3480 J. Med. Chem. 2010, 53, 3480–3488
DOI: 10.1021/jm100093p

Journal of Medicinal Chemistry Article

"Viologen" Dendrimers as Antiviral Agents: The Effect of Charge Number and Distance†

Simona Asaftei*,‡ and Erik De Clercq*,§

‡Institute of Chemistry, University of Osnabrück, Barbarastrasse 7, D-49069 Osnabrück, Germany, and
§Rega Institute for Medical Research, K. U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Received November 13, 2009

A series of "viologen" derivatives (4,4′-bipyridinium salts) carrying between 1 and 90 charges per molecule have been prepared and investigated for their activity against human immunodeficiency virus (HIV), herpes simplex virus (HSV), vesicular stomatitis, Punta Toro virus, Sindbis virus, Reovirus, and respiratory syncytial viruses. In general, most of the compounds showed good activities against HIV-1 (strain III_B). In particular, compound **36** exhibited the highest in vitro activity and selectivity index against HIV-1 (strain III_B) ($EC_{50} = 0.26 \pm 0.08\ \mu M$, SI = 75.7) in MT-4 cells. The results imply that the antiviral activity requires an optimal number and distance of the positive charges.

Introduction

The dendrimers' architecture offers a high local concentration of a given functionality, cooperative effects, and polyvalent effects, and sometimes a polycationic structure can be utilized to design effective antimicrobial agents and efficient microbiocide delivery systems.[1] In general, antiviral dendrimers function as artificial mimics of the anionic cell surfaces, thus dendrimers are generally designed having anionic surface groups such as acid- or sulfonate residues (heparan sulfate, sulphated glycosaminoglycans, etc.), which are exposed on eukaryotic cell surfaces and extracellular matrix (ECM[a]). Several viruses such as human immunodeficiency virus, herpes virus, and cytomegalovirus interact with host cells by binding to sulphated residues on cell surfaces and ECM.[2] In other words, the dendritic drug competes with the cellular surface for binding of the virus, leading to a lower cell-virus infections probability.[3] Polylysine dendrimers are known as viral inhibitors for Herpes Simplex virus in vitro,[4] and PAMAM dendrimers show also antiviral activity against HIV.[5,6] The dendrimers work as an inhibitor for virus entry and in the late stages of virus replication, but the exact mechanism of action has not been described in detail.

Through a systematic analysis of structure−activity relationships of a series of "viologen" derivatives (4,4′-bipyridinium salts) carrying between 1 and 90 charges per molecule (Charts 1 and 2), we report here a new biological aspect of polycationic "viologen"-based dendrimers as novel HIV-1 replication inhibitors.

We found four polycationic "viologen"-based dendrimers (out of **38** derivatives) which significantly enhanced antiviral potency (Chart 2). The highest activity against HIV-1 exhibits **36**; it inhibits the replication of HIV-1(IIIB) in MT-4 cell at a 50% effective concentration (EC_{50}) 0.26 μM. In this context, it is interesting to note that poly(propylene imine) dendrimers with 16 dimethyldodecylammonium end groups exhibited pronounced activity against gram-negative bacteria (4 μg/mL) 2 orders of magnitude higher than their monofunctional analogues.[1] In our study are included: (i) the activity of the compounds in an MT-4 cell line against HIV, (ii) anti-HIV activity in peripheral blood mononuclear cells (PBMC), (iii) the virucidal effect of the compounds and inhibition of virus binding. Furthermore, the novel compounds were tested against vesicular stomatitis, Punta Toro virus, Sindbis virus, Reovirus, and respiratory syncytial viruses.

Results and Discussion

Chemistry. The synthesis of the biologically active compounds **35**−**38** is described in Schemes 1 and 2. The compounds **35** and **37** with a phenyl core were synthesized, following the divergent dendrimers' synthesis method, according to the method of Ardoin and Astruc[7] and Heinen et al.[8] from the corresponding initiator core **3** (Scheme 1).

4,4′-Bipyridine was converted to the intermediarily formed compound 1,1′-bis(2,4-dinitrophenyl)4,4′-bipyridinium salt (**28**) by a nucleophilic substitution reaction with 1-chloro-2,4-dinitrobenzene.[9] The resulting dinitro intermediate is a well-known starting material for the synthesis of diphenylbipyridinium salts, and was further reacted with 3,5-di(hydroxymethyl) aniline, synthesized according to the literature[10] by reaction of 5-amino-isophthalic acid dimethylester and lithium−aluminum hydride. The intermediate PF_6^- salt of compound **26** is highly soluble in hydrobromic acid/acetic acid and can be further converted to the tetra-bromide **27**, followed by ion exchange of the PF_6^- salt in good yield (78%). From the intermediary product **27**·$2PF_6^-$, the desired compound **35**, a dendrimer with phenyl core, was obtained by reaction of *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**)[11] followed by ion exchange (route Ia, Scheme 1).

The first generation phenyl core dendrimer was obtained by reaction with *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate, prepared by reaction of

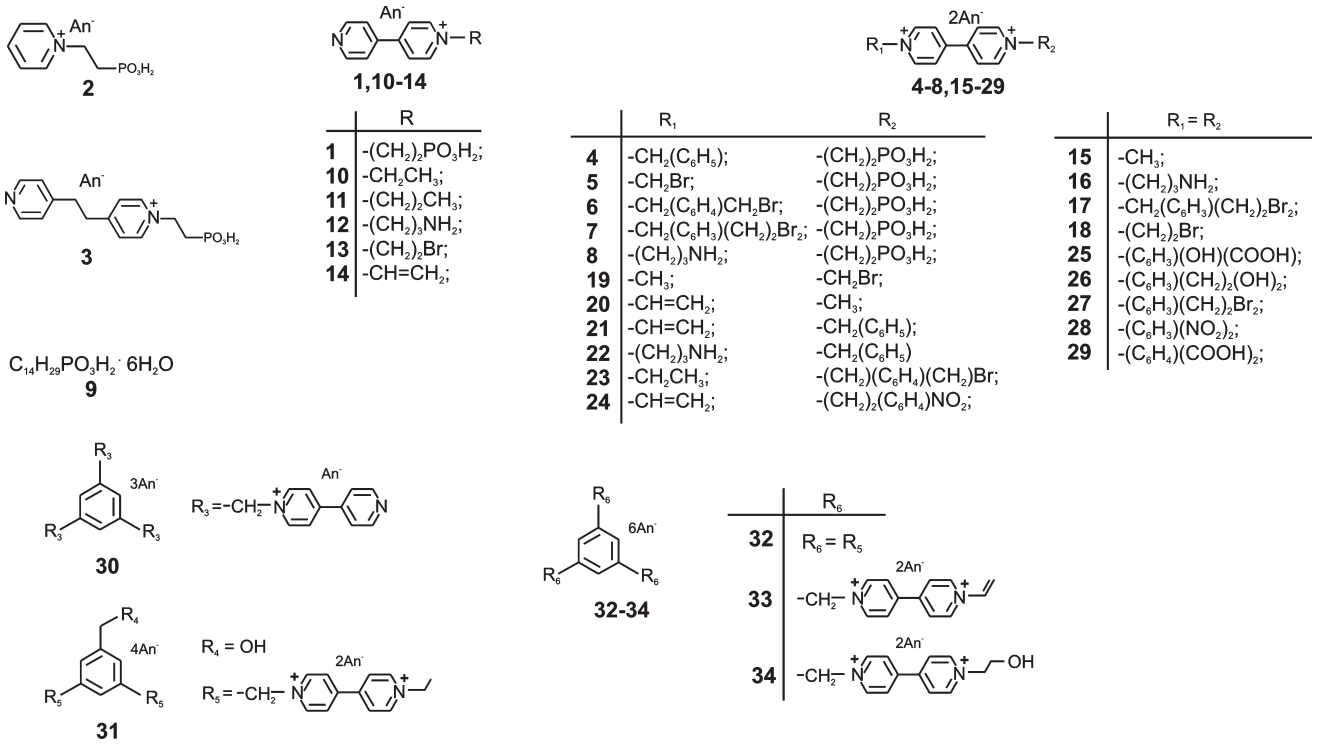
†Dedicated to Professor Dr. Lorenz Walder, University Osnabrück, on the occasion of his 60th birthday.

