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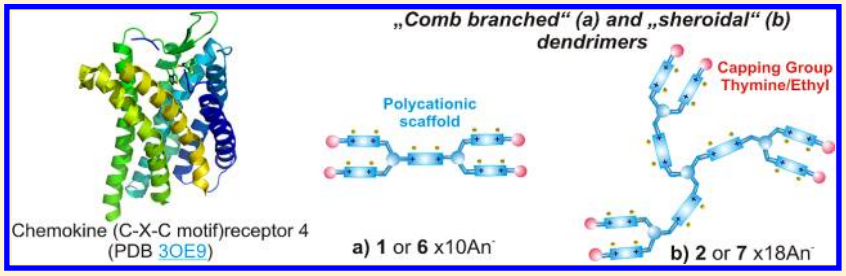
HIV-1 X4 Activities of Polycationic "Viologen" Based Dendrimers by Interaction with the Chemokine Receptor CXCR4: Study of Structure−Activity Relationship

Simona Asaftei,*[,†] Dana Huskens, and Dominique Schols[‡]

[†]Institute of Chemistry, University of Osnabrück, Barbarastrasse 7, D-49069 Osnabrück, Germany

[‡]Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Supporting Information



ABSTRACT: A series of "viologen" based dendrimers with polycationic scaffold carrying 10, 18, 26, 42, and 90 charges per molecule were used to determine the structure−activity relationship (SAR) with regard to HIV-1 inhibitory activity. The studies involved five compounds with a high activity against HIV-1 already utilized in our previous study[1] and five new dendrimers. Such dendrimers block HIV-1 entry into the cell, indicating that they bind to HIV-1 surface proteins and/or on the host cell receptors required for entry. The increasing positive character of dendrimers leads to more cytotoxicity. The 10 charges dendrimers (**1**, **6**) have less influence on the cell viability but low inhibition of the binding of the CXCR4 mAb clone 1D9. Thus, dendrimers with 18 charges (**2**, **7**) are the most promising CXCR4 imaging probes. We report the design, synthesis, and biological activity of new HIV-1 inhibitors that are conceptually distinct from those of the existing HIV-1 inhibitors.

INTRODUCTION

Dendrimers (dendri = tree, mer = part) are a class of macromolecules characterized by highly branched, well-defined, three-dimensional structures that are being developed as drug delivery vehicles and as therapeutic agents. In fact, their branches can be capped with different surface groups that can impart distinct biological and pharmacological properties.[2−4] In contrast, with small molecule drugs that tend to make monovalent contacts, dendrimers can bind to their target in a multivalent manner.[5]

N-Alkylated 4,4′-bipyridinium units, so-called "viologens",[6] are classic organic building blocks to build polycationic dendrimers that are utilized in a variety of fields, such as electrochemistry,[7] photochemistry,[8] electrical conductivity,[6,9] solar-energy conversion,[10] electron transfer,[11] charge transfer,[12] ligands in metallosupramolecular assemblies,[13] molecular wires,[14] and biocide.[15] In addition, we have previously shown that some polycationic "viologen" based dendrimers carrying between 10 and 90 charges per molecule were active against the human immunodeficiency virus type 1 (HIV-1) in T cell lines and primary cell cultures.[1] In the beginning we assumed that these polycationic compounds bind by electrostatic interactions to the polysaccharide heparan sulfate (HS), a negatively charged structure expressed on the surface of the cells of the T cell lines. However, one polycationic compound was active against HIV-1 in peripheral blood mononuclear cells (PBMCs) and these cells have a low expression of HS.[1] This observation initiated the search for the exact mechanism of action of polycationic dendrimers.

The entry of HIV into the host cell starts with the binding of the envelope glycoprotein gp120 to its primary host cell receptor CD4. Subsequently, HIV has to bind to a co-receptor, the chemokine receptor CCR5 for R5 viruses or the chemokine receptor CXCR4 for X4 viruses.[16,17] CXCR4 is the receptor for the CXC-chemokine CXCL12 or stromal-cell-derived factor 1α (SDF-1α),[18,19] whereas CCR5 is recognized by the CC-chemokines CCL3 or macrophage inflammatory protein 1α (MIP-1α), CCL4 or MIP-1β and CCL5 or regulated upon activation normal T-cell expressed and secreted (RANTES).[16,20] These chemokines can block HIV-1 infection by binding to the co-receptor and inducing internalization of the receptor from the cell surface.[16,18,19] For the treatment of HIV-1-infected patients, one CCR5 antagonist, maraviroc, was approved by the Food and Drug Administration (FDA)[21,22] However, until now, no clinically useful drug is available for the treatment of HIV-1 infected patients, although several CXCR4 antagonists have been previously reported. In particular 1,1′-[1,4-phenylenebis-

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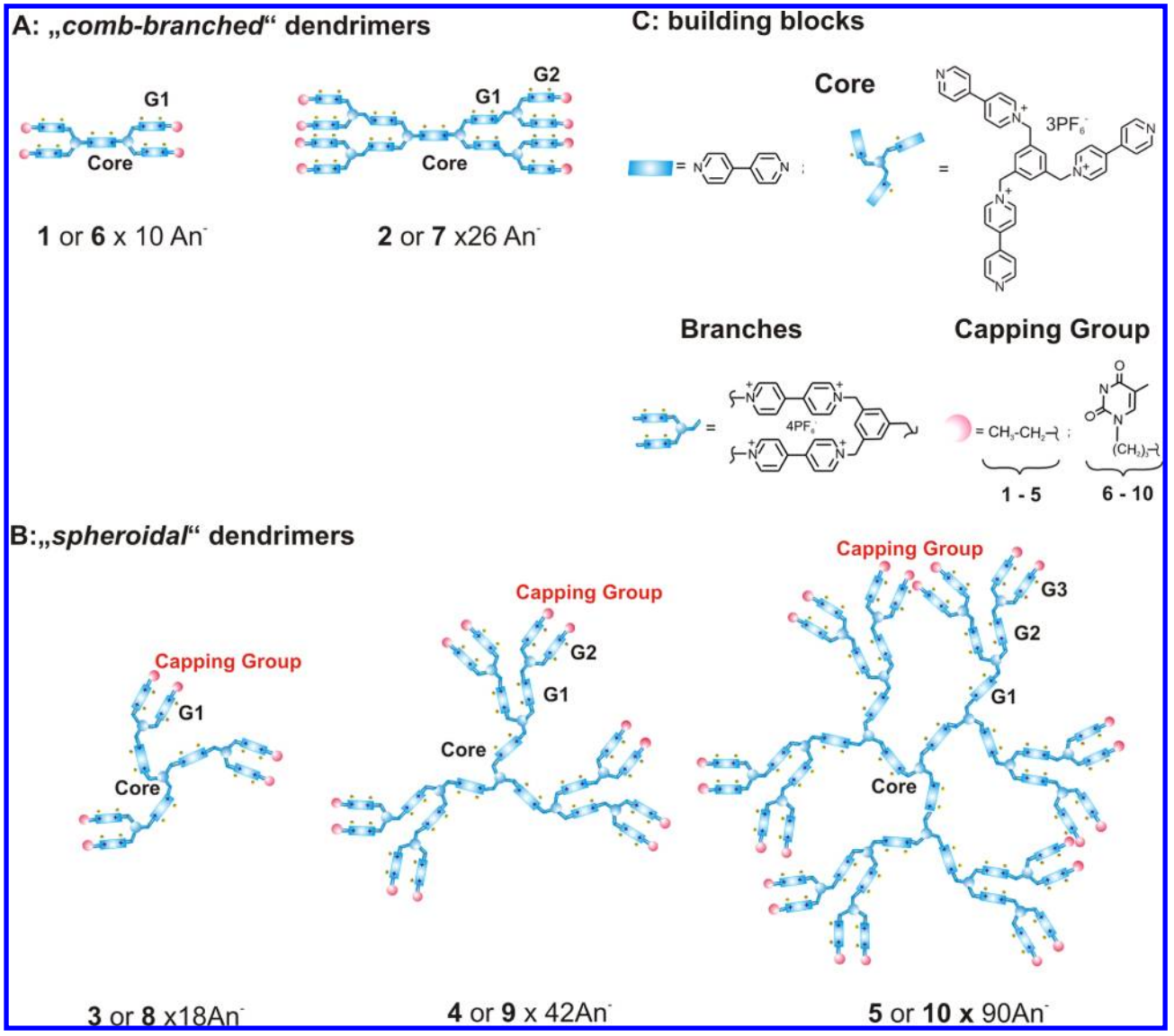
Chart 1. Structures of Ethyl/Thymine "Viologen" Based Dendrimers 1−10[a]



