Orally Bioavailable Isothioureas Block Function of the Chemokine Receptor CXCR4 In Vitro and In Vivo

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The interaction of the chemokine receptor CXCR4 with its ligand CXCL12 is involved in many biological processes such as hematopoesis, migration of immune cells, as well as in cancer metastasis. CXCR4 also mediates the infection of T-cells with X4-tropic HIV functioning as a coreceptor for the viral envelope protein gp120. Here, we describe highly potent, selective CXCR4 inhibitors that block CXCR4/CXCL12 interactions in vitro and in vivo as well as the infection of target cells by X4-tropic HIV.

Introduction

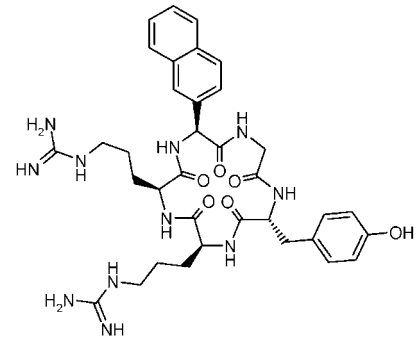
The chemokine CXCL12, also known as stromal cell-derived factor-1 (SDF-1[a]) is the natural agonistic ligand identified for the homeostatic, heptahelical G-protein coupled receptor (GPCR) CXCR4, which is expressed on hematopoietic stem cells, leukocytes, endothelial cells, platelets, and tumor cells.[1–3] Specific interactions of CXCR4 with CXCL12 regulate the migration of primordial germ cells[4] and are involved in cell migration in the immune and nervous systems[5] as well as in cancer metastasis and angiogenesis.[6] Furthermore, CXCR4 mediates infection of T-cells by the X4-tropic human immunodeficiency virus (HIV) functioning as a coreceptor for the viral envelope protein gp-120.[7] Thus, low molecular weight antagonists of CXCR4 are of high interest because of their therapeutic potential in autoimmune diseases, cancer, and AIDS.

Several potent CXCR4 inhibitors have been described to date, among them CXCL12-derived peptides,[8] the bicyclam AMD3100[9a–d] (and simplified analogues thereof;[9e] Chart 1), and the 14-residue truncated polyphemusin peptide analogue T140[10] as well as a T140-derived cyclic penta- (Chart 1), tetra-, and pseudopeptides.[11] However, many of these potent compounds might exhibit poor biopharmaceutical properties.[12]

Here we present a novel series of orally bioavailable, highly potent, selective CXCR4 inhibitors that block both, human and rodent CXCR4.

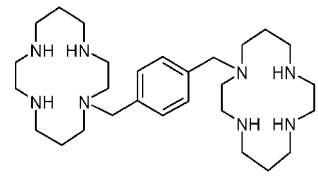
Chemistry. All isothioureas **1a**−**1u** were readily available by refluxing a 1:1 mixture of the corresponding thioureas **2** and the appropriate halides **3** in acetonitrile (Scheme 1; for R-groups, see Tables 2 and 3). A single crystallization gave the pure hydrochlorides of **1**. Halide **3p** is commercially available, compounds **3a**,[13] **3r**,[14] and **3s**[13] have been prepared as previously described, and compounds **3q** and **3t** were synthesized from 1,3-dichloro-propan-2-one (**4**) and thioureas **5q** and **5t**, respectively (Scheme 2). In the case of **5t**, the regioisomer **3t** with geminal methyl groups at C-6 was exclusively formed. The structure was confirmed by NMR measurements. Strong NOEs were observed between H-5 and H-10,11 as well as between H-5 and H-9 but no NOE could be detected between H-9 and H-10,11 (for numbering, see Scheme 2). The regiosisomer with geminal methyl groups at C-5 would have led to strong NOEs between H-10,11 and H-9. The X-ray analysis of crystalline **1t** finally confirmed the structure (Chart 2).