*To whom correspondence should be addressed. For S.A.: phone, +49-541-969 2802; fax, + 49-541-969 2370; E-mail: sasaftei@uos.de. For E.D.: phone, 32-16-33.73.67; fax, 32-16-33.73.40; E-mail: erik.declercq@rega.kuleuven.be.

[a]Abbreviation: ECM, extracellular matrix ; HIV, human immunodeficiency virus; HSV, herpes simplex virus; PBMCs, peripheral blood mononuclear cells; THF, tetrahydrofuran; MeCN, acetonitrile; DS5000, dextran sulfate 5000; AZT, 3′-azido-3′-deoxythymidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; GFP, green fluorescent protein; CPE, cytopathic effect.

 Published on Web 04/08/2010

Chart 1. "Viologen" Monomers Carrying One to Six Charges Used in This Study



3,5-di(hydroxymethyl)-benzylbromide with 4,4′-bipyridine in freshly distilled tetrahydrofuran (THF),[12] followed by substitution of −OH by −Br in compound **27a** with 5.7 M HBr in conc acetic acid at room temperature and alkylation with *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**) (route Ib, Scheme 1).

The synthetic pathways for the dendrimers of first and third generation with a benzyl core were performed according to the method of Heinen et al.[13] (Scheme 2). The three-charge initiator core 1,3,5-tris[(4,4′-bipiridinium)methyl]benzene trihexafluorophosphate (**30**) was prepared by reaction of 1,3,5-tris bromomethyl benzene[14] with an excess of 4,4′-bipyridine in MeCN according to the literature.[15]

The three peripheral nitrogens can be alkylated quantitatively with primary alkylhalides such as 1,3-di(hydroxymethyl)-benzyl bromide to the hexavalent alcohol (**30a**). This succeeded by substitution of −OH for −Br, yielding **30a** with 5.7 M HBr in conc acetic acid and subsequent ion exchange to the intermediate **30b** as PF_6^- salt. The compound **36** dendrimer with 18 charges per molecule was available from intermediate **30b** as tetrabromide via alkylation with *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**) in MeCN (route Ia, Scheme 2). The dendrimers of the third generation were prepared by reaction of the precursor **30b**·$6PF_6^-$ with *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate to give the intermediate **30d**·$18PF_6^-$. The resulting polyalcohol was activated by conversion to the polybromide. This reaction step was repeated once to obtain the intermediate **30f**·$42PF_6^-$. For solubility reasons, an ion exchange was carried out after each reaction step. In the last reaction step, the intermediate **30f**·$42PF_6^-$ reacted with *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**) in MeCN to afford the compound **38** in good yields (route **Ib**, Scheme 2).

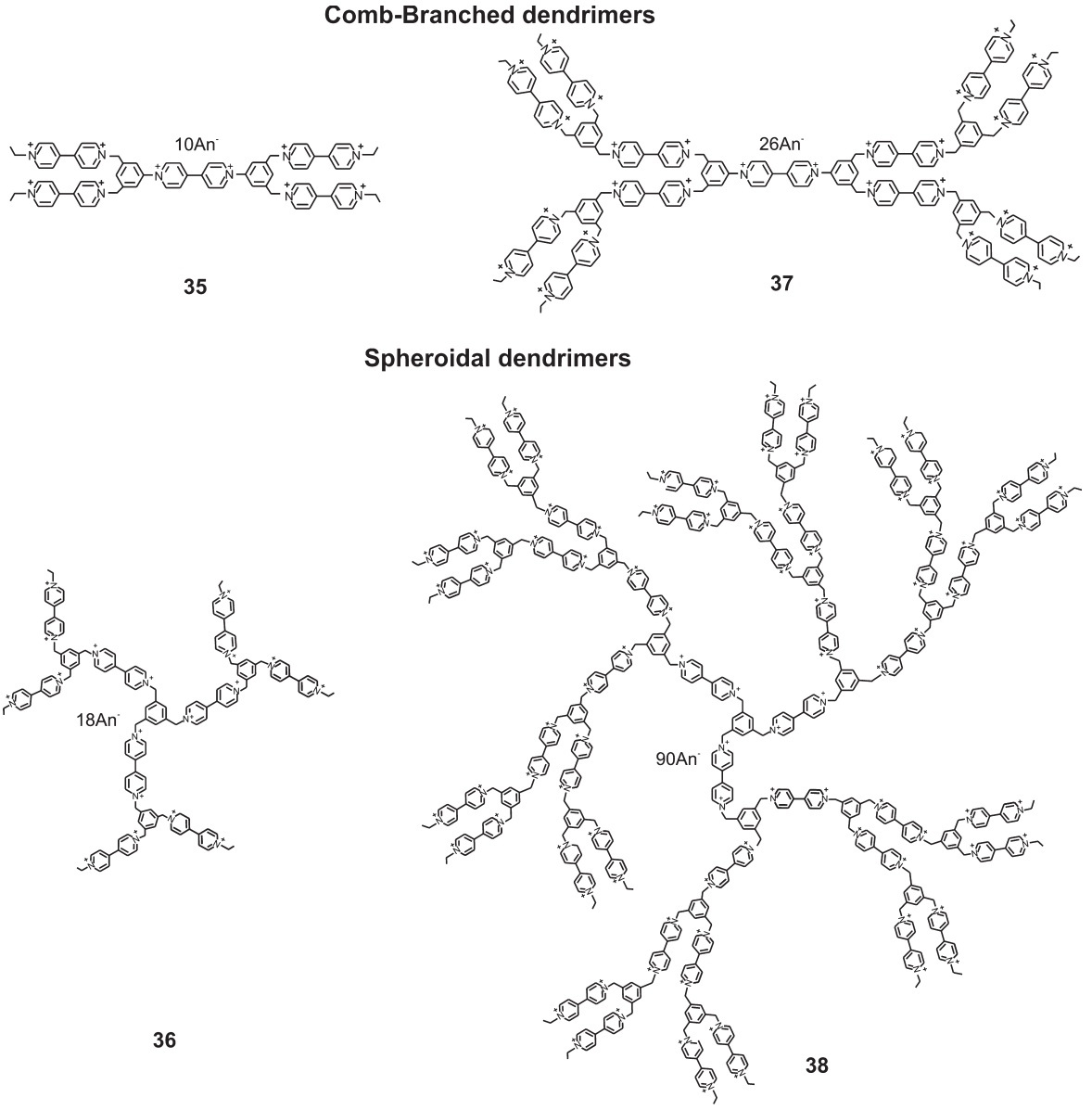
All the target compounds were characterized by ^{1}H-, ^{13}C-NMR, and IR spectroscopy as well as by elemental analysis.