[a](A) Model representation of "comb-branched" dendrimer structure showing central core and branches G_1 and G_2 with 10 and 26 charges, respectively, and surface groups of 4 and 8 ethyl or thymine units, respectively, denoted as red spheres. (B) "spheroidal" "viologen" based dendrimers showing central core and branches G_1, G_2, and G_3 with 18, 42, and 90 charges, respectively, and surface groups of 6, 12, and 24 ethyl or thymine units, respectively. (C) Structures of the cores, branches, and capping groups of the "comb-branched" and "spheroidal" dendrimers.

(methylene)]bis-1,4,8,11-tetraazacyclotetradecane (AMD 3100) has clinical use for patients with non-Hodgkin's lymphoma and multiple meloma.[23] The key players in the interaction of HIV-1 with CXCR4 are the V3 loop of gp120 together with the N-terminal region and extracellular domains of CXCR4.[24,25] It is known that the overall positive charge of the V3 loop is correlated to the usage of CXCR4 and CCR5 (a high positive charge promotes CXCR4 usages and a low positive charge promotes CCR5 usages).[26] More specifically, if the glycosylation motif ($N^6X^7T^8|S^8X^9$, where X ≠ Pro and N is the glycosylation site) is absent from the V3 loop sequence, the virus will show preference toward CXCR4 as co-receptor.[27] If the $N^6X^7T^8|S^8X^9$ motif is present, the co-receptor selection will be influenced by the amino acids at positions 11, 24, and 25 (of the "11/24/25" rule). If any of these amino acids are not positively charged, the virus will show a preference toward CCR5.[28] De Victoria et al. proposed that if the $N^6X^7T^8|S^8X^9$ glycosylation motif is present and any of the amino acids at positions 11, 24, and 25 are positively charged, the co-receptor preference will be governed by the net charge of the V3 loop sequence. If the net charge of the V3 loop is >5, the virus will show a preference toward CXCR4.[27]

The chemokine ligand/receptor pair SDF-1/CXCR4 appears to play a central role in the metastasis of several types of cancer and certain inflammatory autoimmune disorders such as rheumatoid arthritis.[29−33] Therefore, chemokine receptor antagonists may be attractive drug candidates not only for anti-HIV therapy. AMD3100, the lead compound of the bicyclams, was developed as a stem cell mobilizing agent that culminated in the approval of plerixafor in 2008 for the mobilization of hematopoietic stem cells in patients with non-Hodgkin's lymphoma and multiple myeloma.[34,35] However, initially AMD3100 was described as the first low-molecular-weight agent that inhibits virus fusion and infectivity through interaction with the HIV-1 co-receptor CXCR4.[36] Mutational studies have

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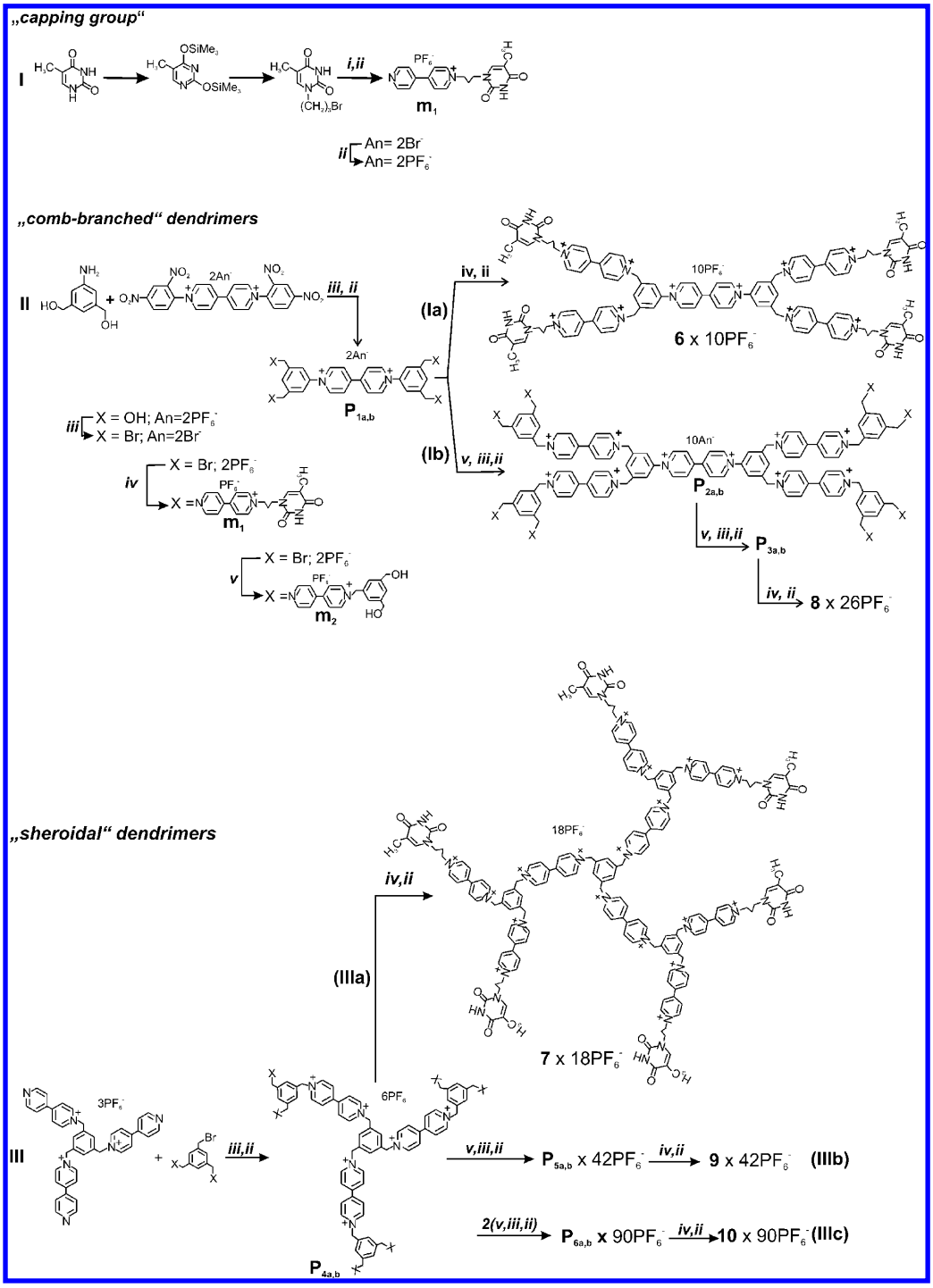
Scheme 1. Synthesis of the Dendrimers 1−10[a]