Chart 1. Structures and Potencies of CXCR4 Antagonists AMD3100, T140-Derived Cyclic Pentapeptide, and Compound **1a**



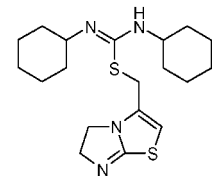
Cyclic Pentapeptide

	IC_{50} (bind.)
hu	109 nM
rat	1440 nM



AMD3100

	IC_{50} (bind.)
hu	245 nM
rat	108 nM



1a

	IC_{50} (bind.)
hu	48 nM
rat	55 nM

Results and Discussion

Isothiourea **1a** (Chart 1) was discovered as a single hit by screening the Novartis compound collection against CXCR4 using a radioligand binding assay. The initial hit was resynthesized and profiled in a series of in vitro assays including radioligand binding assays using membranes prepared either from CEM cells, a T lymphoblast cell line expressing human CXCR4, or from IR983F cells, a rat cell line endogenously

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[a] Abbreviations: SDF-1, stromal cell-derived factor-1; GPCR, g-protein coupled receptor; HIV, human immunodeficiency virus; NOE, nuclear Overhauser effect; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FLIPR, fluorometric imaging plate reader.

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Table 1. Properties of Compounds **1a** and **1t**

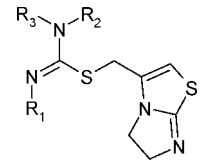
assay	**1a**	**1t**
hu CXCR4 (binding), IC_{50}, nM	48 ± 3	8.0 ± 2
rat CXCR4 (binding), IC_{50}, nM	55 ± 9	11 ± 4
hu CXCR4 (Ca^{2+}), IC_{50}, nM	11 ± 2	1.1 ± 0.1
hu CXCR4 (CXCL12-induced migration), IC_{50}, nM	58 ± 28	n.t.
rat CXCR4 (CXCL12-induced migration), IC_{50}, nM	29 ± 12	n.t.
CXCL12-induced actin polymerization (hu), IC_{50}, nM	32 ± 15	n.t.
CXCL12-induced actin polymerization (rat), IC_{50}, nM	86 ± 81	n.t.
CYP1A2, IC_{50}, nM	>10000	>10000
CYP2C9, IC_{50}, nM	>10000	>10000
CYP2C19, IC_{50}, nM	>10000	5200
CYP2D6, IC_{50}, nM	6780	400
CYP3A4, IC_{50}, nM	3860	>10000
hERG (cellular), IC_{50}, nM	>20000	>20000
hERG (binding), IC_{50}, nM	8500	13240
pK_a	9.5, 7.7	9.6, 8.0
LogP	3.5	4.5
LogD	−0.2	1.2
intrinsic clearance (*hu*), μL min^{-1} mg^{-1}	51	149
intrinsic clearance (*rat*), μL min^{-1} mg^{-1}	411	342
solubility (pH 1.0), g/L	>5	>5
solubility (pH 6.8), g/L	>5	>5
Caco-2	efflux	passive
protein binding (hu), free fraction, %	29	20
protein binding (rat), free fraction, %	54	46

expressing rat CXCR4. As functional in vitro readouts, the inhibition of CXCL12 induced Ca^{2+}-mobilization in CEM cells (human CXCR4) and IR983F cells (endogenously expressing rat CXCR4) were determined. Inhibition of CXCL12 induced cell migration was assessed using either Jurkat cells (endogenously expressing human CXCR4) or IR983F cells (rat CXCR4). Functional inhibitory activity of **1a** in whole blood was demonstrated in an assay that measures the inhibition of CXCL12 induced actin polymerization in blood neutrophils. Fluorescence labeled phalloidin, which specifically recognizes polymeric F-actin but not monomeric, globular actin, was used as a probe.[16]

Compound **1a** was found to be very potent in all these in vitro assays showing similar activities on human and rat CXCR4. All IC_{50} values were below 100 nM (Table 1). In our assays, both AMD3100 and the T140-derived cyclic pentapeptide were less potent than compound **1a** (Chart 1). Interestingly, the pentapeptide was 10-fold less potent on the rat receptor. Compound **1a** is an antagonist, as it inhibited concentration-dependently the CXCL12 induced Ca^{2+}-release in CXCR4 positive cells. Compound **1a** was tested against a broad panel of >50 GPCRs (including several chemokine receptors, such as CCR4, CCR5, CCR7, CCR9, CXCR2) and ion channels. It was found to be highly selective and did not show IC_{50} values below 3000 nM.