Antiviral Activity in Vitro. The synthesized compounds were initially evaluated for anti-HIV-1 (III_B) and anti-HIV (ROD) activity in MT-4 cells, and cytotoxicity was estimated in parallel by an MTT assay.[16,17] The results, expressed as CC_{50} (50% cytotoxicity), EC_{50} (50% effective concentration), and SI (selectivity index = CC_{50}/EC_{50}) values, were then calculated from the cell viability from each concentration of the compounds and are presented in Table 1. The "viologen" derivatives from **1** to **9** (monoalkylated 4,4′-bipyridinium units with ethylphosphate-residues) showed no significant antiviral activity. The compounds from **10** to **34** exhibited moderate activity against HIV-1 (III_B) and HIV (ROD) but no good selectivity. It is worth noting that polycationic "viologen" **35**, **36**, **37**, and **38** dendrimers were found to be the most active compounds against wild-type HIV-1. Good activity and encouraging SI values were obtained by both the first generation of dendrimers with a phenyl- or benzyl-core, respectively. In particular, compound **36** with 18 charges per molecule and a spheroidal structure was the most potent (EC_{50} value of 1.01 ± 0.09 μM and SI = 75.76), inhibiting HIV-1 replication in MT-4 cells more effectively than **35** (comb-branched structure). To better define the antiviral profile of derivative **35**−**38**, its anti-HIV-1 activity was determined in human PBMC cells. Compound **35** showed better activity and selectivity in the PMBC cells as compared to MT-4 cells but also higher cytotoxicity (Table 2 PBMC) compared with compound **36**.

The HIV-activity of compound **36** was also assayed in acutely infected Jurkat cells (Table 3). In these cells, compound **36** showed potent and selective activity against different strains of HIV-1.

To clarify the viral replication process inhibited by **36**, a time-of-addition assay was performed and the results were compared to reference compounds with a known mode of action. In contrast to the reference inhibitors, dextran sulfate 5000 (DS5000), 3′-azido-3′-deoxythymidine (AZT),

Chart 2. Chemical Structure of the Biologically Active "Viologen"-Based Dendrimers

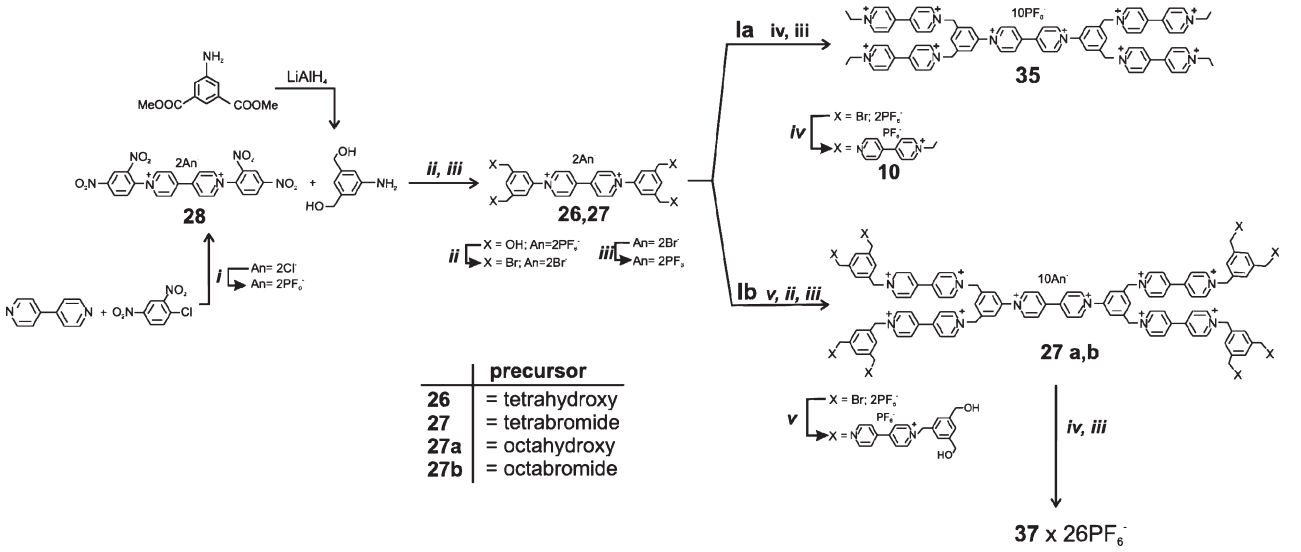


and nevirapine, for which addition can be delayed until 0, 5, and approximately 10 h postinfection, addition of compound **36** could be postponed for only 1 h postinfection before it lost its antiviral activity (Figure 1).

To further investigate the effect of **36** on the HIV-1 entry process VSV-G-pseudotyped (*env*-deficient) GFP-encoding HIV-1 particles were exposed to C8166 cells in the absence (control) or presence of different concentrations of the test compounds (Figure 2). After 48 hours of postinfection, supernatants were analyzed for GFP production. The reverse transcriptase inhibitor nevirapine suppressed dose-dependently GFP expression by the pseudotyped virus-infected cells, while the CXCR4 antagonist AMD3100 and **36** completely lost their inhibitory effect, pointing to a specific interaction of the HIV-1 envelope with susceptible cells as target of antiviral intervention of **36**.