[a]Reagents and conditions: (i) bipyridine, 1 h, 110 °C, nitrobenzene; (ii) ion exchange chromatography with 10% aq NH_4PF_6; (iii) 48 h, under reflux with HBr/AcOH; (iv) *N*-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidine-1(2*H*)-yl)propyl]-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_1}$), MeCN under reflux; (v) *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_2}$), MeCN under reflux.

revealed that the amino acid residues Asp[171], Asp[262], and Glu[288] are key interaction points for AMD3100. It suggested that Asp[171] in transmembrane domain 4 (TM-IV) interacts with one of the bicyclam rings and the other ring is sandwiched between Asp[262] and Glu[288] in TM-VI and TM-VII, respectively.[37,38]

In the context of systematic studies on "viologen" based dendrimers with polycationic scaffold carrying 10 (**1**, **6**), 18 (**2**, **7**), 26 (**3**, **8**), 42 (**4**, **9**), and 90 charges (**5**, **10**) per molecule, we have determined the structure−activity relationship (SAR) of dendrimers with regard to HIV-1 inhibitory activity. We have extended our studies from the five compounds (**1**−**5**) with only 4,4′-bipyridinium units as an integral part of the polycationic scaffold, with high activity against HIV-1 (IIIB) already utilized in our previously study[15] to another five new dendrimers (**6**−**10**) with a recognition function on the surface. We report the synthesis, characterization, and inhibitory activities of five new

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Table 1. Inhibitory Effects of 1−10 on Replication of HIV-1 X4 NL4.3 Virus[a]

		compds, ethyl units					compds, thymine units				
	ref AMD3100	1	2	3	4	5	6	7	8	9	10
IC_{50} (μg/mL), HIV-1 NL 4.3	0.017	1.1	0.9	0.8	1.4	1.3	2.4	1.5	1.8	1.5	1.2
CC_{50} (μg/mL), tox MT-4	>10	>100	34	11	9	9	>100	19	10	9	10

[a]All data represent mean values and standard deviations for at least two separate experiments. IC_{50}: concentration of compound to inhibit 50% HIV-1 (NL4.3) replication in MT4 cells (average of three independent experiments). CC_{50}: concentration of compound to inhibit 50% the viability in MT-4 cells.

"viologen" based dendrimers with polycationic scaffold and 4, 6, 8, 12, and 24 thymine units as a recognition function on the surface (Chart 1). Such dendrimers have a dual advantage. We expect that the polycationic scaffold interacts with the heparan sulfate and the thymine units are capable of hydrogen-bonding to the carboxylate group of the residues Asp^{262}, Glu^{288}, and Asp^{171} of the HIV co-receptor CXCR4. The compounds were evaluated for their ability to inhibit HIV-1 replication and for their interactions with the CXCR4 HIV co-receptor and compared to the specific CXCR4 inhibitor AMD3100.

RESULTS AND DISCUSSION

Chemistry. The main aim of our chemical design was to obtain compounds with double function able to minimize the adhesion of the virus envelope on the host cell by the interaction of polycationic scaffold and HS expressed from the host cell and increasing the binding affinity of dendrimers via hydrogen-bonding between thymine units and the carboxylate group of the residues Asp^{262}, Glu^{288}, and Asp^{171} of HIV co-receptor CXCR4. To achieve this, we prepared polycationic "viologen" based dendrimers, where the periphery labels consist of thymine units (Chart 1). The synthesis of the dendrimers **1−5** was previously published.[1] The synthesis of dendrimers **6−10** is outlined in Scheme 1 and is described in detail in the Experimental Section and Supporting Information.

First, the monomer *N*-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidine-1(2*H*)-yl)propyl]-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_1}$), used as a capping group, was prepared by nucleophilic substitution of 1-(3-bromopropyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione obtained following a known procedure[39] and 4,4′-bipyridine in nitrobenzene in 60% yield (Scheme 1, route I). To synthesize the dendrimers core, we used our established method previously published by Asaftei and De Clercq[1] starting from the corresponding initiator core and following the "divergent dendrimers synthesis method" reported previously by Anroin et al.[40] and Heinen et. al.[41,42]

The conjugated 4-fold nucleophilic phenyl precursors $\mathbf{P_{1a,b}}$ have been prepared according to route II, Scheme 1. First 4,4′-bipyridine was reacted with the intemediate compound *N*,*N*′-bis[2,4-bis(dinitophenyl)-4,4′-bipyridium dichloride by nucleophilic substitution with 1-chloro-2,4-dinitobenzene.[43] The *N*,*N*′-bis[2,4-bis(dinitophenyl)-4,4′-bipyridium dichloride reacted with 3,5-di(hydroxymethyl)aniline, synthesized according to the literature[44] to obtain $\mathbf{P_{1a}}$.

The reaction of 1-(2,4-dinitrophenyl)pyridinium chloride with aniline forming an intermediate pentamethine salt "Zincke salt" and 2,4-dinitroaniline is known as the "Zincke reaction". This process is now the standard route to *N*-arylpyridinium salts that cannot be formed by the direct reaction of electrophilics and pyridine.[45] The mechanism consists of two stages, ring opening and ring closing, and the excess amine plays an important role in assisting proton transfer in the conversion of Zincke salts to Zincke product.