The biopharmaceutical in vitro properties of compound **1a** were assessed in a variety of profiling assays (Table 1). It did not inhibit cytochrome P450 enzymes at concentrations below 3500 nM. In an assay, using a membrane potential dye to determine hERG channel blockade in a recombinant hERG-CHO-K1 cell line, the IC_{50} value was above 20000 nM, whereas in a cell-free, biochemical assay, measuring ^{3}H-dofetilide binding to HEK293 cell membranes expressing human recombinant hERG K^+ channels, the IC_{50} was determined to be 8500 nM. The intrinsic clearance was found to be acceptable when incubated with human liver microsomes but high when treated with rat liver microsomes. The water solubility was very high, and Log P and Log D were acceptable. The binding to both human and rat serum proteins was very low. The Caco-2 assay indicated active transport mechanisms and predicted moderate oral bioavailablity. In conclusion, the overall profile was very promising for a screening hit.

Table 2. Modifications of the Thiourea Portion of **1a**[a]

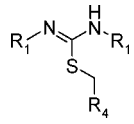


	R_1	R_2	R_3	IC_{50} (bind.)* [nM]	IC_{50} (Ca^{2+})* [nM]
1a			H	48	11
1b			Me	3575	931
1c			H	3835	493
1d			H	>10000	n.t.
1e			H	25	3.1
1f			H	27	5.3
1g			H	28	6.6
1h			H	28	8.0
1i			H	372	173
1j			H	855	75
1k			H	1873	304
1l			H	81	13
1m			H	1533	437
1n			H	>10000	5275
1o			H	10000	2220

[a] * = For potent compounds mean values of at least 3 independent measurements.

Consequently, the in vivo PK properties were assessed in Lewis rats after intravenous and oral administration (Chart 3). The area under the curve (AUC) normalized to a dose of 1 mg/kg was determined to be 567 and 76 ng/mL h, respectively, translating into acceptable 14% oral bioavailability. This was surprising, as compound **1a** is highly protonated under physiological pH (see pK_a in Table 1). Possible explanations are paracellular uptake of this small, highly soluble compound as well as the involvement of active transport mechanisms as indicated by the Caco-2 assay. The maximal concentration (C_{max}) normalized to a dose of 1 mg/kg

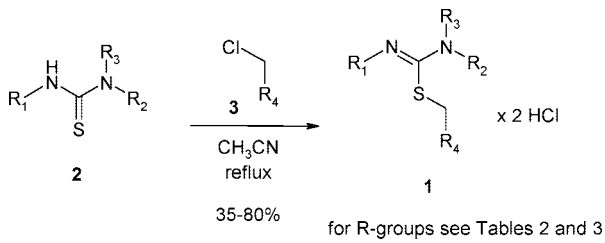
Table 3. Modifications of the Thiourea Portion of **1a**[a]



	R_4	R_1	IC_{50} (bind.)* [nM]	IC_{50} (Ca^{2+})* [nM]
1a			48	11
1p			>10000	>10000
1q			>10000	9130
1r			>10000	8120
1s			185	57
1t			8.0	1.1
1u			8.8	2.1

[a] * = For potent compounds, mean values of at least 3 independent measurements.

Scheme 1. Syntheses of Compounds **1a**−**1u**

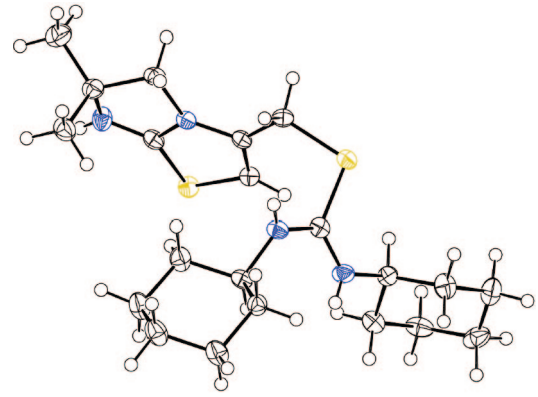


was 8 ng/mL, the time to reach C_{max} (t_{max}) was 0.5 h. The elimination half-life ($t_{1/2}$) was found to be 7.2 h.

Compound **1a** blocked CXCR4 function in vivo. Administration to Lewis rats ($n = 3$) by osmotic pumps (30 mg/kg·d) for 5 days led to constant blood levels of ~1 μM, i.e., a concentration that was >10 fold above the IC_{50} value obtained in our rat whole blood actin polymerization assay. Blood samples were taken on day 5 and analyzed ex vivo. In all samples, we observed >80% reduced actin polymerization compared to blood samples from untreated animals indicating in vivo antagonism of CXCR4.

