactions were started by the addition of the enzyme. Inhibitors were incubated briefly with

the reaction components before the addition of IN. Reactions were allowed to proceed at 37°C for 7 min in the 3′-processing assay and for 1 h in the overall integration assay. Reactions were stopped by the addition of a formamide dye solution, and products were separated in a 15% denaturing polyacrylamide/urea gel. Autoradiography was performed by exposing the wet gel to X-ray film (CURIX RP1, Agfa, Germany). Quantification of the results was performed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Selection of BRI2923-Resistant HIV-1(NL4.3). BRI2923-resistant HIV-1 strains were obtained after sequential passaging of HIV-1(NL4.3) virus in the presence of increasing concentrations of BRI2923, in MT-4 cells. At the start of the selection, NL4.3 virus was inoculated in MT-4 cells in the presence of 0.1 μg/ml BRI2923. When the cytopathic effect (CPE) of HIV was observed, the cell-free culture supernatant was used as inoculum to infect fresh, uninfected MT-4 cells in the presence of equal or higher concentrations of BRI2923. After 20 and 30 passages, we were able to culture resistant virus in the presence of 8 and 20 μg/ml BRI2923, respectively.

PCR Amplification of gp120-Encoding Sequences. MT-4 cells were infected with the HIV-1(NL4.3) BRI2923-resistant strains. DNA extraction of proviral DNA was performed using the Qiagen QIAamp blood kit (Westburg, Leusden, The Netherlands). A 2105-nucleotide-bp fragment (codons 1–445) of gp120 was amplified in a nested PCR using the Expand High Fidelity PCR system (Boehringer Mannheim, Roche, Germany), which is composed of an enzyme mixture containing thermostable *Taq* DNA and *Pwo* DNA polymerase with 3′-5′-exonuclease proofreading capacity. The outer PCR reaction was performed on a Gene Amp PCR system 9600 (Perkin-Elmer, Brussels, Belgium) and the inner PCR reaction was performed on a Biometra Trioblock (Westburg) using the primers AV310 (5′-AGC AGG ACA TAA T/CAA GGT AGG-3′ corresponding to position 5447–5467 of NL4.3) and AV311 (5′-CTA CTT TAT AC/TT TAT ATA ATT CAC TTC TCC-3′ corresponding to position 7630–7659 of NL4.3), followed by the primers AV312 (5′-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3′ corresponding to position 5549–5573 of NL4.3) and AV313 (5′-TCC T/CTC ATA TT/CT CCT CCT CCA GGT C-3′ corresponding to position 7605–7629 of NL4.3). The outer cycling conditions were as follows: a first denaturation step of 3 min at 95°C, followed by 40 cycles of 45 s at 95°C, 30 s at 50°C, 2 min at 72°C. A final extension was performed at 72°C for 10 min. For the inner cycling, the following conditions were used: after 3 min at 95°C, 30 cycles of 45 s at 95°C, 30 s at 58°C, 2 min at 72°C, and 10 min at 72°C extension.

Sequencing of the gp120-Coding Regions. PCR products were purified using the Qiagen PCR purification kit (Westburg). To carry out the sequencing reaction, the ABI PRISM dye terminator cycle-sequencing core kit (Perkin-Elmer) was used. The primers used to sequence the *gp120* gene were: AV304 (5′-ACA TGT GGA AAA ATG ACA TGG T-3′ corresponding to position 6504–6525 of NL4.3), AV305 (5′-CCA TGT GTA AAA TTA ACC CCA CTC-3′ corresponding to position 6552–6575 of NL4.3), AV306 (5′-TGT CAG CAC AGT ACA ATG TAC ACA-3′ corresponding to position 6946–6969 of NL4.3), AV307 (5′-ATG GCA GTC TAG CAG AAG AAG A-3′ corresponding to position 6987–7008 of NL4.3), AV308 (5′-TCC TCA GGA GGG GAC CCA GAA ATT-3′ corresponding to position 7313–7336 of NL4.3), AV309 (5′-TGT GGA GGG GAA TTT TTC TAC TG-3′ corresponding to position 7333–7355 of NL4.3), and AV313 (5′-TCC TC/TC ATA TT/CT CCT CCT CCA GGT C-3′ corresponding to position 7605–7629 of NL4.3). The samples were loaded on the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The sequences were analyzed using the software program Geneworks 2.5.1 (Intelligenetics, Inc., Oxford, UK).