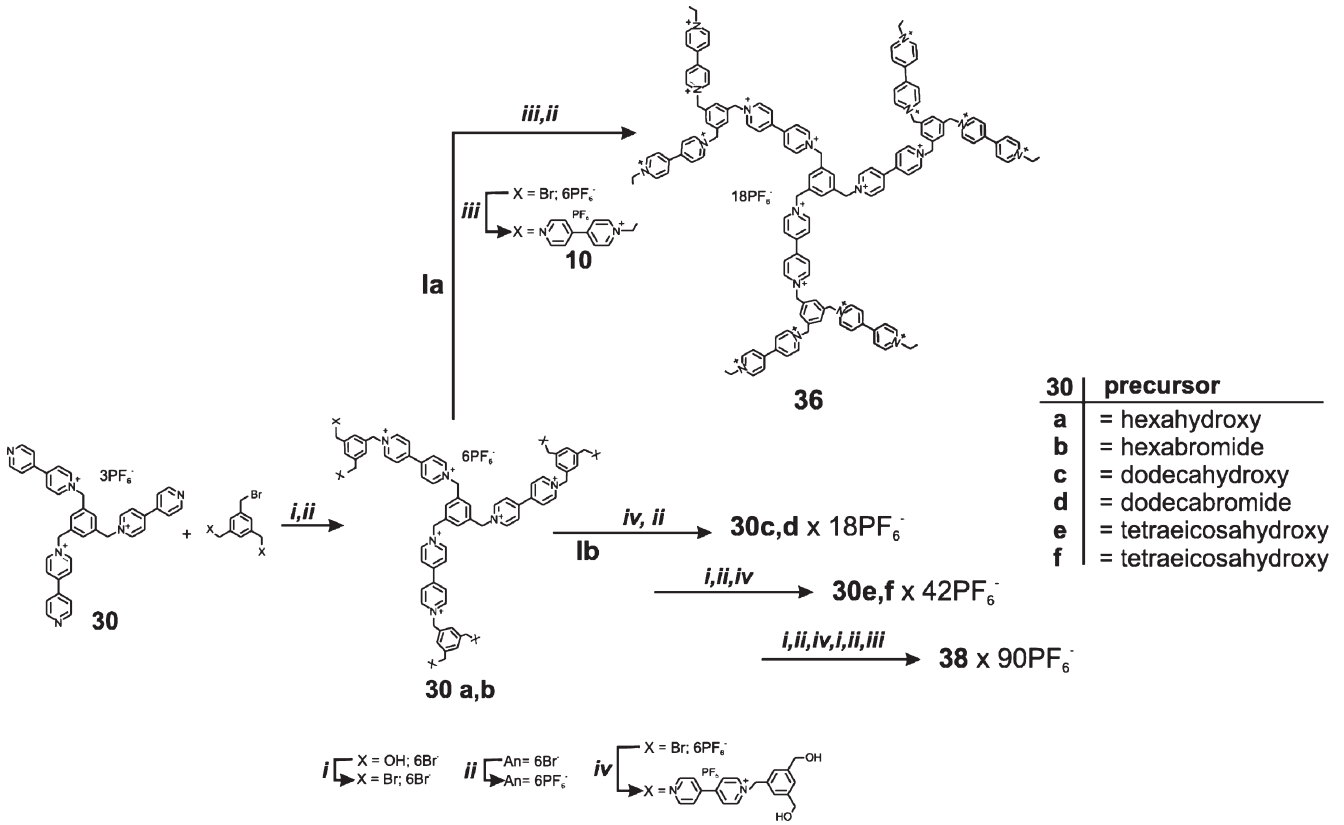
To study in more detail the mechanism of action of **36** on the HIV-1 specific entry, C8166 cell cultures were exposed to HIV-1/NL4.3 virions labeled with GFP-Vpr fusion proteins in the intact virion particles to monitor virion attachment to target cells in the presence of different concentrations of compound **36** and DS-5000. After a 6 h incubation period with a viral input of 15 ng p24 Gag, approximately 4% of CD4-expressing human T cells contained HIV-1 virions. Interestingly, the presence of **36** had no effect on cell-associated GFP fluorescence. In contrast, DS-5000 completely prevented appearance of cell-associated GFP fluorescence (0.03%), pointing to an efficient inhibition of virus adsorption to the cells. The decrease of the antiviral activity and viral selectivity suggest that the $\pi-\pi$ stacking could play a role as well as the dendrimers with aromatic units in the structure.[18] Compounds **35**−**38** were also evaluated for their activity against HSV-1 (KOS), Herpes simplex virus-2, vaccinia virus, vesicular stomatitis virus (VSV), herpes simplex virus-1 TK^-, respiratory syncytial virus (RSV), parainfluenza-3 virus, reovirus-1, Sindbis virus, and Punta Toro virus in E_6SM, Vero, and HeLa cells. Table 4 clearly shows that all polycationic viologen dendrimers exhibit almost no activity against the viruses mentioned above and that these compounds are only selective against HIV-1.

Scheme 1. Synthetic Scheme of the 10 and 26-Fold Charged Monomers; Core Type Phenyl- (**35**; **37**)[a]



[a] Reagents: (i) MeCN, 24 h, reflux and ion exchanged with NH_4PF_6; (ii) MeOH, 23 h, reflux; HBr/AcOH, 3 days, rt; (iii) ion exchanged with NH_4PF_6; (iv) *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**), MeCN, reflux, 1 week, extracted with NH_4PF_6, purified by CC, Sephadex LH-20, MeOH: MeCN 1:1; (v) *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate, MeCN, 10 days, reflux, extracted with NH_4PF_6; purified by CC, Sephadex LH-20, MeOH: MeCN 1:1.

Scheme 2. Synthetic Scheme of the 18- and 90-Fold Charged Monomers; Core Type Benzyl- (**36**; **38**)[a]



[a] Reagents: (i) MeCN, 45 h, reflux and HBr/AcOH, 3 days, rt (ii); ion exchanged with NH_4PF_6; (iii) *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**), MeCN, reflux, 1 week, purified by extraction from $MeNO_2/H_2O$ 1 week; (iv) *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate, MeCN, 1 week, reflux.

Future Aspects. Further experiments will be focused on the preparation of dendritic compounds built up from units which contain positive charges in a larger distance as well as of those which are furnished with heterocyclic nucleobases. Such compounds and those described in this manuscript should also be evaluated as CXCR4 antagonists and as possible inhibitors of X4 HIV-1 strains and as stem cell mobilizers which depend on their antagonization with CXCR4 to mobilize stem cells.

Table 1. Antiretroviral Activity and Cytotoxicity of HIV Inhibitors in MT-4[a]

	EC_{50}[b] [μM]					
compd	HIV-1_{IIIB}	HIV-2_{ROD}	SIV_{MAC251}	SO6141	CC_{50}[c] [μM]	SI
10	> 281.8	> 281.8	NT	NT	281. ± 11.98	< 1
11	> 363.4	> 363.4	NT	NT	> 363.4	< 1
12	> 346.3	> 346.3	NT	NT	> 346.4	< 1
13	> 79.2	> 79.2	NT	NT	79.2 ± 18.0	< 1
14	> 15.9	> 15.9	NT	NT	15.9 ± 2.7	< 1
15	> 25.2	> 25.2	NT	NT	25.2 ± 1.2	< 1
16	> 3.3	> 3.3	NT	NT	3.3 ± 0.4	< 1
17	> 12.1	> 12.1	NT	NT	12.1 ± 1.0	< 1
18	5.88 ± 3.08	> 20.6	> 20.6	NT	20.6 ± 3.1	3.5
19	> 5.1	> 5.1	NT	NT	5.1 ± 0.4	< 1
20	> 26.1	> 26.1	NT	NT	26.1 ± 1.3	< 1
21	> 2.7	> 2.7	NT	NT	2.7 ± 1.0	< 1
22	> 0.9	> 0.9	NT	NT	0.9 ± 0.1	< 1
23	> 0.9	> 0.9	NT	NT	0.9 ± 0.2	< 1
24	> 0.4	> 0.4	NT	NT	0.4 ± 0.4	< 1
25	> 0.2	> 0.2	NT	NT	0.2 ± 0.0	< 1
26	> 2.7	> 2.7	NT	NT	2.7 ± 0.2	< 1
27	> 148.5	> 148.5	NT	NT	> 148.5	< 1
28	≥42.2	> 107.4	NT	NT	107.4 ± 10.8	≤2.54
29	> 124.0	> 124.0	NT	NT	124.0 ± 4.1	< 1
30	58.8 ± 4.6	> 151.5	NT	> 151.5	> 151.5	2.57
31	> 10.6	> 10.6	NT	NT	10.6 ± 0.6	< 1
32	> 81.1	> 81.1	NT	NT	> 81.1	< 1
33	> 33.4	> 33.4	NT	NT	33.4 ± 12.9	< 1
34	33.3 ± 3.7	> 104.2	37.7 ± 13.2	> 104.2	> 104.2	3.11
35	**1.01 ± 0.09**	**> 49.0**	**> 49.0**	**> 49.0**	**> 49.0**	**48.51**
36	**0.26 ± 0.08**	**> 19.7**	**0.46 ± 0.37**	**≥1.55**	**19.7 ± 2.1**	**75.76**
37	1.40 ± 0.50	9.82 ± 1.30	≥0.49	1.23 ± 0.99	> 18.6	13.28
38	0.066 ± 0.002	> 0.490	≥0.052	≥0.075	0.490 ± 0.002	7.42