The tetrahydroxy derivative $\mathbf{P_{1a}}$ was converted to tetrabromide $\mathbf{P_{1b}}$, using hydrobromic acid/acetic acid, followed by ion exchange of the PF_6^- salt in good yield (78%). From the intemediate product $\mathbf{P_{1b}}$ (PF_6^-), the desired dendrimer **6** was obtained by reaction of monomer $\mathbf{m_1}$ followed by ion exchange (route **IIa**, Scheme 1).

The dendrimer **8** phenyl core was obtained by reaction with monomer *N*-(3,5-di(hydroxymethyl)benzyl)-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_2}$) synthesized according to the literature,[46] with the intermediate $\mathbf{P_{1b}}$, followed by substitution of −OH by −Br in compound $\mathbf{P_{2a}}$ with 5.7 M HBr/acetic acid at room temperature to obtain precursor $\mathbf{P_{2b}}$. This reaction step was repeated once to obtain the precursors $\mathbf{P_{3a}}$ and $\mathbf{P_{3b}}$, respectively. In the last reaction step, the precursor $\mathbf{P_{3b}}$ was reacted with monomer $\mathbf{m_1}$ in MeCN to obtain the compound **8** in 60% yield (route **IIb**, Scheme 1).

The dendrimers **7**, **9**, and **10** containing 6, 12, and 24 thymine units, respectively, as capping groups on the peripheries were built from precursor core 1,1′,1″-{[(benzene-1,3,5-triyltris-(methylene)][tris(3,5-di(bromomethyl)benzene]-(4,4′-bipiridinium) }hexafluorophosphate $\mathbf{P_{4b}}$, reported in the literature.[1,40−42] (scheme 1, route III).

The trifunctional cross-linker 1,3,5-tris[(-4,4′-bipiridinium)-methyl]benzene trihexafluorophosphate consisting of a mesithyl derivative linked to three 4,4′-bipyridine units was prepared by reaction of 1,3,5-tris(bromomethyl)benzene with an excess of 4,4′-bipyridine in MeCN and further alkylated with 1,3-di(hydroxymethyl)benzyl bromide to the hexavalent alcohol $\mathbf{P_{4a}}$. This was succeeded by substitution of −OH for −Br, yielding $\mathbf{P_{4a}}$ with 5.7 M HBr/acetic acid and a subsequent ion exchange to the intermediate $\mathbf{P_{4b}}$ as PF_6^- salt. The compound **7** dendrimer with 18 charges per molecule was available from intermediate $\mathbf{P_{4b}}$ as tetrabromide via alkylation with monomer $\mathbf{m_1}$ in 61% yield (route IIIa, Scheme 1). Dendrimers of **9** and **10** were prepared by reaction of precursor $\mathbf{p_{4b}}$ with monomer $\mathbf{m_2}$, to give the intemediate $\mathbf{P_{5a}}$ $18PF_6^-$ (routes IIIb and IIIc, Scheme 1). The resulting dodecahydroxyalcohol $\mathbf{P_{5a}}$ was converted into dodecabromide $\mathbf{P_{5b}}$ by treatment with 5.7 M HBr/acetic acid. This reaction step was repeated once to obtain the intermediates $\mathbf{P_{6a}}$ and $\mathbf{P_{6b}}$, respectively. In the latter step the corresponding polybromide intemediates $\mathbf{P_{5b}}$ and $\mathbf{P_{6b}}$ were closed to **9** and **10**, respectively, by reaction with monomer $\mathbf{m_1}$ in MeCN in 50% and 63% yields respectively.

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

Antiviral Activity in Vitro. The anti-HIV-1 activity of dendrimers **1−10** was determined with respect to the number of charges in the scaffold and the ethyl (**1−5**) or thymine units (**6−10**) on the periphery (Chart 1). Dendrimers were evaluated for their capacity to inhibit replication of NL4.3, a HIV-1 strain that utilizes the chemokine co-receptor CXCR4 (X4 strains) for entry, in MT-4 cells (Table 1) and in human peripheral blood

Table 2. Effect of Charges and Number of Thymine Units on Surfaces of 1−10 and Inhibitory Effects (IC_{50}) on Replication of HIV-1 X4 NL4.3[a]

		compds, ethyl units					compds, thymine units				
	ref AMD3100	1	2	3	4	5	6	7	8	9	10
IC_{50} (μg/mL), HIV-1 NL 4.3	0.002	5.6	0.12	0.34	1.07	1.79	4.62	0.41	0.89	2.32	>4
CC_{50} (μg/mL), tox PBMC	>10	>100	100	20	20	20	>100	20	20	20	20

[a]All data represent the mean values and standard deviations for at least three separate experiments. IC_{50}: concentration of compound to inhibit 50% HIV-1 (NL4.3) replication in human PBMC cells (average of two independent experiments). CC_{50}: concentration of compound to inhibit 50% the viability in human PMBC cells.

Table 3. Inhibitory Effects of 1−10 on Chemokine Induced Calcium Signaling in U87.CD4.CXCR4 and U87.CD4.CCR5 Cells and Inhibition of CXCL-12^{AF647} Binding to CXCR4$^+$ SupT1 Cells[a]

	IC_{50} (μg/mL)										
			compds, ethyl units					compds, thymine units			
	ref AMD3100	maravioc	1	2	3	4	5	6	7	8	9	10
CXCL12-induced calcium signaling[b]	0.17	nd	61.15	3.61	1.37	0.97	1.19	65.26	2.13	1.53	0.48	0.48
CCL3L1-induced calcium signaling[b]	nd	0.0024	>100	80.92	22.64	5.9	5.9	>100	28.6	10.25	21.95	5.57
CXCL12^{AF647} binding[c]	0.057	nd	14.81	1.91	1.44	1.10	1.17	10.57	1.62	1.27	1.55	1.06

[a]All data represent mean values and standard deviations for at least three separate experiments. [b]Concentration of compound to inhibit 50% CXCL12-induced or CCL3L1-induced calcium signaling in U87.CD4.CXCR4 cells or U87.CD4.CCR5 cells, respectively. [c]Concentration of compound to inhibit for 50% CXCL-12^{AF647} binding in SupT1 cells.

Table 4. Inhibitory Effects of 1-10 on Anti-CXCR4 mAbs Binding in SupT1 Cells[a]

	IC_{50} (μg/mL) anti-CXCR4 mAb binding inhibition										
		compds, ethyl units					compds, thymine units				
	ref AMD3100	1	2	3	4	5	6	7	8	9	10
clone 12G5	0.02	1.96	2.31	1.21	1.21	1.56	2.25	1.84	1.93	2.23	>4
clone 173	0.02	1.39	1.36	1.76	1.18	1.74	2.05	1.64	2.21	2.40	2.06
clone 1D9	>1	8.59	2.99	2.16	1.96	2.57	9.56	2.55	2.83	2.92	>10

[a]All data represent mean values and standard deviations for at least three separate experiments. Concentration of compound to inhibit 50% anti-CXCR4 mAb binding in SupT1 cells.

mononuclear cells (PBMC) (Table 2). The cytotoxicity of dendrimers was evaluated in the same assay (see Experimental Section).

