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The molecular pharmacology of AMD11070: An orally bioavailable CXCR4 HIV entry inhibitor

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ABSTRACT

In order to enter and infect human cells HIV must bind to CD4 in addition to either the CXCR4 or the CCR5 chemokine receptor. AMD11070 was the first orally available small molecule antagonist of CXCR4 to enter the clinic. Herein we report the molecular pharmacology of AMD11070 which is a potent inhibitor of X4 HIV-1 replication and the gp120/CXCR4 interaction. Using the CCRF-CEM T cell line that endogenously expresses CXCR4 we have demonstrated that AMD11070 is an antagonist of SDF-1α ligand binding (IC_{50} = 12.5 ± 1.3 nM), inhibits SDF-1 mediated calcium flux (IC_{50} = 9.0 ± 2.0 nM) and SDF-1α mediated activation of the CXCR4 receptor as measured by a Eu-GTP binding assay (IC_{50} = 39.8 ± 2.5 nM) or a [^{35}S]-GTPγS binding assay (IC_{50} = 19.0 ± 4.1 nM), and inhibits SDF-1α stimulated chemotaxis (IC_{50} = 19.0 ± 4.0 nM). AMD11070 does not inhibit calcium flux of cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5 or CCR7, or ligand binding to CXCR7 and BLT_1, demonstrating selectivity for CXCR4. In addition AMD11070 is able to inhibit the SDF-1β isoform interactions with CXCR4; and N-terminal truncated variants of CXCR4 with equal potency to wild type receptor. Further mechanistic studies indicate that AMD11070 is an allosteric inhibitor of CXCR4.

1. Introduction

The introduction of highly active antiretroviral therapy (HAART) has dramatically changed the outcome for HIV-1 infected persons with improvements in morbidity and mortality. However, there are challenges associated with chronic use of reverse transcriptase inhibitors and protease inhibitors, the components of HAART, including emergence of resistance and chronic toxicities which limit patient compliance such as diarrhea, anemia and lipodystrophy [1]. There is therefore a need for new agents with different molecular targets. The inhibition of viral entry has emerged as a validated target for HIV infection and the first approved entry inhibitor was enfuvirtide which acts by blocking gp41-mediated fusion [2]. The discovery that HIV requires both host CD4 and a chemokine receptor as a co-receptor for cell entry stimulated research into chemokine receptor inhibitors as a new class of HIV drugs [3,4]. Chemokines are 8–10 kDa proteins defined by the number and relative spacing of cysteine residues at the N-terminal end of the protein. The two major families are CC and CXC in which there are two cysteine residues that are either adjacent (CC) or separated by one amino acid residue (CXC). They are mediators of hematopoiesis and inflammation, regulating lymphocyte development, homing and trafficking. Their receptors belong to the class A family of seven transmembrane (7 TM) G-protein coupled receptors (GPCR) [5,6].

HIV uses two chemokine receptors, CCR5 and CXCR4, proven targets for HIV drugs [3,7]. CCR5 is the principle co-receptor for viral transmission in the early, clinically latent stage of the disease, whereas CXCR4 usage emerges in the later stages and is associated with a decrease in CD4 cell count and accelerated disease progression [4]. Virus that uses CCR5 as a co-receptor is characterized as M (macrophage)-tropic, CCR5-using or R5, and virus that uses CXCR4 as T (T lymphocyte)-tropic, CXCR4-using or X4. The CCR5 inhibitor Selzentry® (maraviroc) was approved by the FDA for use in treatment experienced patients in August 2007 [8–10], and in treatment naïve patients in November 2009 [11,12]. The bicyclam Mozobil® (plerixafor) is a selective antagonist of CXCR4 [13,14] and is an inhibitor of T-tropic, X4 virus [15–17], and was demonstrated to reduce the X4 viral load in HIV-1 infected subjects [18]. Inhibition of CXCR4 by plerixafor also leads to leukocytosis and mobilization of hematopoietic stem cells (HSC) from the bone marrow in both healthy volunteers and in patients

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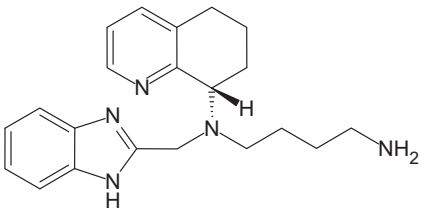

Fig. 1. Structure of AMD11070.

with non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) [19,20] and improves mobilization when combined with the cytokine G-CSF [21–23]. Plerixafor was evaluated in two successful randomized Phase III clinical trials and was subsequently approved by the U.S.F.D.A. in 2008 in combination with G-CSF to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with NHL and MM. The EMA approved Mozobil in 2009 [24–26].