[a] All data represent mean values and standard deviations for at least two separate experiments. [b] Concentration required to inhibit 50% of virus-induced cytopathic effect. [c] Concentration that reduced cellular viability by 50%

Table 2. Anti-HIV Activity and Cytotoxicity of Selected Viologens in PBMC[a]

	HIV-1 III_B		
compd	EC_{50} (μg/mL)[b]	CC_{50} (μg/mL)[c]	SI
35	0.79 ± 0.09	26.81 ± 3.33	**34**
36	1.62 ± 0.33	4.04 ± 0.10	2.5
37	> 0.52	0.52 ± 0.05	< 1
38	0.25 ± 0.03	0.61 ± 0.02	2.5

[a] All data represent mean values and standard deviations for at least two separate experiments. [b] Concentration required to inhibit 50% of the level of the HIV-1 p24 produced in the absence of drugs. [c] Concentration that reduced cellular viability by 50%

Conclusion

Dendrimers consisting of viologen units such as **35**−**38** inhibit HIV-1 (strain III_B) replication in MT-4 cells. These compounds also inhibit the SIV (strain MAC251) replication but to a lesser extent. No inhibitory activity was observed on HIV-2 (strain ROD) replication. The reason why we asked for this specific structure is that we expected these polycationic compounds to bind to heparan sulfate structures expressed on the surface of the host cells. Logically, one might expect that if heparan sulfate would be the target for blocking HIV replication, the active compounds should also block herpes simplex virus replication in vitro, as heparan sulfate also plays an important role in the cellular entry of this virus. When assaying the anti-HIV activity of the most active compounds (**35**−**38**) in human PBMC, we observed no inhibitory activity, which can be explained by the low expression of heparan sulfate on these cells. The specificity of the inhibition clearly indicates that the mechanism of action of polycationic compounds is more refined than just a general block of the electrostatic interaction between cell surface and virus.

We assume that these polycationic compounds bind to heparan sulfate structures expressed on the surface of the host cells. This is corroborated by the fact that the positive charge distance of the 4,4′-bipyridine moiety (∼7.6 Å) fits roughly to the negative charge distance (∼6 Å) calculated from an energy-minimized heparane sulfate disaccharide units (3D-optimized structure of a heparane sulfate disaccharide unit using ChemSkech, 3D viewer version 12.0 Advanced Chemistry Developments, Inc., Toronto, Canada, http://www.acdlabs.com).

We have tested two types of compounds: spheroidal dendrimers **36**, **38**, and comb-branched dendrimers **35**, **37**. The spheroidal dendrimers are effective in inhibiting virus replication and the adhesion on the virus envelope is better than for the comb-branched dendrimers **35**, **37**. These showed only moderate antiviral activities. The main intention of this paper is to show that there is a correlation between the number of charges as well as of their distance with the antiviral activity. To corroborate this point, we included the data of other similar compounds (Charts 1 and 2 in the Introduction) showing no activity because lack of charges.

Experimental Section

4.1. Chemistry. Materials and Methods. Without further mention, the chemicals were analytical grade from Aldrich, Merck, or Fluka and used as received. Ethyl acetate, diethylether, THF, methanol, and petrol ether were distilled before use. Organic solutions were dried over anhydrous Na_2SO_4 or $MgSO_4 \cdot 2H_2O$ and concentrated with a Heidolph rotary evaporator

Table 3. Antiretroviral Activity and Cytotoxicity of Compound **36** in A72 Jurkat Cells[a]

	EC_{50}[b] [μg/mL] HIV strain								
compd	HIV-1_{IIIB}	HIV-2_{ROD}	NL4.3	AZT^R	S056	S067	S07	CC_{50}[c] [μg/mL]	SI
36	0.685 ± 0.8	21.53 ± 6.9	< 0.2	5.9 ± 1.9	< 2	< 0.2	< 0.2	102 ± 30	148.9
Nevirapine	0.008 ± 0.002	> 2	0.008 ± 0.0003	0.005 ± 0.0002	< 0.2	0.016 ± 0.0008	0.012 ± 0.007	> 2	< 250

[a] All data represent mean values and standard deviations for at least two separate experiments. [b] Concentration required to inhibit 50% of the level of GFP expression obtained in the absence of drug. [c] Concentration that reduced cellular viability by 50%.

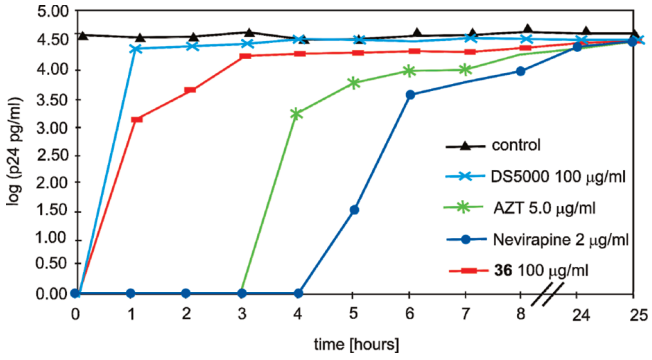


Figure 1. Time-of-addition experiment. MT-4 cells were infected with HIV-1 IIIb at an moi of 0.5 and test compounds were added at different times post infection. Viral p24 Ag production was determined at 31 h post infection and is expressed as the $\log_{10}$ of the p24 Ag content in pg/mL.

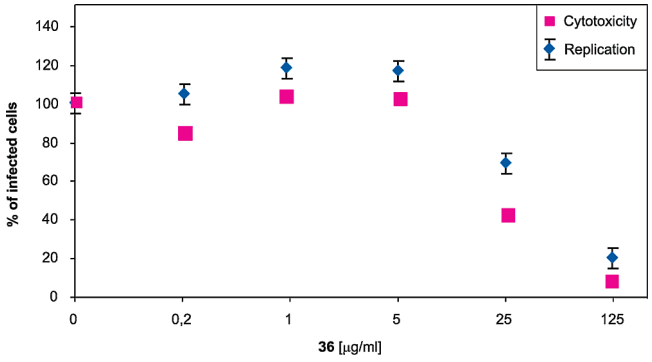


Figure 2. Compound **36** is inactive against VSV-G pseudotyped HIV. C8166 cells were infected in vitro with GFP-encoding HIV-1 pseudotyped with VSV-G and treated with the respective compounds at different concentrations as indicated. Infection was assayed for GFP expression 48 h postinfection. One hundred percent of GFP expression was defined for the infection control (no compound). Toxicity of the compounds was measured in parallel using the MTT-based method.

at reduced pressure. Yields are of purified products and were not optimized. All reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel $60F_{254}$ (Merck) plated and visualized by using UV erasing. Flash column chromatographic separations were carried out on silica gel Baker 60 (mesh 30−60) or Sephadex LH-20, Fluka (25−100 μm). ^{1}H NMR, DEPT135, and ^{13}C NMR spectra were recorded in CD_3CN (δ = 1.93), Me_2SO-d_6 (δ = 2.50), CD_3OD-d_6 (δ = 4.87) (internal Me_4Si), respectively, at 250 MHz and at 63 MHz (Bruker Avance DPX-250). Chemical shifts are given in ppm (δ), and the spectral data are consistent with the assigned structures. IR spectra were recorded with a Bruker Vector 22 FTIR spectrophotometer. Elemental analyses were performed on a Vario Micro Cube analyzer, and the data for C, H, and N are within ±0.32% of the theoretical values.