Encouraged by these result, we prepared a variety of analogues of **1a** with modifications in the thiourea portion **1b**−**1o** (Scheme 1; Table 2). Methylation of the thiourea led to an almost complete loss of potency (**1b**). A series of symmetrical (**1c**−**1f**) and unsymmetrical thioureas (**1g**−**1m**) revealed that cyclic, aliphatic residues (6−8 membered) are optimal for potency resulting in compounds **1e**−**1h** with IC_{50} values of

Chart 2. Structure of Compound **1t** in the Crystal[15] (Atomic Displacement Ellipsoids Drawn at the 50% Probability Level, Hydrogen Atoms Drawn as Spheres of Arbitrary Radius)



25−28 nM in binding and <10 nM in Ca^{2+} mobilization assays. Like **1a**, these compounds cross reacted with rat CXCR4. Smaller ring sizes (**1c**, **1i**) and acyclic aliphatic groups led to significant loss of potency (**1d**, **1j**). An adamantyl group linked via a secondary C atom was tolerated (**1k**), whereas linkage via a tertiary C-atom caused reduced potency. Aromatic groups led to the poorly active derivatives **1n** and **1o**.

Next we modified the dihydro-imidazothiazole of **1a** by keeping the dicyclohexylthiourea constant (Table 3). The phenyl derivative **1p** was found to be inactive. Opening of the dihydro-imidazole ring gave the inactive derivative **1q**. Replacement with benzoimidazothiazole led to inactive derivative **1r**. Expansion of the dihydro-imidazole ring gave the moderately active dihydro-5*H*-thiazolo[3,2-*a*]pyrimidine **1s**. Introduction of two methyl groups led to the highly potent analogue **1t** with IC_{50} values of 8.0 nM in binding and 1.1 nM in Ca^{2+}-mobilization assays. Compound **1u** was designed by combining the most promising modifications of both series, but no additional improvement over **1t** could be achieved.

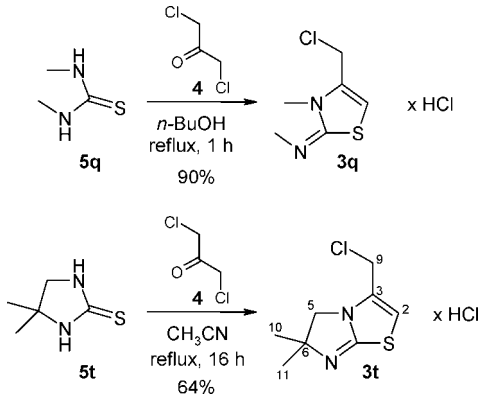
The therapeutic potential of our CXCR4 antagonists as anti-AIDS drugs was evaluated in an assay measuring the inhibition of X4-tropic HIV-1_{IIIB} attachment (Table 4). A small series of compounds covering a broad CXCR4 potency range was selected and compared with AMD3100. We observed a very good correlation between inhibition of CXCR4 and HIV attachment. The most potent CXCR4 antagonist **1t** showed an IC_{50} of 7 and an IC_{90} of 100 nM, whereas the modest CXCR4 antagonist **1b** was found to be inactive. Very interestingly, AMD3100, which was 30-fold less potent than **1t** in our CXCR4 binding assay, was found to be similarly potent in the HIV attachment assay. We conclude that AMD3100 and **1t** bind to nonidentical binding sites of CXCR4.[17] As a consequence AMD3100 may predominantly interfere with the CXCR4/gp120 interaction, whereas **1t** may block both CXCR4/gp120 as well as CXCR4/CXCL12 contacts.

The in vivo PK properties of the most potent compound **1t** were assessed and found to be superior to **1a** (Chart 3). After po-dosing, the area under the curve (AUC) normalized to a dose of 1 mg/kg was determined to be 105 ng/ml h translating into 32% oral bioavailability. The maximal concentration (C_{max}) normalized to a dose of 1 mg/kg was 18 ng/mL, the time to reach C_{max} (t_{max}) was 0.5 h. The elimination half-life ($t_{1/2}$) was found to be 7.6 h.

Conclusion

In conclusion, we have discovered a novel series of orally bioavailable, highly potent, selective CXCR4 antagonists that

Scheme 2. Syntheses of Compounds **3q** and **3t**



block the interaction of the chemokine receptor with both CXCL12 and the HIV envelope protein gp120. As these compounds block CXCR4 function in vivo, they can be used as tools to further explore the role of CXCR4 and its chemokine ligand CXCL12 in disease models.

