The role of N-glycosylation sites on the CXCR4 receptor for CXCL-12 binding and signaling and X4 HIV-1 viral infectivity

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Abstract

The chemokine receptor CXCR4 functions as one of the HIV-1 coreceptors and can be considered as an attractive target for the development of novel anti-HIV drugs. Here, we investigated the effect of its two known N-glycosylation sites g1 (NYT) and g2 (NVS) on the antiviral potential of several classes of entry inhibitors. The lack of g1 clearly affected the binding of the amino-terminal directed 2B11 mAb, but not the 12G5 mAb. No dramatic effects on CXCL-12 binding and CXCL-12-induced intracellular calcium responses were observed. Importantly, the anti-HIV-1 activity and antagonistic activity of the prototype compound of CXCR4 inhibitors, AMD3100, were not affected by the presence or absence of the CXCR4 N-glycans.

Since CXCR4 N-glycans play a less important role in viral entry compared to the N-glycans on the HIV envelope, cells expressing CXCR4 N-glycosylation mutants might be no relevant alternative to allow HIV-1 escape from antivirals.

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Introduction

Binding of the gp120 envelope protein of HIV-1 to the cellular CD4-receptor induces conformational changes and results in the exposure of the coreceptor-binding sites. Then gp120 further interacts with one of the two chemokine receptors, CCR5 or CXCR4, to achieve the final conformational changes that are required for efficient membrane fusion. HIV-1 gp41 unfolds, inserts in the cell membrane and fusion can occur (Alkhatib et al., 1996; Berson et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996).

The chemokine receptor CXCR4 belongs to the seven-transmembrane-domain G-protein-coupled receptor family (Deng et al., 1996; Feng et al., 1996). Its natural ligand is the chemokine stromal cell-derived factor (SDF-1/CXCL-12) (Bleul et al., 1996; Oberlin et al., 1996). CXCR4 is the major coreceptor for X4 HIV-1 viruses that appear at later stages of HIV-1 infection and their appearance is associated with accelerated CD4+ T-cell decline and clinical progression towards AIDS (Connor et al., 1997; Malkevich et al., 2001; Schramm et al., 2000). The V3 loop of gp120 interacts with the N-terminal region and extracellular domains of CXCR4 (Cocchi et al., 1996). Changes in these regions of the CXCR4 receptor and in the V3 loop of HIV-1 can influence virus entry. 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) (Zhong et al., 1995). Both gp120 and CXCR4 are subject to N-glycosylation. The complete gp120 is a highly glycosylated protein which contains between 20 and 29 potential N-linked glycosylation sites and the carbohydrate content constitutes about 50% of the mass of gp120 (Leonard et al., 1990). Lack of some N-glycan structures within the gp120 V3 loop of the HIV-1 NL4.3 strains resulted in an enhanced infectivity in CXCR4-expressing cells (Pollakis et al., 2001; Polzer et al., 2001). It is hypothesized that the
glycan structure on gp120 is shielding some positively charged amino acids. It was also proposed that the carbohydrates act as a shield to protect the virions from the humoral immune response (Wyatt et al., 1998). Thus, compounds interacting with the carbohydrate domains on the virion envelope might provide effective ways to inhibit viral infection. The broadly neutralizing mAb 2G12, the mannose-specific plant lectins derived from *Galanthus nivalis* (GNA) and *Hippeastrum* hybrid (HHA) and the N-acetylglucosamine-binding protein from *Urtica dioica* (UDA) bind to the HIV envelope protein in a carbohydrate-dependent manner and significantly inhibit HIV-1 replication (Balzarini et al., 2004, 2005; Balzarini, 2006; Calarese et al., 2003; Scanlan et al., 2002).