General Procedure for the Synthesis of 10 and 26-Fold Charged "Comb-Branched" Dendrimers (35−37). A mixture of the phenylic dendrimer precursor **27** and the headgroup *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**) (Scheme 1, route Ia) or the cross-linker *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate (Scheme 1, route Ib) in acetonitrile was heated at 70 °C for 1 week. The reaction mixture was worked up and purified as reported in the description of the single compounds.

General Procedure for Synthesis of 18- and 90-Fold Charge "Spheroidal" Dendrimers (36, 38). A solution of the benzylic dendrimer precursor **30b** in acetonitrile and *N*-ethyl-4,4′-bipyridinium hexafluorophosphate (**10**) as headgroup (Scheme 2, route Ia) or the cross-linker *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate (Scheme 2, route Ib) was refluxed for 1 week. The cross-linker equipped with a reactive pyrimidine nitrogen and two benzylic alcohols was used for the preparation of the intermediate **30d·18PF$^-_6$** and **30f·42PF$^-_6$**, respectively, by repetitive application of the reaction steps v and iii (Scheme 2, route Ib). The resulting compound **38** was worked up and purified as reported in the description of the single compound in supporting materials. Compound **36** was obtained according to a procedure described as follows.

Benzyl Core Dendrimer·18 PF^-_6 (36). The synthesis was performed according to a literature procedure.[13] 1,1′,1″-[Benzene-1,3,5-triyl-tris(methylene)tris](4-pyridin-4-yl-pyridinium trihexafluorophosphate (**30**) (1 g, 0.98 mmol) and 900 mg (3.9 mmol) of 3,5-di(hydroxymethyl)benzylbromide were dissolved in MeCN (50 mL) and stirred for 44 h at 70 °C under reflux. After this period, the precipitate was filtered, washed with ether, and dried in vacuo to yield 1.24 g (0.72 mmol, 73%) as yellow powder of hexahydroxy precursor (**30a**). ^{1}H NMR (250 MHz, CD_3CN): δ 8.99 (d, J (H,H) = 6.6 Hz, 6 H), 8.93 (d, J (H,H) = 6.7 Hz, 6H), 8.42−8.37 (m, 12H), 7.68 (s, 3H), 7.44 (s,3H), 7.41 (s, 6H), 5.85 (s, 12H), 4.64 (d, J (H,H) = 5.1 Hz, 12 H), 3.44 (t, J (H,H) = 5.3 Hz, 6 H).

The hexahydroxy precursor (**30a**) (1.063 g, 0.10 mmol) was dissolved in 320 mL of HBr (5.7 M) in acetic acid under argon and stirred for 3 days at rt. The reaction mixture was evaporated, and the residue was dissolved in 2 mL of water, and 3 mL of a 10% aq NH_4PF_6 solution were added. The brown precipitate was filtered, washed with water, and dried in HV. The hexabromide precursor (**30b**) was obtained as a white powder 1.23 g (yield: 0.54 mmol, 87%). ^{1}H NMR (250 MHz, CD_3CN): δ 8.97 (d, J (H,H) = 6.5 Hz, 6 H), 8.95 (d, J (H,H) = 6.5 Hz, 6H), 8.40 (d, J(H,H) = 6.5 Hz, 12 H), 7.68 (s, 3H), 7.49 (s, 6H), 5.83 (s, 6H), 5.80 (s, 6H), 4.58 (s, 12H).

The hexabromide precursor (**30b**) (300 mg, 0.131 mmol) and 330 mg (1 mmol) of *N*-ethyl-4,4′-bipyridinium-hexapfluorophosphate (**10**) were dissolved in MeCN (25 mL) and stirred for 4 days at 70 °C under reflux. The resulting precipitate was filtered off, and the mother liquor was evaporated. The crude product was dissolved in $MeNO_2$ and extracted with an aq NH_4PF_6 solution. The product formed (**36**) was dried in vacuo to yield 285 mg (0.061 mmol, 47%) as brown powder ^{1}H NMR (250 MHz, CD_3CN): δ 8.93−8.90 (m, 36H), 8.42−8.36 (m, 36H), 7.66 (s, 9H), 7.64 (s, 3 H), 5.83 (s, 24H), 4.67 (q, J (H,H) = 7.3 Hz, 12 H), 1.65 (t, J (H,H) = 7.3 Hz, 18 H). ^{13}C NMR (63 MHz,

Table 4. Antiviral Activity and Cytotoxicity Evaluation of Compounds **35**−**38**

	EC_{50}[a] [μg/mL]							
	E_6SM					HeLa		MCC[b] [μg/mL]
compd	HSV-1 (KOS)	HSV-2(G)	vaccinia virus	VSV	TK^- KOS ACV^r	VSV	RSV	E_6SM ; HeLa
35	6	50	> 50	> 50	30	> 50	50	> 50
36	**2**	**10**	**> 50**	**> 50**	**10**	**> 50**	**50**	**> 50**
37	6	50	> 50	> 50	30	> 50	50	> 50
38	4	4	> 4 (12)	> 4	4	> 20	> 20	20
c	0.384	80	9.6	> 400	> 400	> 400	> 400	> 400
d						400	> 400	> 400
e	> 400	> 400	48	> 400	> 400	48	16	> 400

	EC_{50}[a] [μg/mL] Vero				
compd	para-influenza-3	Reo-virus	Sindbis virus	Punta Toro virus	MCC[b] [μg/mL] Vero
35	> 50	> 50	> 50	> 50	> 50
36	**> 50**	**> 50**	**> 50**	**> 50**	**> 50**
37	> 50	> 50	> 50	> 50	> 50
38	> 4	> 4	> 4	> 4	20
c	> 400	> 400	> 400	> 400	> 400
d	400	> 400	> 400	> 400	> 400
e	48	80	> 400	48	> 400

[a] Effective concentration required to inhibit virus-induced cytopathicity by 50%. [b] Minimal cytotoxic concentration required to elicit a microscopically visible alteration of cell morphology. HSV: Herpes simplex virus, VSV: vesicular stomatitis virus; TK^-: thymidine kinase deficient; RSV: respiratory syncytial virus, ACV^r: Acyclovir-resistant, E_6SM embryonic skin-muscle cells, [c] Brivudin. [d] (*S*)-2,3-Dihydroxypropyladenine(*S*)-DHPA. [e] Ribavirin.