Plerixafor is administered subcutaneously for HSC mobilization whereas an orally bioavailable drug is preferred for HIV treatment. This prompted the development of an orally available CXCR4 antagonist resulting in the discovery of the non-cyclam molecule AMD11070 (Fig. 1) [27]. AMD11070 is a potent inhibitor of CXCR4, and inhibits T-tropic viral infectivity. In clinical trials in healthy volunteers AMD11070 was found to be well tolerated and orally bioavailable [28]. In a proof of concept clinical trial in HIV infected patients harboring X4 virus 4/9 patients had a greater than $1\log_{10}$ reduction in X4 viral level [29].

In this paper we present in detail the molecular pharmacology and mechanism of CXCR4 inhibition by AMD11070. We demonstrate that AMD11070 is a potent inhibitor of X4 HIV replication. Confirmation that AMD11070 inhibits viral infection via CXCR4 blockade is provided by the inhibition of cell/cell fusion in an assay that mimics the binding of gp120 to CXCR4. Using a T cell line that naturally expresses CXCR4 we show that AMD11070 inhibits the binding of CXCL12 (SDF-1), the cognate ligand for CXCR4, and inhibits subsequent receptor activation and downstream signaling. In addition AMD11070 was shown to have no activity against other chemokine receptors demonstrating selectivity for CXCR4. Mechanistic studies further probing the molecular interactions of AMD11070 with CXCR4 demonstrate the allosteric nature of inhibition.

2. Materials and methods

2.1. Cell lines

All parent cell lines were obtained from the ATCC (Manassas, VA). CCRF-CEM cells, which naturally express CXCR4, CCR4 and CCR7, were cultured in RPMI 1640 containing 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum. HEK293F cells were transfected to express CCR1, CCR2b, CXCR3, CCR4, or CCR5. Cells expressing CXCR3 and CCR4 were also co-transfected with a chimeric Gαqi5 protein to improve signaling. CHO-S cells were transfected to express the LTB_4 receptor BLT_1. Canine thymus Cf2Th cells transfected with CXCR7 were maintained in DMEM containing 1 mM sodium pyruvate, 4 mM L-glutamine and 20% FBS. All other cells were cultured in DMEM containing 1 mM sodium pyruvate, 4 mM L-glutamine, 0.1 mM non-essential amino acids and 10% fetal bovine serum and 800 μg/mL geneticin. Hygromycin B was added to cultures expressing the chimeric G-protein.

Canine thymus Cf2Th cells were transiently transfected with the CXCR4 C-terminus truncated variants R334X and E343X. The CXCR4 variants, R334X and E343X, were constructed by site directed mutagenesis of the cloned wild type CXCR4 using the Strategene Quikchange mutagenesis kit (Agilent Technologies, Cedar Creek, TX, USA). The primers used for the mutagenesis were 5′-caagatcctctccaaaggaaag**t**gaggtggacattcatctg-3′ (for R334X) and 5′-gtggacattcatctgtttccact**t**agtctgagtcttcaag-3′ for (E343X), with the respective reverse complement primers. After the mutagenesis, DNA sequencing was performed on the mutants to confirm that the target sequence was obtained. An endotoxin-free preparation of the mutated DNA was prepared by the Qiagen endofree plasmid kit (Qiagen, Mississauga, Ontario). The canine thymus cell line, Cf2Th, was transiently transfected with either R334X or E343X using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For control experiments Cf2Th cells were transiently transfected with wild type CXCR4.

2.2. Compounds and chemokines

AMD11070 was synthesized as previously described [27]. All chemokines were provided by the late I. Clark-Lewis (University of British Columbia, Vancouver, BC).

All chemicals and buffer salts were obtained from Calbiochem (now EMD Biosciences, Rockland, MA) unless otherwise stated. Cell culture media was obtained from Hyclone Inc., Logan, UT. Other chemicals, including Eu-GTP and [^{35}S]-GTPγS, were purchased from Perkin-Elmer Life Sciences, USA.