Studies have shown that the CXCR4 coreceptor is present in different antigenically distinct conformations *in vivo* and that the heterogeneity observed is cell-type-dependent (Baribaud et al., 2001; Baribaud and Doms, 2001). N-Glycosylation is also cell-type-dependent, thus it is conceivable that the various forms of CXCR4 can be the result of changes in N-glycosylation. CXCR4 contains only two potential N-glycosylation sites at amino acid positions 11–13 (g1) and 176–178 (g2) (Fig. 1). It has been shown previously that glycosylation influences the ability of CXCR4 to serve as a coreceptor for T-tropic (X4) and dual-tropic (R5X4) HIV-1 strains. Chabot et al. (2000) found that removal of a site of N-linked glycosylation allows CXCR4 to serve as a more universal coreceptor, allowing efficient entry of several HIV-1 isolates that normally use only CCR5. Thordsen et al. (2002) found a similar permissiveness for an X4 virus and no exchange in coreceptor specificity that allows infection of an R5 virus. However, removal of glycan g1 significantly enhanced the permissiveness for a dual-tropic R5/X4 virus. If g1 is masking some of the positively charged amino acids on CXCR4, the deletion of g1 would therefore reduce the overall negative charge of the CXCR4 coreceptor, increasing the infectivity of dual-tropic virus.

In this study, we further investigated the role of the N-linked glycosylation sites of CXCR4. Several experiments were performed to test the binding of CXCL-12 and anti-CXCR4 monoclonal antibodies (mAbs) to the mutant CXCR4 receptors and the inhibitory effect of the CXCR4 antagonist AMD3100 was also investigated (Donzella et al., 1998; Hatse et al., 2002; Schols et al., 1997b). The infectivity of the NL4.3 virus and several *in vitro* generated entry inhibitor resistant NL4.3 viruses was also investigated.

**Results and discussion**

**Establishment of the U87.CD4.CXCR4 glycosylation mutants**

Because of its complete lack of endogenous CXCR4 expression (and many other chemokine receptors), we used the U87.CD4 cell line as a starting point to create the CXCR4 glycosylation mutants. The pBABE-puro vectors with mutated CXCR4 genes (i.e. CXCR4 [WT], CXCR4 [N11Q], CXCR4 [N176Q], CXCR4 [N11Q, N176Q], CXCR4 [T13A] and CXCR4 [S178A] (Fig. 1)) were transfected into the U87.CD4 cells using FuGENE 6 Transfection Reagent and selected further with puromycin. We evaluated the CXCR4 expression with the anti-CXCR4 mAb clone 12G5 and the CXCR4 glycosylation mutants stained 82%–97% positive with this mAb as measured.

![Fig. 1. Amino acid sequence and membrane organization of the chemokine receptor CXCR4 with indication of the positions of the mutated amino acids that remove the N-glycosylation sites g1 (AA N11Q-T13A) and g2 (AA N176Q-S178A).](image-url)
by flow cytometry (Fig. 2). The wild-type CXCR4 U87.CD4 mutant, used as a control for the transfection procedure and for further experiments, was 89% CXCR4-positive.

**Effect of CXCR4 glycans on antibody recognition**

HIV-1 entry can be influenced by receptor density and potentially by receptor conformation. It has been shown that CXCR4 can exist in antigenically distinct conformations on certain cell types and it is conceivable that the various forms could be the result of changes in N-glycosylation. Therefore, we evaluated the antibody binding capacities in the different CXCR4 mutants using 2 CXCR4-specific mAbs i.e. the clone 12G5 and 2B11. The mAb 12G5 mainly targets the second extracellular loop while the 2B11 mAb binds the amino-terminal loop of the CXCR4 receptor.

Table 1 shows the mean fluorescence intensities (MFI) for 12G5 mAb and 2B11 mAb binding. The MFI values for 12G5 mAb binding were comparable for all the generated CXCR4 mutants, so the lack of N-glycosylation sites did not noticeably

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**Table 1**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>12G5 MFI (Mean)</th>
<th>2B11 MFI (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 WT</td>
<td>89.12%</td>
<td></td>
</tr>
<tr>
<td>CXCR4 [N11Q]</td>
<td>86.59%</td>
<td></td>
</tr>
<tr>
<td>CXCR4 [N176Q]</td>
<td>91.87%</td>
<td></td>
</tr>
<tr>
<td>CXCR4 [N11Q, N176Q]</td>
<td>88.82%</td>
<td></td>
</tr>
<tr>
<td>CXCR4 [T13A]</td>
<td>81.68%</td>
<td></td>
</tr>
<tr>
<td>CXCR4 [S178A]</td>
<td>96.67%</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2**. Flow cytometric analysis of the CXCR4 membrane expression of the different U87.CD4 transfected cell lines. The right panel represents CXCR4-specific staining by 12G5 mAb. The left panel represents the background fluorescence of the non-specific binding of an isotype mAb. The percentage of CXCR4⁺ cells is presented in the upper-right corner of each dot plot.
influence the binding of the 12G5 mAb. The results for the 2B11 binding were not comparable for all the CXCR4 mutants. The MFI values for both the CXCR4 [N11Q] mutant and the [T13A] mutant were lowest compared to the WT CXCR4 receptor mutant cell line. So, the lack of the g1 N-glycosylation site clearly disturbed the binding of the 2B11 mAb to the amino-terminal loop of CXCR4. Chabot et al. (2000) also demonstrated that the binding of 12G5 mAb (2B11 mAb was not used in their study) was comparable for the different N-glycosylation CXCR4 mutants.