CD_3CN): δ 151.1, 151.0, 150.0, 146.3, 146.2, 145.8, 135.3, 135.2, 132.3, 132.2, 127.9, 127.8, 127.6, 64.0, 58.2, 16.0. IR (KBr) 3140, 1640, 1562, 1508, 1453, 1224, 1176, 834, 558 cm^{-1}, Anal. calcd for $C_{138}H_{138}N_{18}P_{18}F_{108}\cdot 3H_2O$ (4658.10 + 54) C 35.18, H 3.08, N 5.35; found C 35.13, H 3.21, N 5.32.

4.2. Biological Activity. Dextran sulfate [DS, average molecular weight (MW) 5000] was purchased from Sigma (Bornem, Belgium). Nevirapine was obtained from Boehringer Ingelheim (Ridgefield, CT). 3′-Azido-3′-deoxythymidine (AZT) was synthesized according to the method described by Horwitz et al.[19] Ritonavir (ABT538) was obtained from Abbott Laboratories (Abbott Park, IL).

Cells and Virus. MT-4[20] and C8166[21] cells were grown and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% sodium bicarbonate, and 20 μg gentamicyne per mL.

The HIV-1(III_B) strain was provided by R. C. Gallo and M. Popovic.[22] The NL4-3.GFP11 strain expressing an enhanced version of GFP[23] instead of Nef has been described previously.[24] The HIV-1 G89 V, NL4-3_{A92E}, and NL4-$3_{P90A\text{-}A924E}$ mutants were a kind gift of Dr J. Luban.[25,26] For all tests described, NL4-3.GFP11 virus was obtained from transfection of 293T cells with the molecular clone. Then, 1 mL of virus-containing supernatant was used to infect 8×10^6 MT-4 cells in 40 mL of culture medium. Three days after infection, the supernatant was collected and used as inoculum in the respective assays.

The vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped HIV-1 was generated by transient cotransfection of recombinant pNL4-3-Δ*E*-GFP (Zhang et al., 2004)[27] (a kind gift of Dr R. Siliciano, Johns Hopkins University, Baltimore, MD) or pNL4-3.Nef-GFP-G89 V (a kind gift of Dr J. Luban) and pVSV-G using calcium phosphate in 293T cells. Supernatants containing VSV-G-pseudotyped HIV-1 were collected 60 h after transfection. Cell debris was removed from the supernatant by centrifugation (450*g* for 10 min), and the supernatant was used for infection or stored at −80 °C.

Inhibitory effects of the compounds on HIV-1 and HIV-2 replication were monitored by inhibition of virus-induced cytopathic effect in MT-4 cells and were estimated by MTT assay.[16,17] Briefly, 50 μL HIV-1(III_B) and HIV-2 ROD at 100−300 $CCID_{50}$ (50% cell culture infective doses) were added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effects of test compounds on uninfected cells in order to assess the cytotoxicity of the test compounds. The MT-4 cells were added at a final concentration of 6×10^5 cells/mL, and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock-and HIV-infected cells was examined spectrophotometrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The absorbances were read in an eight-channel computer-controlled spectrophotometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The 50% cytotoxic concentration (CC_{50}), the 50% effective concentration (EC_{50}), and the selectivity index ($SI = CC_{50}/EC_{50}$) were then calculated from the cell viability from each concentration of the compound. Anti-HIV activity was also investigated in PBMCs infected with HIV-1IIIB and cultured with various concentrations of the test compounds. The activity was evaluated by the level of inhibition of the p24 core antigen in the culture supernatant, assessed with the HIV-1 p24 core antigen in the culture supernatant, assessed with the HIV-1 p24 core profile enzyme-linked immunosorbent assay (NEN, Paris, France).

Evaluation of the antiviral activity of the compounds against NL4-3.GFP11 in C8166 cells was performed using flow cytometry (see below) and was described earlier.[28] HIV-1 core antigen (p24 Ag) in the supernatant was quantified by the p24 Ag enzyme-linked immunosorbent assay (PE, Brussels, Belgium).

Evaluation of the antiviral activity of the compounds against VSV-G pseudotyped viruses in C8166 cells was performed using flow cytometry. Briefly, 3×10^4 C8166 in 200 μL medium were infected with VSV-G pseudotyped NL4.3 or NL4.$3_{G89\ V}$ in the presence of different concentrations of the test compound.

Forty-eight hours later, the infection was monitored by measuring the virus-associated GFP expression using flow cytometry. Toxicity of the compounds was assessed using an MTT-based method.

Flow cytometric analysis was performed on a FACSCalibur flow cytometer equipped with a 488 nm argon-ion laser and a 530/30 nm bandpass filter (FL1: detection of GFP associated fluorescence) (Becton Dickinson, San Jose, CA). Before acquisition, cells were pelleted at 1000 rpm for 10 min and fixed in a 3% paraformaldehyde solution. Acquisition was stopped when 10000 events were counted. Data analysis was carried out with Cell Quest software (BD Biosciences). Cell debris was excluded from the analysis by gating on forward versus side scatter dot plots.

Selection of 36 Resistant HIV-1_{IIIB}. Compound **36** resistant HIV-1 strain was obtained after sequential passaging of HIV-1_{IIIB} virus in the presence of increasing concentrations of **36** in MT-4 cells. At the start of the selection, the HIV-1_{IIIB} virus was inoculated on MT-4 cells in the presence of 0.2 μM **36**. When the cytopathic effect (CPE) was observed, cell culture supernatant was used as inoculum to infect new MT-4 cells at the same concentration of compound. The second time CPE was observed, the concentration of **36** was increased by a factor of 1.5. After serial passaging, we were able to culture resistant viruses in the presence of 10.7 μM **36**.

Time-of-Addition Experiment. To determine more specifically at which stage of the HIV replication cycle a compound interferes, we performed time-of-addition experiments.Time-of-addition experiments were adapted from Pauwels et al.[29] Briefly, MT-4 cells were infected with HIV-1 (III_B) at an moi of 0.5. Following a 1 h adsorption period, cells were distributed in a 96-well tray at 45000 cells/well and incubated at 37 °C. Test compounds were added at different times (0, 1, 2, 3, 4, 5, 6, 7, 8, 24, and 25 h) after infection. Viral p24 antigen production was determined at 31 h postinfection using an enzyme-linked immunosorbent assay (PE, Brussels, Belgium). The reference compounds were used at the following concentrations: dextran sulfate: 100 μg/mL; AZT: 0.5 μg/mL; nevirapine: 2 μg/mL. **36** was used at 100 μg/mL.

Acknowledgment. We express our thanks to Prof. Christophe Pannecouque (Rega Institute for Medical Research) for performing the experiments with HIV, and Leentje Persoons (Rega Institute for Medical Research) for the experiments with the other viruses.