2.3. Anti-HIV activity

Inhibition of HIV-1 replication in MT-4 cells ($CD4^+CXCR4^+$ lymphocytic cell line) was performed as previously described. Anti-HIV-1 activity was determined by assaying the viability of HIV-1 NL4.3 X4 virus infected MT-4 cells with increasing concentrations of AMD11070. The IC_{50} was defined as the concentration of AMD11070 required to inhibit viral cytopathicity by 50% [30]. PBMCs were isolated from healthy donors by density gradient centrifugation and stimulated with PHA at 1 μg/mL (Sigma Chemical Co., Bornem, Belgium) for 3 days at 37 °C. The activated cells (PHA-stimulated blasts) were washed three times with PBS, and infected with HIV-1 NL4.3 X4 virus. HIV-infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/mL of IL-2 and varying concentrations of AMD11070. Supernatant was collected at day 10, and HIV-1 core antigen in the culture supernatant was analyzed by the p24 viral Ag ELISA kit (Perkin-Elmer, Zaventem, Belgium) [15,17].

2.4. CXCR4-mediated cell fusion

P4-R5 MAGI cells express endogenous CD4 and CXCR4 on the cell surface and have also been stably transformed with β-galactosidase that is under the control of HIV-LTR, which is transactivated by HIV or SIV Tat protein. CHO-K1 cells (>95% confluent) were transiently transfected with an endotoxin free preparation of the plasmid pDOLHIVenv using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) accordingly to supplier's protocol. Plasmid pDOLHIVenv contains a 3108 bp *Sal*I–*Xho*I fragment encompassing the open reading frames of the *env*, *tat*, *rev* coding regions from pNL4-3. Twenty-four hours after the transfection, cells were lifted and resuspended at 4×10^5 cells/mL and mixed with P4-R5 MAGI cells (1:1 ratio) in white 96-well plates in the presence of AMD11070. Cells were incubated for 16–20 h at 37 °C, 5% CO_2. Interaction of CXCR4 expressed on P4-R5 MAGI cells and gp120 on the transiently-transfected CHO-K1 cells induced fusion of the two cell types and resulted in the transactivation of the β-galactosidase gene. Fusion level was monitored using the Galscreen substrate (Applied Biosystems, Carlsbad, CA, USA), with luminescence measured by Victor 2 spectrophotometer (Perkin-Elmer, USA) [31].

2.5. Receptor binding assays

The SDF-1 ligand binding assay against CXCR4 was performed using the CCRF-CEM cells, and for CXCR7 using Cf2Th cells transfected to express CXCR7 as previously described [13,32]. Briefly 500,000 cells were incubated for 3 h at 4 °C in binding buffer (PBS containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25% BSA, pH 7.4) with 100 pM [^{125}I]-SDF-1α (Perkin-Elmer, 2200 Ci/mmol) on Millipore DuraporeTM (Millipore, Billerica, MA, USA) filter plates. AMD11070 was included in the incubation mix at appropriate concentrations as required. Unbound [^{125}I]-SDF-1α was removed by washing with cold 50 mM HEPES, 0.5 M NaCl pH 7.4. The competition binding assay against BLT_1 was performed on membranes from CHO-S cells expressing recombinant BLT_1. The membranes were prepared by mechanical cell lysis followed by high speed centrifugation, re-suspended in 50 mM HEPES, 5 mM $MgCl_2$ buffer and flash frozen. The membrane preparation was incubated with AMD11070 for 1 h at room temperature in an assay mixture containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 4 nM LTB_4 mixed with 1 nM [^{3}H]-LTB_4 (195.0 Ci/mmol, Perkin-Elmer Life Sciences) and 8 μg membrane. The unbound [^{3}H]-LTB_4 was separated by filtration on Millipore Type GF-C filter plates. The bound radioactivity was counted using a LKB Rackbeta 1209 Liquid Scintillation Counter.