To find out whether the N-glycosylation sites have an influence on the activity of the CXCR4 inhibitor AMD3100, we measured the IC50 values of AMD3100 on the 12G5 mAb binding. The IC50 values of AMD3100 are shown in Table 2 and are comparable for all the different CXCR4 mutants. Therefore, the binding of AMD3100 to CXCR4 is not influenced by the N-glycosylation sites. AMD3100 does not interfere with the binding of the 2B11 mAb, as described previously on untransfected CXCR4+ cells (Schols and De Clercq, 2001).

**Effect of CXCR4 glycans on chemokine-induced intracellular calcium mobilization assays**

After binding to the receptor, chemokines trigger an intracellular signal transduction cascade comprising a transient cytosolic calcium mobilization. To examine the binding of CXCL-12 to the mutated CXCR4 receptors we performed intracellular calcium mobilization assays on these mutants and compared their responses to those of the wild-type U87.CD4.CXCR4 cells. Again we evaluated the inhibitory effect of the CXCR4 antagonist AMD3100. After loading the cells with the fluorescent calcium indicator Fluo-3, the cells were incubated with CXCL-12 and in order to investigate the effect of AMD3100 the cells were pre-incubated for 10 min with the antagonist before chemokine stimulation.

Fig. 3 shows the intracellular calcium flux in the different CXCR4 mutants. In all the mutants, after stimulation with CXCL-12 at 100 ng/ml, the calcium fluxes were comparable to the wild-type cells (a fluorescence change from 2000 to 3000 counts) except for the CXCR4 [N11Q] mutant who had always a higher calcium flux signal (about 5000 counts). The antagonist AMD3100 completely blocked the CXCL-12-induced intracellular calcium flux at 1 μg/ml in all mutant cell lines (Fig. 3). Calcium signaling was inhibited in a dose-dependent manner by AMD3100, with 50% inhibitory concentrations of 61 ng/ml, 27 ng/ml, 101 ng/ml, 63 ng/ml, 143 ng/ml and 29 ng/ml for CXCR4 [WT], CXCR4 [N11Q], CXCR4 [N176Q], CXCR4 [N11Q, N176Q], CXCR4 [T13A] and CXCR4 [S178A] respectively (Table 2). These values are quite comparable with the values achieved with the CXCL-12AF647 binding assay.

**Effect of CXCR4 glycans on X4 HIV-1 infection**

To investigate whether the U87.CD4.CXCR4 mutant cells supported HIV-1 replication, cells were infected with various inputs of the laboratory HIV-1 X4 NL4.3 strain. At several days after infection (day 3, 5 and 7), the cytopathic effect (CPE) was consistent and reproducible manner. Also, when the infectivity of three resistant HIV-1 NL4.3 viruses, NL4.3 resistant to the wild-type CXCR4 mutants were then incubated with different concentrations of the AlexaFluor647-labeled CXCL-12 AF647 (100 ng/ml, 20 ng/ml and 4 ng/ml) for 30 min. The fluorescence increased gradually with increasing concentrations of CXCL-12AF647 (data not shown). We compared the mean fluorescence intensity (fluorescence increase for the different concentrations of compound AMD3100 and its IC50 values). The IC50 values were comparable for all the mutant CXCR4 receptors (Table 2), but in general the concentration of AMD3100 needed to block CXCL-12-binding was higher than the concentration needed to block 12G5 mAb binding.