Bioassays. Serum concentrations of the compounds (after i.v. and oral administration to rabbits) were determined by a bioassay, as described by Witvrouw et al. (1990). The rabbits received an i.v. injection (in the ear vein) or an oral administration (via gastric intubation) of BRI2923 at a dose of 0.1 g/kg. At different times after injection, blood was collected from the rabbit's other ear and kept at 4°C for 12 h, after which the serum was collected. The samples were stored at −20°C until assayed. The compound concentrations in the serum were determined by measuring the inhibitory effect of a serial dilution of the serum samples on HIV-1-induced cytopathicity in MT-4 cells.

Results

Antiretroviral Activity Spectrum. The carboxylated dendrimer BRI6195 and the sulfonated dendrimer BRI2923 were found to inhibit HIV-1(III_B) replication at 0.1 and 0.3 μg/ml, respectively, in MT-4 and C8166 cells, with selectivity indices up to 2500. The anti-HIV activity of the dendrimers was confirmed for various T-tropic strains of HIV-1 (III_B, NL4.3, and RF), clinical HIV isolates (HE and L1), various HIV-2 strains (ROD and EHO), and SIV(MAC_{251}) (Table 1) at EC_{50} values ranging from 0.01 to 3.5 μg/ml in MT-4 and C8166 cells. Whereas HIV-2(EHO) was the most sensitive virus to the inhibitory effect of BRI2923, (EC_{50}, 0.01 μg/ml), the clinical isolate HIV-1(HE) showed the lowest sensitivity to the anti-HIV activity of BRI6195 (EC_{50}, 3.5 μg/ml). The sulfated polysaccharide DS showed comparable activities against these viruses (EC_{50} values, 0.04–10.6 μg/ml). BRI2923 and BRI6195 inhibited the replication of HIV-1(III_B) in PBMCs at an EC_{50} of 2.2 and 9.1 μg/ml, respectively.

Inhibitory Activity of Dendrimers against Mutant HIV Strains. The compounds were evaluated for their inhibitory effects on a variety of mutant HIV strains (Table 2). The dendrimers lost 4- to 25-fold activity against NL4.3 virus

TABLE 1
Antiretroviral activity of dendrimers BRI2923 and BRI6195 and DS

Virus	Strain	Cells	EC_{50}[a] (μg/ml)		
			BRI2923	BRI6195	DS
HIV-1	III_B	MT-4	0.3 ± 0.2	0.1 ± 0.06	0.4 ± 0.3
		C8166	0.3 ± 0.01	0.1 ± 0.04	0.2 ± 0.2
		PBMC	2.2 ± 1.5	9.1 ± 4.2	0.4 ± 0.2
	NL4.3	MT-4	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.4
	RF	MT-4	0.1 ± 0.02	0.1 ± 0.02	0.8 ± 0.3
	HE	MT-4	1.0 ± 0.4	3.5 ± 2.8	2.1 ± 0.5
	L1	MT-4	0.9 ± 0.9	0.5 ± 0.1	3.3 ± 4.0
HIV-2	ROD	MT-4	0.7 ± 0.5	1.7 ± 0.6	0.04 ± 0.03
	EHO	MT-4	0.01 ± 0.008	2.1 ± 0.7	10.6 ± 4.8
SIV	MAC_{251}	MT-4	0.8 ± 0.2	1.2 ± 0.6	3.5 ± 1.9

[a] Concentrations of each compound required to inhibit the CPE of retroviruses in cell culture by 50%.

strains resistant to agents that block virus entry, i.e., NL4.3 strains resistant to DS, AMD3100, and AR177. BRI2923 showed the highest cross-resistance to the NL4.3/AR177 mutant virus, whereas BRI6195 showed the highest cross-resistance to the NL4.3/AMD3100-selected virus (Table 2).