2.6. Calcium flux assays

Chemokine-stimulated calcium flux was assayed on the following cells: CCRF-CEM for CXCR4 and CCR7, HEK293F cells expressing CCR1, CCR2b, CXCR3, CCR4 or CCR5, and Cf2Th cells expressing the CXCR4 C-terminal truncated variants R334X and E343X. Cells were loaded with the calcium-indicator Fluo-4-AM (Molecular Probes Inc.). The loaded cells were then washed and resuspended in HBSS containing 20 mM HEPES, 0.2% BSA, 2.5 mM probenecid, pH 7.4. Before the assay, cells were pre-incubated for 15 min at 37 °C with AMD11070 at appropriate concentrations. Changes in intracellular calcium concentration upon addition of chemokine were monitored by fluorescence, E_{ex} = 485 nM, E_{em} = 525 nM, using a FLEXstation fluorescent plate reader (Molecular Devices, CA, USA). Chemokine ligand concentrations used were 2.5 or 10 nM SDF-1α (CXCL12) for inhibition studies, and a concentration range of 0.2 nM–1 μM for mechanistic studies, and 10 nM SDF-1β for CXCR4, 10 nM MIP-1α (CCL3) for CCR1, 13.3 nM MCP-1 (CCL2) for CCR2b, 7 nM TARC (CCL17) for CCR4, 30 nM RANTES (CCL5) for CCR5, 200 nM MIP-3β (CCL19) for CCR7, and 40 nM IP10 (CXCL10) for CXCR3 [13,32]. Results were normalized with respect to a control without AMD11070.

2.7. GTP binding assays

For the GTPγS binding studies, a concentration range of AMD11070 was incubated for 1 h at 30 °C in an assay mixture containing 5 mM GDP, 30 mM NaCl, 10 nM SDF-1α, 1 nM [^{35}S]-GTPγS and 8 mg CCRF-CEM membrane. The unbound [^{35}S]-GTPγS was separated by filtration on Millipore Type GF-C filter plates and the data analyzed as described above [13].

The CXCR4 Eu-GTP assay was performed as previously described [33]. A concentration range of AMD11070 was incubated for 1 h at 30 °C on Acrowell plates (Pall-Gelman, East Hills, NY, USA) in the presence of 10 μg/well CCRF-CEM membrane, 5 nM SDF-1α in 50 mM HEPES, pH 7.4, 5 μM GDP, 5 mM $MgCl_2$, 10 mM NaCl, 0.1 mg/mL saponin, and 5 nM Eu-GTP (Perkin-Elmer), a nonhydrolyzable, europium-labeled analogue of GTP. Unbound Eu-GTP was separated by filtration and the bound was counted by time-resolved fluorescence, E_{ex} = 340 nM, E_{em} = 615 nM, using a Victor 2 fluorescent plate reader (Perkin-Elmer).

2.8. Chemotaxis assay

For the chemotaxis studies, cells were loaded with 5 mM calcein-AM (Molecular Probes, InVitrogen, Carlsbad, CA, USA). Cells were then washed and resuspended in media (RPMI containing 10 mg/mL BSA). Cells were preincubated for 10 min at 37 °C with a concentration range of AMD11070. In a Corning Transwell plate (5 μm pore 24-well plate), AMD11070 and 10 nM SDF-1α were added to the lower well and the cell-AMD11070 mixture was added to the upper well. Plates were incubated for 3–5 h at 37 °C, 5% CO_2. Migration of the cells towards the SDF-1α containing lower chamber was quantified by fluorescence reading E_{ex} = 485 nM, E_{em} = 525 nM using a FLEXstation (Molecular Devices) [13,32].

2.9. Data analysis

Ligand binding and concentration/response curves were analyzed by nonlinear regression using PRISM 3.0 (GraphPAD Software, San Diego, CA) or XLFit add-in for Microsoft Excel. Results are expressed as mean ± S.E.

3. Results

3.1. AMD11070 is a potent inhibitor of X4 HIV-1 replication

AMD11070 is an inhibitor of X4-tropic HIV, with antiviral activity demonstrated against HIV-1 NL4.3 in both MT-4 cells and PBMC with IC_{50} values of 9.6 ± 0.3 nM and 10.1 ± 0.3 nM respectively (Table 1). The CC_{50} for AMD11070 was around 30 μM in MT-4 cells, giving a selectivity index (SI) of more than 3000. In addition AMD11070 potently inhibited cell fusion between a CHO-K1 cell line expressing gp120 and the P4-R5 MAGI cells which express endogenous CD4 and CXCR4 with an IC_{50} of 1.5 ± 0.3 nM (Fig. 2a). No antiviral activity was observed with AMD11070 against HIV-1 R5 (BaL) in PBMC (IC_{50} > 10 μM).