Supporting Information Available: Experimental details corresponding to the synthesis and analytical data for target compounds **35**, **37** and **38**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chen, C. Z. S.; Cooper, S. L. Recent Advances in Antimicrobial Dendrimers. *Adv. Mater*. **2000**, *12*, 843–846.
(2) Baba, M.; Pauwels, R.; Balzarini, J.; Amout, J.; Desmyter, J.; De Clercq, E. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vito. *Proc. Natl. Acad. Sci. U.S.A*. **1988**, *85*, 6132–6136.
(3) Boas, U.; Heegaard, M. H. Dendrimers in Drug Research. *Chem. Soc. Rev*. **2004**, *33*, 43–63.
(4) Bourne, N.; Stanberry, L. R.; Kern, E. R.; Holan, G.; Matthews, B.; Berstein, D. I. Dendrimers, a New Class of Candidate Topical Microbicides with Activity against Herpes Simplex Virus Infection. *Antimicrob. Agents Chemother*. **2000**, *44*, 2471–2474.
(5) Witvrouw, M; Fikkert, W.; Pluymers, W.; Matthews, B.; Mardel, K.; Schols, D.; Raff, J.; Debyser, Z.; De Clercq, E.; Holan, G.; Pannecouque, C. 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. *Mol. Pharmacol*. **2000**, *58*, 1100–1108.
(6) Witvrouw, M; Weigold, H.; Pannecouque, C.; Schols, D.; De Clercq, E.; Holan, G. Potent anti-HIV (type 1 and type 2) activity of polyoxometalates: structure−activity relationship and mechanism of action. *J. Med. Chem*. **2000**, *43*, 778–783.
(7) Ardoin, N.; Astruc, D. Molecular trees—from syntheses towards applications. *Bull. Chim. Soc. Fr*. **1995**, *132*, 875–909.
(8) Heinen, S.; Meyer, W.; Walder, L. Charge trapping in dendrimers with a viologen skeleton and a radial redox gradient. *J. Electroanal. Chem*. **2001**, *498*, 34–43.
(9) Kamogawa, H.; Satoh, S. Organic-Solid Photochromism by Photoreduction Mechanism—Aryl Viologens Embedded in Poly-(*N*-Vinyl-2-Pyrrolidone). *J. Polym. Sci., Part A* **1988**, *26*, 653–656.
(10) Bergbreiter, D. E.; Osburn, P. L.; Liu, Y. S. Tridentate SCS palladium(II) complexes: new, highly stable, recyclable catalysts for the heck reaction. *J. Am. Chem. Soc*. **1999**, *121*, 9531–9538.
(11) Bruinink, J.; Kregting, C. G. A.; Ponjee, J. J. Modified Viologens with improved electrochemical properties for display applications. *J. Electrochem. Soc*. **1977**, *124*, 1854–1856.
(12) Buhleier, E. W.; Wehner, W.; Vögtle, F. Cascade and nonskid-chain-like syntheses of molecular cavity topologies. *Synthesis* **1978**, 155–158.
(13) Heinen, S.; Walder, L. Generation-dependent intramolecular CT complexation in a dendrimer electron sponge consisting of a viologen skeleton. *Angew. Chem., Int. Ed*. **2000**, *39*, 806–809.
(14) Vögtle, F.; Zuber, M.; Lichtenthaler, R. G. Simplified technique for synthesis of 1,3,5-tris(bromomethyl)benzene. *Chem. Ber*. **1973**, *106*, 717–718.
(15) Amabilino, D. B.; Ashton, P. R.; Belohradsky, M.; Raymon, F. M.; Stoddart, J. F. The controlled self-assembly of a [3]rotaxane incorporating 3 constitutionally different components. *Chem. Commun*. **1995**, 747–750.
(16) Pauwels, R.; De Clercq, E.; Desmyter, J.; Balzarini, J.; Goubau, P.; Herdewijn., P.; Vanderhaeghe, H.; Vandeputte, M. Sensitive and rapid assay on MT-4 cells for the detection of antiviral compounds against the AIDS virus. *J. Virol. Methods* **1987**, *16*, 171–185.
(17) Pannecouque, C.; Daelemans, D.; De Clercq, E. Tetrazolium-based colorimetric assay for the detection of HIV replication inhibitors: revisited 20 years later. *Nat. Protoc*. **2008**, *3*, 427–434.
(18) Razinkov, V.; Gazumyan, A.; Nikitenko, G.; Ellestad, G.; Krishnamurthy, G. RFI-641 inhibits entry of respiratory syncytial virus via interactions with fusion protein. *Chem. Biol*. **2001**, *8*, 645–659.
(19) Horwitz, J. P.; Chua, J.; Noel, M.; Nucleosides, V. The monomesylates of 1-(2-deoxy-beta-D-lyxofuranosyl) thymine. *J. Org. Chem*. **1964**, *29*, 2076–2078.
(20) Miyoshi, I.; Kubonishi, I.; Yoshimoto, S.; Akagi, T.; Ohtsuki, Y.; Nagata, K.; Hinuma, Y. Detection of type C viral particles in a cord T-cell line derived by cocultivation of normal human leukocytes and human leukemic T-cells. *Nature* **1981**, *294*, 770–771.
(21) Salahuddin, S. Z.; Markham, P. D.; Wong-Staal, F.; Franchini, G.; Kalyanaraman, V. S.; Gallo, R. C. Restricted expression of human T-cell leukemia-lymphoma virus (HTLV) in transformed human umbilical cord blood lymphocytes. *Virology* **1983**, *129*, 51–64.
(22) Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **1984**, *224*, 497–500.
(23) Stauber, R. H.; Horie, K.; Carney, P.; Hudson, E. A.; Tarasova, N. I.; Gaitanaris, G. A.; Pavlakis, G. N. Development and applications of enhanced green fluorescent protein mutants. *BioTechniques* **1998**, *24*, 462–471.
(24) Valentin, A.; Lu, W.; Rosati, M.; Schneider, R.; Albert, J.; Karlsson, A.; Pavlakis, G. N. Dual effect of interleukin 4 on HIV-1 expression: implications for viral phenotypic switch and disease progression. *Proc. Natl. Acad. Sci. U.S.A*. **1998**, *95*, 8886–8891.
(25) Braaten, D.; Aberham, C.; Franke, E. K.; Yin, L.; Phares, W.; Luban., J. Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. *J. Virol*. **1996**, *70*, 5170–5176.
(26) Berthoux, L.; Sebastian, S.; Sokolskaja, E.; Luban, J. Cyclophilin A is required for TRIM5{alpha}-mediated resistance to HIV-1 in Old World monkey cells. *Proc. Natl. Acad. Sci. U.S.A*. **2005**, *102*, 14849–14853.
(27) Zhang, W.; Morris, Q. D.; Chang, R.; Shai, O.; Bakowski, M. A.; Mitsakakis, N.; Mohammad, N.; Robinson, M. D.; Zirngibl, R.; Somogyi, E.; Laurin, N.; Eftekharpour, E.; Sat, E.; Grigull, J.; Pan, Q.; Peng, W. T.; Krogan, N.; Greenblatt, J.; Fehlings, M.; van der Kooy, D.; Aubin, J.; Bruneau, B. G.; Rossant, J.; Blencowe, B. J.; Frey,

B. J.; Hughes, T. R. The functional landscape of mouse gene expression. *J. Biol.* **2004**, *3*, 21.1–21.22.
(28) Daelemans, D.; Pannecouque, C.; Pavlakis, G. N.; Tabarrini, O.; De Clercq, E. A novel and efficient approach to discriminate between pre- and post-transcription HIV inhibitors. *Mol. Pharmacol.* **2005**, *67*, 1574–1580.
(29) Pauwels, R.; Andries, K.; Desmyter, J.; Schols, D.; Kukla, M. J.; Breslin, H. J.; Raeymaeckers, A.; Van Gelder, J.; Woestenborghs, R.; Heykants, J.; Schellekens, K.; Janssen, M. A. C.; De Clercq, E.; Janssen, P. A. J. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. *Nature* **1990**, *343*, 470–474.