We observed no cross-resistance against the multinucleoside-resistant virus strains L2, L4, and L6 and an R82913(TIBO)-resistant strain (13MB1; Table 2). The same cross-resistance pattern was seen for DS (Table 2).

Gp120 Binding. We demonstrated that BRI2923 and BRI6195 block recombinant gp120 binding to MT-4 cells (IC_{50} between 0.16 and 0.8 μg/ml; Table 3). Following the same procedures, we found that DS inhibited the binding of recombinant gp120 to MT-4 cells at comparable concentrations (Table 3).

Virus Adsorption. To confirm that the anti-HIV activity of BRI2923 and BRI6195 is due to the inhibition of virus binding to the cells, a virus adsorption assay was performed. DS, a known virus adsorption inhibitor was included as a control. Cells were infected with wild-type HIV-1(NL4.3) strain. DS, BRI2923, and BRI6195 inhibited the binding of HIV-1(NL4.3) to the cells with an IC_{50} of 0.4, 0.9, and 0.3 μg/ml, respectively (Fig. 2).

Time (Site) of Intervention. Time-of-addition experiments (Fig. 3) were performed to pinpoint the possible step(s) of the replicative cycle of HIV-1 that could be inhibited by the test compounds. These results indicated that BRI2923 at 100 times its EC_{50} value (20 μg/ml) and BRI6195 at 100 and 1000 times its EC_{50} (2 and 20 μg/ml), akin to the virus-cell-binding inhibitor DS, interact with the first step of the replication cycle (virus adsorption; Fig. 3). HIV-1 replication, as measured by p24 antigen production, could be inhibited only if the compounds were added at the time of infection. The same result was obtained when we used BRI6195 at 5000 times its EC_{50} value (data not shown). However, when BRI2923 was used at 500 times its EC_{50} (100 μg/ml), addition of the compound could be delayed up to 4 h after infection without loss of inhibitory activity as previously seen for AZT, an inhibitor of the reverse transcription step. The same result was observed when we added BRI2923 at 2500 times its EC_{50} (data not shown). The protease inhibitor ritonavir, as expected, interacted with a late stage of the HIV replicative cycle.

Cellular Uptake. Extracts from 2×10^6 MT-4 cells that had been treated with BRI2923 inhibited the replication of HIV-1(III_B) in MT-4 cells by 98%. An EC_{50} was reached at 30% cell extract. The total intracellular amount of BRI2923 in 2×10^6 MT-4 cells was calculated to be 0.4 μg. Cell extracts from cells treated with BRI6195 and DS did not show any inhibitory effect. We concluded that the polyanionic dendrimer BRI2923, but not BRI6195 and DS, was taken up by MT-4 cells.

Confocal Microscopy. When CEM cells were treated with fluorescein-labeled analogs of BRI2923 and BRI6195, we observed by confocal microscopy that only cells treated with a BRI2923 congener became fluorescent. These results indicated that BRI2923 penetrated the cells, whereas BRI6195 did not.

Anti-RT Activity. BRI2923 and BRI6195 were evaluated for their inhibitory effects on HIV-1 RT. BRI2923 inhibited HIV-1 RT activity by 50% (IC_{50}) at 0.2 μg/ml (Table 4). This was only 4-fold higher than the IC_{50} of 8-chloro-TIBO R86183. BRI6195, however, inhibited HIV-1 RT activity only at a 100-fold higher concentration, as did DS (IC_{50} values, 17.1 and 24.5 μg/ml, respectively; Table 4).