Experimental Section

General. All reactions were carried out under an atmosphere of dry argon. Commercially available absolute solvents were used. The NMR spectra were recorded on a Bruker Avance DPX 400 spectrometer. If not indicated otherwise, the spectra were recorded at ambient temperature. The MS spectra were obtained on a Finnigan MAT 90 mass spectrometer, the HR-MS spectra on Finnigan MAT 900 S or Bruker Daltronics 9.4T APEX-III FT-MS mass spectrometers.

General Procedure for the Synthesis of Compounds 1. A mixture of thiourea (1.0 mmol), halide (1.0−3.0 mmol), and acetonitrile (10 mL) was refluxed for 4−16 h. The precipitate was filtered off and crystallized from methanol/ether to give the dihydrochloride of **1**. The compounds were analyzed by ^{1}H NMR and high resolution MS as well as LC/MS applying two distinct methods. The compounds showed purities >95%.

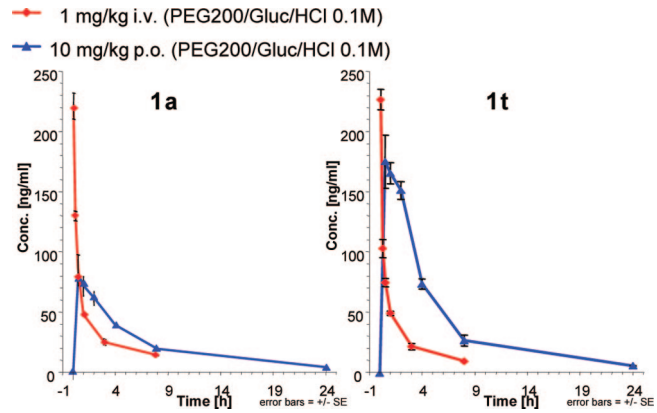
1a. ^{1}H NMR DMSO-d_6/D_2O (10:1) δ = 6.78 (s, 1 H), 4.53 (s, 2 H), 4.48 (m, 2 H), 4.35 (m, 2 H), 3.82 (m, 2 H), 1.87−1.05 (m, 20 H). HR-MS $[M + H]^+$ observed = 379.1992, estimated = 379.1990.

3q. A mixture of *N*,*N*′-dimethyl thiourea (1.04 g, 10.0 mmol), 1,3-dichloro acetone (1.27 g, 10.0 mmol), and *n*-butanol (25 mL) was heated at 140 °C for 1 h. The solvent was removed and the residue crystallized from methanol/ether to give the hydrochloride of **3q** (1.90 g, 90%). ^{1}H NMR DMSO-d_6/D_2O (10:1) δ = 7.36 (s, 1 H), 4.96 (s, 2 H), 3.66 (s, 3 H), 3.11 (s, 3 H). MS/ESI 177 $[M + H]^+$.

3t. A mixture of 4,4-dimethyl-imidazolidine-2-thione (1.0 g, 7.5 mmol), 1,3-dichloro acetone (1.00 g, 7.5 mmol), and acetonitrile (15 mL) was refluxed for 2 h. The colorless precipitate was filtered off, dried, suspended in 1-methoxy-2-(2-methoxy-ethoxy)-ethane, and subsequently heated at 140 °C for 2 h. The precipitate was filtered off and washed with ether to give the hydrochloride of **3t** (1.14 g, 64%) ^{1}H NMR DMSO-d_6/D_2O (10:1) δ = 7.08 (s, 1 H), 4.88 (s, 2 H), 4.2 (s, 2 H), 1.50 (s, 6 H). MS/ESI 203 $[M + H]^+$.

Biological Assays. CXCR4 Radioligand Binding Assay. The assay was based on the scintillation proximity assay (SPA) technology (Amersham Biosciences, Buckinghamshire, UK). Membranes from CEM cells were used as source for human CXCR4 and IR983F cells for rat CXCR4. The cells were homogenized in a buffer containing 20 mM HEPES, 1 mM EDTA pH = 7.4, and protease inhibitors followed by centrifugation at 28000*g*. After a second round of homogenization and centrifugation, the membranes were resuspended in 20 mM HEPES, 0.1 mM EDTA pH = 7.4, and stored in aliquots at −80 °C. Human CXCL12 was obtained from R&D Systems (Minneapolis MN) and ^{125}I labeled CXCL12 from Amersham. For the assay, the following ingredients were added into a 96-well Optiplate: 10 μg of membrane protein in 50 μL of assay buffer (25 mM HEPES, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, 0.5% BSA, and protease inhibitors) followed by 0.5 mg of wheat germ agglutinin coated SPA beads (Amersham) in 50 μL of assay buffer followed by 50 μL of compound or buffer or 400 nM unlabeled CXCL12. Finally 50 μL of ^{125}I labeled CXCL12 in assay buffer was added (final concentration 20−25 pM). The plates were incubated for 2 h at room temperature under continuous shaking. Following the incubation, the plates were centrifuged for 10 min and subsequently measured in a TopCount NXT instrument (Packard Instruments, Boston, USA). Data were analyzed using GraphPad Prism software.