### Table 1

Mean fluorescence intensities (MFI) of the binding of the chemokine CXCL-12AF647 and two different anti-CXCR4 mAbs 12G5 and 2B11

<table>
<thead>
<tr>
<th>MFI a</th>
<th>CXCL-12AF647 binding</th>
<th>12G5 mAb binding</th>
<th>2B11 mAb binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 WT</td>
<td>23.8±5.8</td>
<td>63.6±10.9</td>
<td>16.7±9.3</td>
</tr>
<tr>
<td>CXCR4 [N11Q]</td>
<td>21.3±6.7</td>
<td>64.0±17.7</td>
<td>5.9±1.9</td>
</tr>
<tr>
<td>CXCR4 [N176Q]</td>
<td>28.0±2.8</td>
<td>55.4±9.6</td>
<td>7.8±4.7</td>
</tr>
<tr>
<td>CXCR4 [N11Q, N176Q]</td>
<td>13.7±3.5</td>
<td>81.2±11.5</td>
<td>18.3±13.9</td>
</tr>
<tr>
<td>CXCR4 [T13A]</td>
<td>14.2±0.5</td>
<td>20.2±6.4</td>
<td>1.6±1.5</td>
</tr>
<tr>
<td>CXCR4 [S178A]</td>
<td>37.3±2.6</td>
<td>54.7±19.5</td>
<td>11.0±3.6</td>
</tr>
</tbody>
</table>

# References

Chabot et al. (2000). Calcium signaling was inhibited in a dose-dependent manner by AMD3100, with 50% inhibitory concentrations of 61 ng/ml, 27 ng/ml, 101 ng/ml, 63 ng/ml, 143 ng/ml and 29 ng/ml for CXCR4 [WT], CXCR4 [N11Q], CXCR4 [N176Q], CXCR4 [N11Q, N176Q], CXCR4 [T13A] and CXCR4 [S178A] respectively (Table 2). These values are quite comparable with the values achieved with the CXCL-12AF647 binding assay.

Table 2

Inhibitory effects of the CXCR4 inhibitor AMD3100

<table>
<thead>
<tr>
<th>IC50 a (ng/ml)</th>
<th>CXCL-12AF647 binding</th>
<th>12G5 mAb binding</th>
<th>Ca2+ signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 WT</td>
<td>63.4</td>
<td>2.5</td>
<td>61.4±8.6</td>
</tr>
<tr>
<td>CXCR4 [N11Q]</td>
<td>21.9</td>
<td>2.9</td>
<td>26.8±1.8</td>
</tr>
<tr>
<td>CXCR4 [N176Q]</td>
<td>30.8</td>
<td>6.3</td>
<td>100.7±68.6</td>
</tr>
<tr>
<td>CXCR4 [N11Q, N176Q]</td>
<td>21.8</td>
<td>2.7</td>
<td>63.5±14.4</td>
</tr>
<tr>
<td>CXCR4 [T13A]</td>
<td>40.2</td>
<td>3.8</td>
<td>142.9±49.6</td>
</tr>
<tr>
<td>CXCR4 [S178A]</td>
<td>23.2</td>
<td>3.4</td>
<td>29.1±6.0</td>
</tr>
</tbody>
</table>

a 50% inhibitory concentration of AMD3100 to inhibit for 50% CXCL-12AF647 binding, 12G5 mAb binding and CXCL-12-induced calcium signaling in the different U87.CD4.CXCR4 mutants. The values are the mean of two experiments (for CXCL-12AF647 binding and 12G5 mAb binding) or the mean ±S.D. of three separate experiments (for Ca2+ signaling).
chemokine CXCL-12, NL4.3 resistant to the CXCR4 antagonist AMD3100 and NL4.3 resistant to the neutralizing 2G12 mAb was evaluated, no differences were detected in viral replication (as evaluated by CPE and p-24 viral Ag production) of these viruses in the different U87.CD4.CXCR4 mutant cells. Thus, the infectivity of NL4.3 and several entry inhibitor resistant viruses (to CXCL-12, AMD3100 and the 2G12 mAb) was not affected by the presence or absence of the glycosylation sites in CXCR4. When we evaluated the R5 laboratory strains JR-FL, ADA, Ba-L and SF-162 no CPE or any p-24 viral Ag production could be detected. Chabot et al. (2000) described that R5 viruses were able to infect some of the N-linked CXCR4 mutants (expressed on U373 cells) whereas Thordsen et al. (2002) reported that R5 viruses were not able to infect these mutants (expressed on GHOST cells).