IN Assays. IN catalyzes two subsequent reactions: 3′-processing and DNA strand transfer. BRI2923 and BRI6195 inhibited the 3′-processing reaction of HIV-1 IN at IC_{50} values ranging from 0.3 to 0.4 μg/ml (Fig. 4). The IC_{50} values obtained for the overall integration reaction (3′-processing plus DNA strand transfer) were similar to the values obtained for the 3′-processing reactions (IC_{50} values, 0.2 and 0.3 μg/ml). DS was found to be equally active in the 3′-processing assay (IC_{50}, 0.4 μg/ml).

Selection of HIV-1(NL4.3) Mutant Strains. HIV-1 strains resistant to BRI2923 were selected in MT-4 cells by passaging the virus in the presence of increasing concentrations of BRI2923. An HIV-1(NL4.3)-resistant strain, NL4.3/BRI2923A, was able to grow in the presence of 8 μg/ml BRI2923 after 20 passages. NL4.3/BRI2923A proved to be 15-fold resistant to BRI2923 and 90-fold resistant to DS (Table 5). NL4.3/BRI2923B was obtained after 10 additional passages (30 passages) in the presence of increasing concentrations of BRI2923 (final concentration, 20 μg/ml). NL4.3/BRI2923B was also 15-fold resistant to BRI2923 and >625-fold resistant to DS (Table 5). Proviral DNA of these resistant viruses was isolated and sequenced.

The genotypic analysis of the gp120-coding gene of the BRI2923-resistant viruses revealed several mutations spread over the gp120 molecule (V2, V3, C3, V4, and C4; Table 6). Most of the observed mutations have already been

TABLE 2
Antiviral activity of BRI2923, BRI6195, DS, and bicyclam AMD3100 against mutant HIV-1 strains

Mutant Strain	Wild-Type Strain	Resistance Developed to	EC_{50}[a] in μg/ml (-fold increase)[b]			
			BRI2923	BRI6195	DS	AMD3100
Resistant to agents blocking entry						
NL4.3/DS	NL4.3	DS	1.6 ± 0.6 (4)	3.7 ± 1.5 (9.3)	>125 (>313)	0.01 ± 0.007 (1.4)
NL4.3/AMD3100	NL4.3	AMD3100	4.8 ± 2.8 (12)	10.1 ± 6.7 (25.3)	>125 (>313)	1.2 ± 1.2 (171)
NL4.3/AR177	NL4.3	Zintevir	6.5 ± 2.8 (16.3)	1.3 ± 0.8 (3.3)	19.3 ± 7.1 (48.3)	0.04 ± 0.02 (5.7)
Resistant to RT inhibitors						
L2	L1	NRTIs	1.1 ± 0.6 (1.2)	1.0 ± 0.2 (2)	3.3 ± 4.0 (1)	N.D.
L4	L1	NRTIs	1.3 ± 0.5 (1.4)	1.0 ± 0.3 (2)	5.0 ± 6.5 (1.5)	N.D.
L6	L1	NRTIs + NNRTIs	0.8 ± 0.4 (0.9)	0.4 − 0.2 (0.8)	3.4 ± 4.1 (1.0)	N.D.
13MB1	III_B	NNRTIs	0.6 ± 0.3 (2)	0.2 ± 0.1 (2)	1.1 ± 1.0 (2.8)	N.D.

N.D., not determined.
[a] EC_{50}, or concentration required to inhibit the viral CPE by 50% in cell culture.
[b] Fold increase in EC_{50} value, compared to activity against wild-type HIV-1 strain.

described as resistance mutations of strains selected in the presence of other entry inhibitors (De Vreese et al., 1996; Esté et al., 1997, 1998; Schols et al., 1998). In general, the gp120 protein of the BRI2923-resistant strains contained less basic and more acidic amino acid residues in comparison to the wild-type virus. Moreover, a gradual shift toward more negatively charged amino acids was noted when the observed resistance was more pronounced. In the V2 loop of the BRI2923-resistant strain, the mutation F147L appeared, a substitution reported to emerge in HIV strains selected in the presence of the CXCR4 antagonist AMD3100 and the natural CXCR4 ligand SDF-1α. Close to this region a novel amino acid mutation, V154V/E, was found. The K292Q point mutation in the V3 loop has also been described for the polyanionic G quartet oligonucleotide AR177 resistant strain. In the C3 region the substitution F323Y was found. A five-amino acid deletion in the V4 loop emerges in strains selected in the presence of BRI2923. This deletion is associated with resistance development toward most known HIV entry inhibitors. The CD4-binding region of gp120 of the BRI2923-resistant virus contained two substitutions, namely, R389G (which evolved to R389E when the selection pressure continued) and F393V (Table 6). The RT and IN genes of the resistant strains were sequenced as well, but no significant mutations were found in these genes.