All dendrimers showed comparable antiviral activity against HIV-1 NL4.3 with IC_{50} values between 0.8 and 2.4 μg/mL. However, differences in cytotoxicity of the dendrimers in MT-4 cells were observed. The dendrimers **1** and **6** were not toxic at 100 μg/mL, while the CC_{50} of the other dendrimers ranged between 9 and 34 μg/mL. In addition, no significant differences were observed between the dendrimers with or without thymine units as surface groups (Table 2). In fact, there is a trend toward a decrease in antiviral activity when the thymine units are present.

To identify the mode of action of the different dendrimers, we investigated their interaction with the HIV-1 co-receptors CXCR4 and CCR5 by using calcium signaling assays. After binding to the receptor, chemokines trigger an intracellular signal transduction cascade comprising a transient cytosolic calcium mobilization. The chemokine receptors CCR5 and CXCR4 were transfected into human atroglioma U87.CD4 cells (see Experimental Section), and these cells were used to investigate the agonistic and antagonistic activity of the different dendrimers. None of the compounds could induce calcium signaling by itself, and these compounds thus have no detectable agonistic properties (data not shown). When CXCL12-induced calcium mobilization was measured in U87.CD4.CXCR4 cells, the dendrimers showed a dose-dependent decrease of the Ca^{2+} flux with IC_{50} values between 0.4 and 65 μg/mL (Table 3). To investigate the CCR5 antagonistic activity of these dendrimers, calcium signaling was induced in U87.CD4.CCR5 cells by CCL3L1. Again, a dose-dependent decrease of the Ca^{2+} flux was measured; however, overall, the IC_{50} values increased and compounds **1** and **6** at 100 μg/mL could not inhibit the Ca^{2+} flux with 50% (Table 3). No differences were detected between thymine dendrimers and dendrimers without thymine. As controls, the antagonists AMD3100 and maraviroc were used for CXCR4 and CCR5, respectively. Next, SupT1 cells were incubated with Alexa-fluor647-labeled CXCL12 (CXCL12^{AF647}) in the presence of the dendrimers. Here again, they all inhibited the binding of CXCL12^{AF647} equally (IC_{50} ranging between 1 and 2 μg/mL) except for dendrimers **1** and **6**, which were less potent in inhibiting CXCL12^{AF647} binding (IC_{50} ranging between 10 and 15 μg/mL) (Table 3).

To further investigate the interaction of the "viologen" dendrimers with the CXCR4 receptor, we examined the inhibition of the binding of anti-CXCR4 mAbs directed against different epitopes of CXCR4: clone 1D9 recognizing the N terminus, clone 173 recognizing mainly ECL2, and clones 12G5 recognizing ECL1 and ECL2. All dendrimers dose-dependently inhibited the binding of these three mAbs, and there were no differences observed between thymine dendrimers and dendrimers without thymine (Table 4). Only the "comb-branched" dendrimers **1** and **6** were not as effective in inhibiting the binding of the CXCR4 mAb clone 1D9, and the complex "spheroidal"

dendrimer **10** could not inhibit the binding of the anti-CXCR4 mAbs clone 12G5 and 1D9 (Table 4).

CONCLUSIONS

In this study, we have carried out the first structure−activity relationship (SAR) study for a novel class of compounds, polycationic "viologen" based dendrimers that inhibit HIV-1 (strain IIIB) replication in MT-4 cells by interactions with the CXCR4 HIV co-receptor. We previously demonstrated that the specificity of the inhibition clearly indicates that the mechanism of action of such compounds is more refined than electrostatic interaction between cell surface and virus without inhibitory activity of the enzyme's reverse transcriptase (RT). Furthermore, we synthesized and evaluated the capacity to inhibit replication of NL4.3, a HIV-1 strain that utilizes the chemokine co-receptor CXCR4 (X4 strains) for entry, in MT-4 cells and in human peripheral blood mononuclear cells (PBMC) of 10 compounds, 5 with ethyl as capping group, **1**−**5**, used in a previous study. In addition five new compounds with thymine as capping group, **6**−**10**, have been established. The cytotoxicity of dendrimers was evaluated in the same assay. The CXCR4 receptor affinity was evaluated and compared to the specific CXCR4 inhibitor AMD3100 at different concentrations. In vitro studies regarding the binding affinity are the most reliable point of comparison for the polycationic "viologen"-based dendrimers.

The trend that the increasing positive character of the dendrimers **3**−**5** and **8**−**10** leads to more cytotoxicity than has previously been observed by us and is most likely to be a charge-related effect.[1] The dendrimers **1** and **6** are +10 charged, and the dendrimers **2** and **7** are +18 charged. They were not toxic at 100 and 34 μg/mL, while the CC_{50} of the other dendrimers ranged between 9 and 11 μg/mL. We reason that this increase in positive charge may result in charge-driven nonspecific membrane binding and causes cytotoxicity, similar to that reported for positively charged PAMAM dendrimers.[47] In contrast in the calcium signaling assays low charged denrimers **1** and **6**, +10 charges, were 10-fold less potent in inhibiting $CXCL12^{AF647}$ binding (IC_{50} ranging between 10 and 15 μg/mL) compared with dendrimers **3**−**5** and **8**−**10** (IC_{50} ranging between 1 and 2 μg/mL). Only the dendrimers **1** and **6**, +10 charges, were not as effective in inhibiting the binding of the CXCR4 mAb clone 1D9, and the complex "spheroidal" dendrimer **10** with thymine capping group could not inhibit the binding of the anti-CXCR4 mAbs clones 12G5 and 1D9.

The CXCR4 affinity of dendrimers **1**−**5** ethyl capping and **6**−**10** thymine capping is comparable. A bivalent effect based on electrostatic interaction between polycationic scaffold and heparan sulfate corroborates hydrogen bonding between thymine units, and that of carboxylate group residues of HIV co-receptor CXCR4 for dendrimers **6**−**10** was not observed but rather a slight decrease in the affinity of **10**.

Our results clearly demonstrate that with the use of highly positively charged compounds some polycationic "viologen" based dendrimers should be avoided to suppress the cytotoxicity and the nonspecific cell and tissue binding. However, the dendrimers **1** and **6**, with +10 charges, and **2** and **7**, with +18 charges, have less influence on the cell viability. The **1** and **6**, +10 charges, were not so effective in inhibiting the binding of the CXCR4 mAb clone 1D9. Thus, dendrimers **2** and **7**, with +18 charges, are the most promising CXCR4 imaging probes. These compounds have potential for development as inhibitors of other nonviral pathogens such as human tumor cells. This study provides an important framework for the rational design of more potent inhibitors, which are conceptually distinct from any other existing HIV-1 inhibitors.