The antiviral activity of AMD3100, the plant lectins GNA, HHA and UDA and the 2G12 mAb was determined for HIV-1 NL4.3 in the different U87.CD4.CXCR4 mutant cell lines (Table 3). AMD3100 showed a potent and consistent antiviral activity against HIV-1 NL4.3 with an IC50 slightly varying from 3 to 11 ng/ml. The plant lectins were less active than AMD3100, but no significant differences in anti-HIV activity were observed. The 2G12 mAb was not active at a concentration of 2 μg/ml in these mutant cells. Thus, no difference was observed in the antiviral activity of AMD3100 and the plant lectins in the different CXCR4 mutants and we can conclude that the N-glycosylation sites have no effect at all on the antiviral activity of these classes of entry inhibitors.

**Conclusion**

CXCR4 is one of the two major coreceptors for HIV. Therefore it is important to study the characteristics of the CXCR4 receptor in the multifaceted mechanism of HIV...
influence on its coreceptor function for HIV-1 NL4.3 wild-type and several entry inhibitor-resistant viruses generated in vitro (such as CXCL-12, AMD3100 and the 2G12 mAb). Furthermore, our data point to the potent and consistent antiviral and antagonistic activity of AMD3100 being independent for the presence of g1 or g2 on CXCR4 and also of the broad antiviral activity of the lectins GNA, HHA and UDA. Because the different forms of the CXCR4 receptor present in vivo are possibly the result of changes in N-glycosylation it is important to show that these different forms have no influence on the infectivity of X4 viruses and various entry inhibitor-resistant X4 viruses. Even more important is that the antivirals preserve their antiviral activity against X4 viruses, so the N-glycosylation mutations in CXCR4 are no alternative to allow an escape route for the virus.

Materials and methods

Cell cultures and viruses

Human astroglialoma U87 cells expressing human CD4 (U87. CD4) were a kind gift of Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York, NY, USA). These cells were transfected with the CXCR4 mutants (as described below) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS) and 2% FBS and for each sample 0.3 × 10⁶ cells were resuspended in 100 l PBS/FBS 2%. Thereafter, antibodies were added and samples were incubated for 45 min at room temperature. After incubation the cells were washed and resuspended in PBS containing 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

HIV-1 NL4.3 resistant strains to the bicyclam AMD3100 and to the chemokine CXCL-12 have previously been established and were characterized in our laboratory (de Vreese et al., 1996; Schols et al., 1997a, 1998). HIV-1 NL4.3 resistant to the mAb 2G12 has also been established in our laboratory (Huskins et al., in press).

Compounds and chemokines

The bicyclam AMD3100 was synthesized as described previously (Bridger et al., 1995). The mannose-specific plant lectins from G. nivalis (GNA) and Hippeastrum hybrid (HHA) and the N-acetylgalcosamine-specific lectin from U. dioica (UDA) were derived and purified from the bulbs/roots of these plants, as described before (Balzarini, 2006; Kaku et al., 1990; Van Damme et al., 1987, 1988, 1991). The human chemokine CXCL-12 (SDF-1) was purchased from PeproTech (London, United Kingdom) and CXCL-12Af647 (a human CXCL-12 carrying an Alexa-Fluor647 moiety at its second to last amino acid position) was obtained from CCS Albachem (United Kingdom) (Hatse et al., 2004).

Construction of CXCR4 glycosylation mutants

The pBabe-puro vectors (containing a puromycin resistance gene) with mutated CXCR4 genes in their unique EcoRI restriction sites were described in detail (Polzer et al., 2002; Thorsden et al., 2002). These pBabe-puro vectors were stably transfected into the U87.CD4 cells using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. Puromycin selection (1 μg/ml) was started after 24 h and puromycin-resistant cell cultures were established after approximately 3 weeks.

Flow cytometric analyses

The antibodies used in this study were: PE-conjugated mouse anti-human CXCR4 mAb clone 12G5 (R&D Systems Europe, Oxen, United Kingdom) and PE-conjugated rat anti-CXCR4 mAb clone 2B11 (BD Pharmingen, San Diego, CA, USA). As a control for a specific background staining, the cells were stained with Simultest Control, γ₁/γ₂a FITC/PE (Becton Dickinson). After trypsin digestion the CXCR4-transfected U87.CD4 cells were incubated for at least 1 h at room temperature to allow re-expression of receptor proteins on the cell surface. Then, the cells were washed in PBS with 2% FBS and for each sample 0.3 × 10⁶ cells were resuspended in 100 μl PBS/FBS 2%. Thereafter, antibodies were added and samples were incubated for 45 min at room temperature. After incubation the cells were washed and resuspended in 300 μl PBS containing 1% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