Initial Pharmacokinetic Results. When BRI2923 was administered i.v. to rabbits at a dose of 0.1 g/kg, compound concentrations of 0.1 mg/ml were found in the serum 15 min after injection (data not shown). Under similar conditions, DS reached a concentration of 0.5 mg/ml at the same time (Witvrouw et al., 1990). Six hours after i.v. injection, serum concentrations of BRI2923 were below the minimum detectable concentrations. Serum concentrations were below the minimum detectable concentration at any time after oral administration of 0.1 g/kg BRI2923 via gastric intubation of rabbits.

TABLE 3
Inhibitory effects of BRI2923, BRI6195, and DS on recombinant gp120 [HIV-1(III_B)] binding to MT-4 cells

Concentration μg/ml	Inhibition of Binding (% of Control)		
	BRI2923	BRI6195	DS
100	100	100	100
20	92	89	89
4	100	93	71
0.8	69	58	74
0.16	42	41	38
0.032	19	27	N.D.

N.D., not determined.

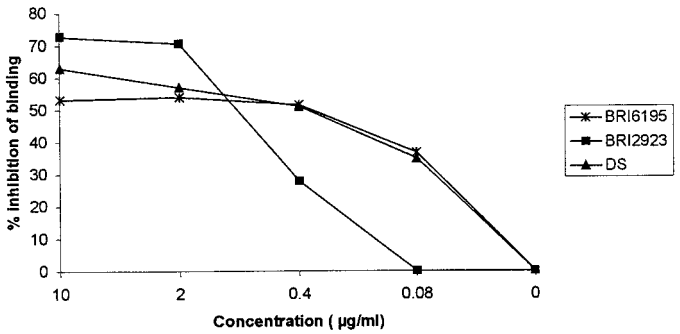


Fig. 2. Inhibitory effects of BRI2923, BRI6195, and DS on HIV-1(NL4.3) binding to MT-4 cells.

Discussion

We have shown that the phenyldicarboxylic acid BRI6195 and the naphthyldisulfonic acid BRI2923 dendrimers inhibit