EXPERIMENTAL SECTION

Chemistry. Materials and Methods. The chemicals were analytical grade from Aldrich, Merck, or Fluka and used as received except ethyl acetate, diethyl ether, THF, methanol, and petroleum ether, which 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 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 500 and at 125 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 FT-IR spectrophotometer. UV−vis spectra were obtained with an 8453 UV−visible spectophotometer (Agilent, Germany), with λ_{max} in nm (ε in M^{-1} cm^{-1}). The UV−vis spectra from dendrimers in their oxidized (V^{+2}) and reduced ($V^{\bullet+}$) state have been recorded spectroelectrochemically in $MeCN/NaClO_4$ (0.1), and the corresponding λ_{max} values and extinction coefficients (ε) are reported. The reference for UV/vis spectra of dendrimers was the pure solvent/electrolyte. The purity of tested compounds was verified by elemental analyses on a vario Micro cube analyzer, and the data for C, H, and N are within ±0.4% of the theoretical values.

General Procedure for the Synthesis of 10 and 20 6-Fold Charged "Comb-Branched" Dendrimers (6, 8). A mixture of the phenylic dendrimer precursor $\mathbf{P_{1b}}$ and the headgroup 1-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4yl)-pyridinium hexafluorophosphate ($\mathbf{m_1}$) or the cross-linker *N*-(3,5-di(hydroxymethyl)-benzyl)-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_2}$) (Scheme 1, route II) 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-, 42-, and 90-Fold Charge "Spheroidal" Dendrimers (7, 9, 10). A solution of the benzylic dendrimer precursor $\mathbf{P_{4b}}$ in acetonitrile and 1-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4-yl)-pyridinium hexafluorophosphate ($\mathbf{m_1}$) as capping group was refluxed for 1 week (Scheme 1, route IIIa) to obtain **7**. The cross-linker *N*-(3,5-di(hydroxymethyl)benzyl)-4,4′-bipyridinium hexafluorophosphate ($\mathbf{m_2}$) was used for the preparation of the intermediates $\mathbf{P_{5b}}$ and $\mathbf{P_{6b}}$ by repetitive application followed in the last reaction step by coupling $\mathbf{m_1}$ capping group monomer to close the **9** and **10**, respectively . The resulting compounds were worked up and purified as reported in the description of the single compounds. The purity checks by mass spectrometry using the MALDI-TOF method were not very successful because highly charged molecules are very difficult to measure (see Supporting Information). Heinen et al. also had no success in MALDI-TOF measurements of similar compounds.[42] Stoddart and Balzani also synthesized dendrimers on bipyridine basis (between 6 and 42 charges per molecule) and could obtain MS analysis results for low charged molecules.[48]

Synthesis of Dendrimer 6. Amounts of 80 mg (0.082 mmol) of tetrabromide precursor $\mathbf{P_{1b}}$ and 230 mg (0.49 mmol) of 1-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4-yl)-pyridinium hexafluorophosphate ($\mathbf{m_1}$) were dissolved in 20 mL of MeCN. The mixture was refluxed at 70 °C for 5 days. The cooled reaction mixture was filtered, washed with MeCN (10 mL) and ether, successively, and dried. The mother liquor was evaporated, and the residue was dried. The residue was dissolved in 7 mL of nitromethane and extracted with 3 mL of a NH_4PF_6 solution (10% in water). The organic phase was washed one more time with water and evaporated to dryness. The residue was put in water, filtered, washed several times with

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water, and dried in a vacuum for 12 h. The product was purified by chromatography (Sephadex LH-20, column 2.5 cm × 25 cm, MeCN/MeOH, 1:1, as eluent), giving 160 mg of dendrimer **6** (0.051 mmol, 62% yield) after drying in a vacuum at 40 °C for 24 h. ^{1}H NMR (500 MHz, CD_3CN): δ = 9.15−9.10 (m, 8H), 8.98 (d, 8H, ^3J[H−H] = 5.5 Hz), 8.94 (d, 8H, ^3J[H−H] = 6.0 Hz), 8.63 (d, 4H, ^3J[H−H] = 4.0 Hz), 8.41 (d, 8H, ^3J[H−H] = 5.5 Hz), 8.37 (d, 8H, ^3J[H−H] = 5.5 Hz), 7.97 (s, 2H), 7.90 (s, 4H), 7.20 (s, 4H), 5.98 (s, 8H). 4.65 (t, 8H, ^3J[H−H] = 7.2 Hz), 3.77 (t, 8H, ^3J[H−H] = 6.2 Hz), 2.36 (t, 8H, ^3J[H−H] = 6.7 Hz), 1.82 (s, 12H). ^{13}C NMR (125 MHz, CD_3CN): δ = 165.2, 152.6, 152.0, 151.0, 147.1, 147.0, 146.8, 144.5, 141.7, 137.3, 135.0, 128.7, 128.3, 128.1,111.6, 64.2, 60.4, 45.3, 31.4, 12.4. IR (cm^{-1}): 3650.5, 3073.0, 2977.6, 1674.8, 1637.4, 1452.4, 1366.1, 1223.2, 820.1, 552.9. UV−vis (MeCN, partial reduced): V^{+2} λ_{max}, 264 (644 65), 422 (4352); $V^{\bullet+}$ at −0.5 V λ_{max}, 402 (70 253), 608 (27 740). Anal. Calcd for $C_{98}H_{98}F_{60}N_{18}O_8P_{10}$·4.4$CH_4O$·1.85$C_2H_3N$ (3105.5 + 216): C 38.36, H 3.68, N 8.37. Found: C 38.32, H 3.70, N 8.40.