To evaluate the effect of AMD3100 on antibody binding, the cells were pre-incubated in PBS/FBS 2% for 15 min at room temperature with the chemokine receptor antagonist AMD3100

Table 3

Anti-HIV-1 activity of AMD3100, the plant lectins HHA, GNA and UDA and the anti-carbohydrate mAb 2G12

<table>
<thead>
<tr>
<th></th>
<th>AMD3100</th>
<th>HHA</th>
<th>GNA</th>
<th>UDA</th>
<th>2G12</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 WT</td>
<td>0.005±0.010</td>
<td>0.56±0.5</td>
<td>1.7±0.9</td>
<td>1.9±0.5</td>
<td>&gt;2</td>
</tr>
<tr>
<td>CXCR4 [N11Q]</td>
<td>0.007±0.005</td>
<td>0.36±0.9</td>
<td>1.1±0.5</td>
<td>1.5±0.7</td>
<td>&gt;2</td>
</tr>
<tr>
<td>CXCR4 [N176Q]</td>
<td>0.003±0.005</td>
<td>0.29±0.5</td>
<td>0.33±0.2</td>
<td>1.1±0.6</td>
<td>&gt;2</td>
</tr>
<tr>
<td>CXCR4 [N11Q, N176Q]</td>
<td>0.009±0.007</td>
<td>0.35±0.2</td>
<td>0.95±0.5</td>
<td>1.5±0.5</td>
<td>&gt;2</td>
</tr>
<tr>
<td>CXCR4 [T13A]</td>
<td>0.011±0.008</td>
<td>0.96±0.5</td>
<td>0.75±0.9</td>
<td>1.4±0.5</td>
<td>&gt;2</td>
</tr>
<tr>
<td>CXCR4 [S178A]</td>
<td>0.004±0.010</td>
<td>0.25±0.9</td>
<td>1.7±0.5</td>
<td>1.4±0.4</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

* 50% inhibitory concentration to inhibit HIV-1 NL4.3-induced CPE by 50% in the different U87.CD4.CXCR4 mutants. The values are the mean±S.D. from 3 to 5 separate experiments.
diluted in PBS and washed in PBS/FBS 2% before incubation with the antibodies. For the calculation of the mAb binding, the mean fluorescence intensity (MFI) of each sample was expressed as percentage of the MFI of control cells and the IC50 values of the compound were then calculated.

The CXCL-12AF647 binding assay has been described by Hatse et al. (2004). Briefly, CXCR4-transfected U87.CD4 cells were washed once in assay buffer (Hanks’ balanced salt solution with 20 mM HEPES buffer and 0.2% bovine serum albumin, pH 7.4) and incubated with CXCL-12AF647 diluted in assay buffer 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). To evaluate the inhibitory effect of AMD3100 on the binding of CXCL-12AF647 the cells were incubated with AMD3100 diluted in assay buffer for 15 min at room temperature, washed and then incubated with CXCL-12AF647 (100 ng/ml) for 30 min at room temperature.

Calcium signaling assays

The U87.CD4.CXCR4 mutant cells were digested by trypsin and seeded in gelatin-coated (0.2%) black-wall 96-well microplates (Costar, Cambridge, MA) at 2 × 104 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 for 45 min at 37 °C. Then cells were washed with assay buffer (see above) and incubated with 150 μM AMD3100 at different concentrations, diluted in assay buffer, for 10 min at 37 °C. The intracellular calcium signaling in response to 100 ng/ml CXCL12 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 (Princen et al., 2003).

HIV infection assays

The U87.CD4.CXCR4 transfectants were seeded in 24-well plates (2 × 103 cells per well) and different concentrations of HIV-1 virus (5000–1.6 pg/ml p24 antigen (Ag)) were added. The cytopathic effect of virus replication in the cell cultures (syncytium formation) was evaluated microscopically every day up to 7 days after infection. To examine the antiviral activity of AMD3100, plant lectins and 2G12 mAb the cells were seeded out in 24-well plates already containing these agents at various concentrations and then virus was added. After 7 days the cytopathic effect (CPE) was evaluated microscopically.

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