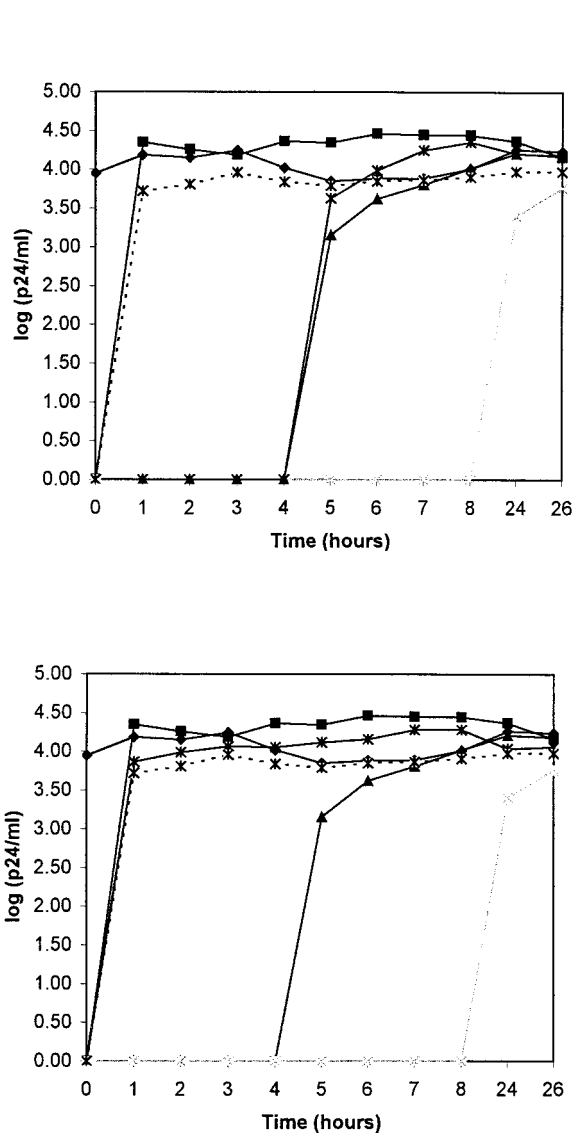


Fig. 3. Time-of-addition experiment. MT-4 cells were infected with HIV-1(III_B) at m.o.i. = 1, and the test compounds were added at different times postinfection. Viral p24 antigen production was determined 31 h postinfection. A, BRI2923: --⁎--, 20 and –⁎–, 100 μg/ml; B, BRI6195: --⁎--, 2 and –⁎–, 20 μg/ml; A and B, ■, DS (mol. wt. 5000; 100 μg/ml); ▲, AZT (0.5 μg/ml); × (gray), ritonavir (2 μg/ml); ◆, nontreated control.

TABLE 4
Sensitivity of HIV-1 RT to the inhibitory effects of the dendrimers and reference compounds

Compound	IC_{50}[a] μg/ml
BRI2923	0.2 ± 0.1
BRI6195	17.1 ± 1.2
DS	24.5 ± 8.6
R86183	0.04 ± 0.03

[a] IC_{50} or concentration required to inhibit the HIV-1 RT activity using poly(C) · oligo(dG) as template/primer by 50%.

the replication of different strains of HIV-1 (III_B, NL4.3, and RF), clinical HIV-1 isolates (HE and L1), various HIV-2 strains (ROD and EHO), and SIV(MAC_{251}) and HIV strains resistant to RT inhibitors, in different cell lines at EC_{50} values ranging from 0.01 to 3.5 μg/ml. These compounds were not toxic for MT-4 and C8166 cells and PBMCs at concentrations up to 250 μg/ml, thus resulting in a maximum selectivity index of up to >25,000.

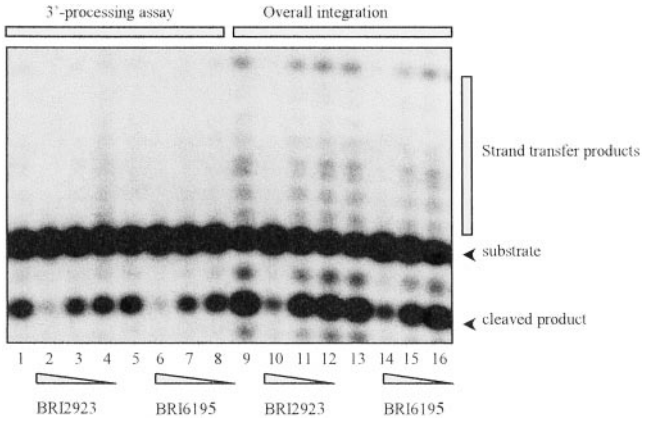


Fig. 4. Inhibition of HIV-1 IN activity by BRI2923 and BRI6195. IN reactions were performed for 7 min in the 3′-processing assay (lanes 1–8) or 60 min in the overall integration assay (lanes 9–16) in the absence of inhibitors (lanes 1, 5, 9, and 13) or in the presence of the inhibitors BRI2923 (lanes 2–4 and 10–12) and BRI6195 (lanes 6–8 and 14–16). The concentrations of the inhibitors were 1 μg/ml (lanes 2, 6, 10, and 14), 0.2 μg/ml (lanes 3, 7, 11, and 15), and 0.04 μg/ml (lanes 4, 8, 12, and 16).