Synthesis of Dendrimer 7. Amounts of 106 mg (0.046 mmol) of hexabromide precursor $\mathbf{P_{4b}}$ and 230 mg (0.49 mmol) of 1-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4-yl)-pyridinium hexafluorophosphate ($\mathbf{m_1}$) were suspended in 20 mL of MeCN. The mixture, protected from light, was refluxed at 75 °C for 6 days. The cooled reaction mixture was filtered and washed with ether, successively, and dried. The mother liquor was evaporated, and the residue was dried. The combined solids were dissolved in 10 mL of nitromethane and extracted with 4 mL of a NH_4PF_6 solution (10% in water). The organic phase was washed one more time with water and evaporated to dryness. The residue was put in water, filtered, washed several times with water, and dried in a vacuum for 12 h. For purification the product was further dissolved in $MeNO_2$ and extracted with water for 1 week in a Ludwig apparatus. The $MeNO_2$ phase was evaporated and dried in HV for 12 h. The residue was washed with water, filtered, and dried in a vacuum to obtain 156 mg of **7** (0.028 mmol, 61% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.29 (s, 6H), 8.98−8.92 (m, 36H), 8.32−8.38 (m, 36H), 7.69 (s, 9H), 7.65 (s, 3H), 7.24 (s, 6H), 5.85 (s, 24H), 4.69 (t, 12H, ^3J[H−H] = 7.5 Hz), 3.81 (t, 12H, ^3J[H−H] = 8.7 Hz), 2.39 (t, 12H, ^3J[H−H] = 6.7 Hz), 1.85 (s, 18H). ^{13}C NMR (125 MHz, CD_3CN): δ = 165.6, 153.1, 152.3, 151.7, 147.4, 142.2, 136.5, 136.5, 133.5, 133.4, 129.1, 129.1, 128.8, 112.1, 65.3, 61.0, 45.8, 31.8, 12.0. IR (cm^{-1}): 3651.4, 3072.2, 2977.9, 1674.8, 1637.9, 1450.9, 1362.6, 1223.4, 1167.7, 813.0, 552.3. UV−vis (MeCN): V^{+2} λ_{max}, 268 (159365), 422 (20177); $V^{\bullet+}$ at −0.5 V λ_{max}, 402 (121 785), 608 (37 488). Anal. Calcd for $C_{174}H_{174}F_{108}N_{30}O_{12}P_{18}$·5$H_2O$ (5486.8 + 90): C 37.47, H 3.33, N 7.53. Found: C 37.2, H 3.35, N 7.39.

Synthesis of Dendrimer 8. Amounts of 100 mg (0.028 mmol) of octabromide precursor $\mathbf{P_{3b}}$ and 150 mg (0.31 mmol) of 1-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4-yl)-pyridinium hexafluorophosphate ($\mathbf{m_1}$) were dissolved in 20 mL of MeCN. The mixture, protected from light, was refluxed at 75 °C for 1 week. The cooled reaction mixture was filtered and washed with ether, successively, and dried. The mother liquor was evaporated, and the residue was dried. The residue was dissolved in 7 mL of nitromethane and extracted with 3 mL of a NH_4PF_6 solution (10% in water). The organic phase was washed one more time with water and evaporated to dryness. The residue was put in water, filtered, washed several times with water, and dried in a vacuum for 12 h. The product was purified by chromatography (Sephadex LH-20, column 2.5 cm × 25 cm, MeCN/MeOH, 1:1, as eluent), giving 140 mg of **8** (0.017 mmol, 60% yield) after drying in a vacuum at 40 °C for 24 h. ^{1}H NMR (500 MHz, CD_3CN): δ = 9.19−9.01 (m, 8H), 8.97−8.92 (m, 48H), 8.68 (s, 4H), 8.44−8.37 (m, 48H), 8.01 (s, 4H), 7.68 (s, 12H), 7.66 (s, 2H), 7.23 (s, 8H), 5.98 (s, 8H), 5.84 (s, 24H), 4.67 (t, 16H, ^3J[H−H] = 7.2 Hz), 4.30 (s, 4H), 3.79 (t, 16H, ^3J[H−H] = 6.0 Hz), 2.38 (qu, 16H, ^3J[H−H] = 6.6 Hz), 1.83 (s, 24H). ^{13}C NMR (125 MHz, CD_3CN): δ = 165.3, 152.7, 151.7, 151.1, 147.1, 146.9, 146.8, 141.8, 137.4, 137.2, 128.6, 128.5, 128.3, 111.6, 64.8, 64.2, 45.3, 31.4, 12.4. IR (cm^{-1}): 3650.0, 3071.9, 2977.7, 1674.0, 1636.9, 1451.6, 1367.3, 1223.1, 1172.2, 811.8, 551.3. UV−vis (MeCN, partial reduced): V^{+2} λ_{max}, 269 (250 850), 424 (16 487); $V^{\bullet+}$ at −0.5 V λ_{max}, 402 (206 665), 608 (78 825). Anal. Calcd for $C_{246}H_{242}F_{156}N_{42}O_{16}P_{26}$·13$CH_4O$·6$C_2H_3N$ (7811.8 + 662): C 37.41, H 3.71, N 7.93. Found: C 38.11, H 4.09, N 7.79.

Synthesis of Dendrimer 9. Amounts of 140 mg (0.022 mmol) of dodecabromide precursor $\mathbf{P_{5b}}$ and 150 mg (0.32 mmol) of 11-[3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propyl]-4-(pyridin-4-yl)pyridinium hexafluorophosphate ($\mathbf{m_1}$) were suspended in 20 mL of MeCN. The mixture, protected from light, was refluxed at 75 °C for 4 days. The cooled reaction mixture was filtered and washed with ether, successively, and dried. The mother liquor was evaporated, and the residue was dried. The combined solids were dissolved in 10 mL of nitromethane and extracted with 4 mL of a NH_4PF_6 solution (10% in water). The organic phase was washed one more time with water and evaporated to dryness. The residue was put in water, filtered, washed several times with water, and dried in a vacuum for 12 h. For purification the product was further dissolved in $MeNO_2$ and extracted with water for 1 week in a Ludwig apparatus. The $MeNO_2$ phase was evaporated and dried in HV for 12 h. The residue was washed with water, filtered, and dried in a vacuum at 40 °C for 24 h to obtain 140 mg of **9** (0.011 mmol, 50% yield). ^{1}H NMR (500 MHz, CD_3CN): δ = 11.24 (s, 12H), 9.37 (s, 84H), 9.14 (m, 84H), 7.79 (b, 30H), 7.49 (s, 12H), 5.92 (s, 60H), 4.74 (s, 24H), 3.78 (s, 24H), 2.35 (s, 24H), 1.77 (s, 36H). ^{13}C NMR (125 MHz, CD_3CN): δ = 164.2, 151.1, 149.1, 148.3, 146.0, 140.8, 135.3, 130.0, 126.8, 126.3, 109.0, 62.7, 58.6, 43.9, 30.2, 11.9. IR (cm^{-1}): 3648.6, 3073.0, 2980.5, 1673.3, 1637.7, 1450.9, 1363.5, 1223.6, 1167.7, 817.0, 553.3. UV−vis (MeCN): V^{+2} λ_{max}, 267 (436 080), 422 (20 119); $V^{\bullet+}$ at −0.5 V λ_{max}, 402 (358 640), 608 (145 420). Anal. Calcd for $C_{396}H_{390}F_{252}N_{66}O_{24}P_{42}$·72.5$CH_4O$·31$C_2H_3N$·12$H_2O$ (12546.3 + 3807): C 38.95, H 4.91, N 8.31. Found: C 38.55, H 5.28, N 8.70.