Chart 3. PK profile of Compounds **1a** and **1t** in Lewis Rat Blood (n=3)



Ca^{2+} Mobilization Assay. CXCL12 induced Ca^{2+} mobilization from intracellular stores was measured in CEM cells loaded with the Ca^{2+}-sensing fluorochrome Fluo-4 (Molecular Probes, Invitrogen). Cells were incubated with 4 μM Fluo-4 for 1 h at 37 °C and subsequently washed in assay buffer. The loaded cells were dispensed into microtiter plates, mixed with antagonist, and incubated for 2 h. Subsequently, the cells were dispensed into wells of 384-well plates in quadruplicates and placed in a fluorescence image plate reader (FLIPR384, Molecular Devices). Fluorescence of the cells was recorded for 20 s, after which CXCL12 was added (final concentration 3 nM) and recording was continued for 215 s. The minimal (F_{min}, baseline) and maximal fluorescence (F_{max}) was used to calculate the inhibitory effect of CXCR4 antagonists. IC_{50} values were expressed as the inhibitor concentration that yielded 50% inhibition of the $F_{max}-F_{min}/F_{min}$ value measured in the absence of inhibitor.

Chemotaxis Assay. Cell migration (chemotaxis) stimulated by CXCL12 was assessed in Transwell tissue culture inserts with porous polycarbonate membranes (Costar, Baar, Switzerland, 5 μM pore size). Target cells (e.g., Jurkat T cells or CEM cells, 500000 cells in 100 μL buffer) were placed in the upper chamber and 600 μL of buffer or chemoattractant (CXCL12, final concentration 10 nM) were placed in the lower chamber. After incubation for 4 h at 37 °C, the migrated cells were counted in a FacsCalibur flow cytometer (BD Biosciences) by acquiring all events for 30 s. When inhibitor was tested, the compound was added to both the upper and the lower compartment to give the desired final concentration.

Actin Polymerization Assay in Whole Blood. Blood was collected in EDTA coated tubes. CXCR4 inhibitors were diluted in phosphate buffered saline (PBS) (GIBCO, Invitrogen) to the appropriate final concentration and typically 3 μL of CXCR4 antagonist solution was mixed with 100 μL of whole blood in a 96-well plate (Costar). After incubation for 10 min at room temperature, 3 μL of CXCL12 solution (to achieve a final concentration of 100 nM) was added to the sample and incubated for 25 s at ambient temperature. Ice-cold FACS lysing buffer (BD Biosciences) was added to the samples, followed by an incubation for 10 min on ice. After several washes with PBS/0.5% BSA (Sigma-Aldrich) on ice, the blood cells were resuspended in 200

Table 4. Inhibition of X4-tropic HIV-1_{IIIB} Attachment

	HIV[a] IC_{90} [nM]	HIV[a] IC_{50} [nM]	CXCR4 IC_{50} [nM]
1a	720	40	48
1b	>100000	>100000	3375
1e	820	50	25
1g	1140	40	28
1i	>100000	470	372
1t	100	7	8.0
1u	130	10	8.8
AMD 3100	50	3	245

[a] * The HIV attachment assay was performed by the Southern Research Institute (www.southernresearch.com).

μL of PBS containing 1% paraformaldehyde (Sigma-Aldrich) and incubated for 5 min on ice. After centrifugation, the cells were resuspended in 100 μL PBS solution containing 0.5% BSA, 1% Alexa 488 phalloidin (Molecular Probes, Invitrogen), and 1% L-α-palmitoyl-lysophosphatidyl-choline, (Sigma-Aldrich) and incubated for 20 min on ice protected from light. Cells were washed 3 times with PBS/0.5% BSA and their fluorescence analyzed using a FacsCalibur flow cytometer (BD Biosciences)

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Supporting Information Available: ^{1}H NMR and HPLC charts for compounds **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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