Because of the structural similarities (negative charges; Fig. 1) of BRI2923 and BRI6195 with polyanionic molecules such as DS and because of the loss of antiviral activity of these compounds against viruses containing mutations in the viral envelope gp120, we assumed that their antiviral activity would be based on the interaction with gp120. Results from a gp120-binding assay and a virus adsorption assay confirmed the interference of these compounds with the first step of the replication cycle of HIV, i.e., virus-cell binding.

In a time-of-addition experiment, the addition of inhibitors can be postponed without loss of anti-HIV activity as long as the target replication step has not taken place. Akin to DS, the dendrimers BRI2923 and BRI6195, at 100 times their EC_{50} (determined at an m.o.i. = 0.01), must be present at the time of virus adsorption to inhibit HIV replication, thus confirming their interaction with the first step (virus adsorption) in the viral replicative cycle. However, BRI2923 at higher concentrations (500–2500 times its EC_{50}) was found to interact at a later stage of the virus replicative cycle, i.e., at the time that reverse transcription takes place.

Because a compound can interfere with RT or IN only after cellular uptake, we studied the penetration of BRI2923 and BRI6195 in CEM and MT-4 cells using two different meth-

TABLE 5
Inhibitory effects of antiviral compounds against a BRI2923-selected HIV-1(NL4.3) strains

Compound	EC_{50}[a] in μg/ml (-fold increase)[b]		
	NL4.3/WT	NL4.3/BRI2923A[c]	NL4.3/BRI2923B[d]
BRI2923	0.3 ± 0.2	4.5 ± 2.2 (15)	4.5 ± 1.0 (15)
BRI6195	0.3 ± 0.1	0.9 ± 0.04 (3)	0.9 ± 0.3 (3)
DS	0.2 ± 0.09	18.0 ± 2.7 (90)	>125 (>625)
AMD3100	0.009 ± 0.003	0.06 ± 0.06 (7)	0.02 ± 0.003 (2.2)
AZT	0.002 ± 0.0005	0.003 ± 0.002 (1.5)	0.002 ± 0.0001 (1)
3TC	0.9 ± 0.6	0.9 ± 0.2 (1)	1.0 ± 0.1 (1.1)
d4T	0.03 ± 0.004	0.05 ± 0.01 (1.7)	N.D.
ddC	0.2 ± 0.04	0.5 ± 0.2 (2.5)	N.D.
ddI	1.1 ± 0.5	3.6 ± 0.5 (3.3)	N.D.
1592U89	1.9 ± 0.2	3.9 ± 3.1 (2.1)	N.D.
BI-RG587	0.02 ± 0.005	0.04 ± 0.01 (2)	0.04 ± 0.01 (2)
PNU-90152T	0.02 ± 0.009	0.06 ± 0.04 (3)	0.05 ± 0.03 (2.5)
DMP266	0.0003 ± 0.00004	0.0004 ± 0.00006 (1.3)	0.0009 ± 0.0002 (3)
Adefovir	1.1 ± 0.3	3.6 ± 0.5 (3.3)	2.6 ± 0.5 (2.4)
Tenofovir	1.1 ± 0.2	2.9 ± 0.9 (2.6)	3.4 ± 1.5 (3.1)
L735524	0.02 ± 0.002	0.01 ± 0.01 (0.5)	0.01 ± 0.001 (0.5)
Ro31-8959	0.01 ± 0.002	0.003 ± 0.002 (0.3)	0.01 ± 0.002 (1)

N.D., not determined.
[a] Concentration required to inhibit the CPE of HIV-1 in MT-4 cell cultures by 50%.
[b] Fold increase in EC_{50}, compared to activity against wild-type NL4.3.
[c] Virus strain selected after 20 passages in MT-4 cells in the presence of increasing concentrations of BRI2923 (up to 8 μg/ml).
[d] Virus strain selected after 30 passages in MT-4 cells in the presence of increasing concentrations of BRI292 (up to 20 μg/ml).