Synthesis of Dendrimer 10. Amounts of 230 mg (0.016 mmol) of tetraeicosabromid precursor $\mathbf{P_{6b}}$ and 225 mg (0.48 mmol) of 1-[3-(6-hydroxy-5-methyl-2-oxo-1,2,3,6-tetrahydropyridin-3-yl)propyl]-4-(pyridin-4-yl)pyridinium were suspended in 15 mL of MeCN. The mixture was refluxed at 75 °C for 1 week. The cooled reaction mixture was filtered and washed with ether, successively, and dried. The mother liquor was evaporated, and the residue was dried. The combined solids were dissolved in 10 mL of nitromethane and extracted with 4 mL of a NH_4PF_6 solution (10% in water). The organic phase was washed one more time with water and evaporated to dryness. The residue was put in water, filtered, washed several times with water, and dried in a vacuum for 12 h. For purification the product was further dissolved in $MeNO_2$ and extracted with water for 1 week in a Ludwig apparatus. The $MeNO_2$ phase was evaporated and dried in HV for 12 h. The residue was washed with water, filtered, and dried in a vacuum at 40 °C for 24 h to obtain 270 mg of **10** (0.010 mmol, 63% yield). ^{1}H NMR (500 MHz, CD_3CN): δ = 9.40 (b signal, 24H), 8.98−8.93 (m, 180H), 8.40−8.39 (m, 180H), 7.68 (b, 66H), 7.25 (s, 24H), 5.86 (s, 132H), 4.68 (t, 48H, ^3J[H−H] = 7.0 Hz), 3.80 (t, 48H, ^3J[H−H] = 5.5 Hz), 2.39 (t, 48H, ^3J[H−H] = 6.0 Hz), 1.84 (s, 72H). ^{13}C NMR (125 MHz, CD_3CN): δ = 165.1, 152.4, 151.5, 150.8, 146.6, 135.7, 132.6, 128.4, 128.3, 128.3, 111.35, 64.5, 60.1, 45.0, 31.2, 12.1. IR (cm^{-1}): 3650.7, 3138.63, 2977.7, 1676.3, 1638.2, 1451.1, 1380.2, 1165.0, 820.7, 553.7. UV−vis (MeCN): V^{+2} λ_{max}, 268 (517 700), 416 (28 088); $V^{\bullet+}$ at −0.5 V λ_{max}, 402 (418 805), 608 (170 175). Anal. Calcd for $C_{841}H_{824}F_{540}N_{138}O_{48}P_{90}$·106$CH_4O$·30$C_2H_3N$·18$H_2O$ (26679.4 + 4946): C 38.24, H 4.38, N 7.64. Found: C 37.94, H 4.76, N 7.72.

Materials and Measurements of Biological Activities. Anti-HIV-1 (X4) NL4.3 Activity of the Dendrimers 1−10. The antiretroviral assays in MT-4 cells have been described in detail earlier by Vermeire et al.[49] Briefly, MT-4 cells (50 μL, 1 × 10^6 cells/mL) were preincubated for 30 min at 37 °C with test compounds (100 μL) in a 96-well plate (Falcon). Then HIV-1 X4 NL4.3 was added according to the $TCID_{50}$ of the viral stock. Cytopathic effect (CPE) was scored microscopically 5 days postinfection, and EC_{50} values were determined using the MTS/PES method by Vermeire et al.[50] (Table 1).

Calcium Signaling Assays. U87.CD4.CXCR4 cells or U87.CD4.CCR5 cells were digested by trypsin and seeded in gelatin-coated (0.2%) black-wall 96-well microplates (Costar, Cambridge, MA) at 2 × 10^4 cells per well the day before the experiment. The next day, the cells were loaded with the fluorescent calcium indicator Fluo-3-acetoxymethyl (Molecular Probes, Leiden, The Netherlands) at 4 μM

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for 45 min at 37 °C. Then cells were washed with assay buffer (see above) and incubated with 150 μL of **1**−**10** and AMD3100 or maraviroc at different concentrations and diluted in assay buffer for 10 min at 37 °C. The intracellular calcium signaling in response to 25 ng/mL CXCL-12 or 10 ng/mL LD78β for U87.CD4.CXCR4 cells or U87.CD4.CCR5 cells, respectively, was measured in all 96 wells simultaneously as a function in time, using the fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA) as described previously by Princen et al.[51] (Table 3).

Inhibition of CXCL-12^{AF647} Binding to CXCR4+ T Cell Line (SUPT-1). The CXCL-12^{AF647} binding assay has been described by Hatse et al.[52] Briefly, SUPT-1 cells, expressing high levels of CXCR4, were washed once in assay buffer and incubated with **1**−**10** or AMD3100 at different concentrations diluted in assay buffer (Hanks' balanced salt solution with 20 mM HEPES buffer and 0.2% bovine serum albumin, pH 7.4) for 15 min at room temperature. Then CXCL-12^{AF647} was added, diluted in assay buffer at a final concentration of 25 ng/mL), and the cells were incubated for 30 min at room temperature. Then the cells were washed, fixed in 1% paraformaldehyde in PBS, and analyzed on the FL4 channel (661/16 nm) of a FACSCalibur flow cytometer equipped with a 635 nm red diode laser (Becton Dickinson) (Table 3).

Inhibition of Anti-CXCR4 mAbs Directed against Different Epitopes of CXCR4. The antibodies used in this study were PE-conjugated mouse anti-human CXCR4 mAb clone 12G5 (BD Biosciences), clone FAB173P (173) (R&D Systems Europe, U.K.), and PE-conjugated rat anti-human CXCR4 mAb clone 1D9 ((BD Biosciences). As a control for specific background staining, the cells were stained with Simultest control, $\gamma_1/\gamma_{2\alpha}$ FITC/PE (Becton Dickinson). SUPT-1 cells were washed in PBS with 2% FBS, and for each sample 0.3 × 10^6 cells were resuspended in 100 μL of PBS/FBS, 2%. Cells were incubated with **1**−**10** or AMD3100 at different concentrations diluted in PBS with 2% FBS for 30 min at 4 °C. Thereafter, antibodies were added and samples were incubated for another 30 min at 4 °C (Table 4). After incubation the cells were washed and resuspended in 300 μL of PBS containing 1% paraformaldehyde and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) (Table 4).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of monomers and precursors, spectroscopic details, and analytical data of target compounds **6**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-541-969 2802. Fax: + 49-541-969 2370. E-mail: sasaftei@uos.de.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HS, polysaccharide heparin sulfate; PBMC, peripheral blood mononuclear cell; SDF-1α, stromal-cell-derived factor 1α; MIP-1α, macrophage inflammatory protein 1α; MIP-1β, macrophage inflammatory protein 1β; RANTES, regulated upon activation normal T-cell expressed and secreted; AMD3100, 1,1′-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetracene; TM-IV, transmembrane domain 4; TM-VII, transmembrane domain 7; MeCN, acetonitrile; U87.CD4, human atroglioma; FDA, Food and Drug Administration

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