TABLE 6
Genotypic analysis of the gp120 genes of HIV-1(NL4.3)-resistant strains

Amino Acid Position (Region) in gp120	NL4.3 WT		NL4.3/BRI2923A		NL4.3/BRI2923B	
	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
147(V2)	TTT	F	TTA	L	TTA	L
154(V2)	GTA	V	GTA	V	GTA/GAA	V/E
292(V3)	AAA	K	AAA	K	CAA	Q
323(C3)	TTC	F	TAT	Y	TAT	Y
365–370(V4)	TTT AAT AGT ACT TGG	FNSTW	deletion		deletion	
389(C4) CD4-binding site	AGA	R	GGA	G	GAA	E
393(C4) CD4-binding site	TTT	F	GTT	V	GTT	V

ods. Results from cellular uptake experiments and confocal microscopy both indicated that BRI2923 penetrated the cells, whereas BRI6195 did not. Because BRI2923 is active in RT and IN assays, both RT and IN are possible targets for interaction.

To identify the molecular target(s) of BRI2923 and BRI6195 in cell culture, HIV strains resistant to the compounds were developed in cell culture. We have previously shown that HIV-1 strains that are resistant to inhibitors targeted at the binding/fusion step of the replication cycle can emerge after sequential passaging of the virions in cell culture in the presence of increasing concentrations of the compounds. Resistance to BRI2923 developed after 20 passages of HIV-1(NL4.3) in MT-4 cells in the presence of this compound. The resistant strain was able to replicate in the presence of 8 μg/ml BRI2923. After 10 additional passages, the resistant strain was able to replicate in the presence of 20 μg/ml BRI2923.

The genotypic analysis of the gp120-coding region of the BRI2923-resistant strain revealed several mutations spread over the gp120 molecule (V2, V3, C3, V4, and C4). In general, the BRI2923-resistant strains contained less basic and more acidic amino acid residues in comparison to the wild-type virus. Moreover, a gradual shift toward negatively charged amino acids was noted when the observed resistance was more pronounced. Most of the mutations observed have already been described as resistance mutations of strains selected in the presence of other binding fusion inhibitors (De Vreese et al., 1996; Esté et al., 1997, 1998; Schols et al., 1998). In addition, novel mutations were found, i.e., V154V/E in the V2 loop as well as R389G/E and F393V positioned at the CD4-binding site. The RT and IN genes were also sequenced, but here no significant mutations were found. Theoretically, it could not be excluded that further passaging of BRI2923-resistant HIV strains in the presence of BRI2923 might eventually lead to the emergence of mutations in the RT (and/or IN) genes.

The polyanionic dendrimers (BRI2923 and BRI6195) have the potential to interact at, at least, three levels: gp120, RT, and IN. This is supported by the results obtained with these compounds in the cell-free assays with these target proteins. That the polycarboxylated dendrimer BRI6195 would interact with RT and/or IN in an intact cell system is unlikely because the compound is not readily taken up by the cell. The polysulfonated dendrimer BRI2923, however, readily permeates the cell and could, therefore, interact, in principle, at either RT or IN, or both. According to the time-of-addition experiments with BRI2923 at higher concentration, the compound seems indeed able to interfere with the RT and/or IN processes. Yet, its primary mode of action must be allocated to interference with the gp120-driven virus-cell-binding process.

Because mutations were found only in the *gp120* gene of BRI2923-resistant virus strains, and because recombinant viruses showed the same phenotypic resistance as the parental resistant strains, we conclude that BRI2923 acts primarily on the binding of viral gp120 with the cells. Synthesis of congeners with higher cell-penetrating properties may be necessary to obtain polyanionic dendrimers that act with a more pronounced activity on intracellular stages of the replication cycle of HIV.

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Send reprint requests to: Myriam Witvrouw, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: Myriam.Witvrouw@uz.kuleuven.ac.be