3.2. AMD11070 inhibits SDF-1α binding to cells expressing CXCR4

The CCRF-CEM T-lymphoblastoid cell line has been reported to naturally express CXCR4 [34]. CXCR4 expression was confirmed by flow cytometry as described previously [13]. This cell line was therefore chosen and used throughout to demonstrate the interactions of AMD11070 with the CXCR4 receptor. AMD11070 was shown to inhibit [^{125}I]-SDF-1α ligand binding to CCRF-CEM cells in a heterologous competition binding assay. A typical result

Table 1
Inhibition of CXCR4-mediated biological responses by AMD11070.

NL4.3:MT4[a]	CC_{50}[b]	SI[c]	NL4.3:PBMC[a,d]	gp120/CXCR4 cell fusion[a]	Ligand binding[a]	Eu-GTP[a]	[^{35}S]-GTP[a]	Calcium flux[a]	Chemotaxis[a]
9.6 ± 0.3	27.5 ± 2.8	3056	10.1 ± 0.3	1.5 ± 0.3	12.5 ± 1.3	39.8 ± 2.5	19.0 ± 4.1	9.0 ± 2.0	19.0 ± 4.0

[a] Results are IC_{50} concentration (nM) giving 50% inhibition expressed as mean ± S.E.
[b] Concentration (μM) causing 50% cytotoxicity of MT-4 cells expressed as mean ± S.E.
[c] Selectivity index: ratio of CC_{50} for MT-4 cells/IC_{50} NL4.3 HIV activity in MT-4 cells.
[d] NL4.3 HIV activity in PBMCs.

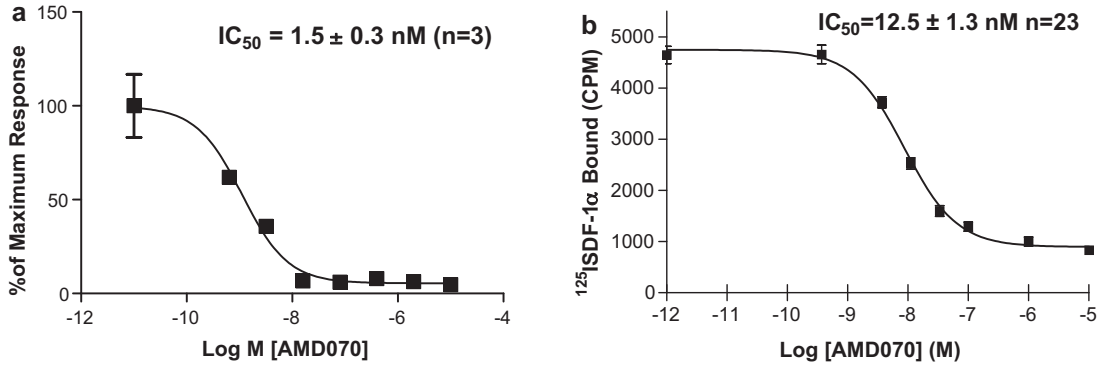

Fig. 2. Inhibition of CXCR4/ligand interactions where the ligands are HIV gp120 and the cognate ligand SDF-1. (a) Inhibition of cell fusion between a CHO-K1 cell line expressing gp120 and the P4-R5 MAGI cells which express endogenous CD4 and CXCR4. (b) AMD11070 inhibition of [^{125}I]-SDF-1α binding to CXCR4^{+} CEM-CCRF cells.

is shown in Fig. 2b. The data was fitted to a single site binding model and gave an IC_{50} of 12.5 ± 1.3 nM (Table 1).

3.3. AMD11070 inhibits SDF-1-mediated cell signaling

Chemokine receptors are G-protein coupled receptors [5,6], i.e. the mechanism of receptor activation is dependent upon coupling to an intracellular heterotrimeric G-protein composed of the Gα, Gβ and Gγ subunits, which in its basal state binds the guanine nucleotide GDP. Upon activation by ligand binding GDP is released and replaced by GTP. This leads to subunit dissociation into a βγ dimer and the α monomer to which the GTP is bound. The GTP is rapidly hydrolysed to GDP resulting in re-association of the receptor and the trimeric G protein complex. This process is assayed using a nonhydrolysable analogue of GTP such as the fluorescently labeled Eu-GTP or radio labeled [^{35}S]-GTPγS, thus trapping the formation of the GTP/G-protein. Typical results are shown in Fig. 3a and b. AMD11070 inhibited CXCR4 activation with IC_{50} values of 39.8 ± 2.5 and 19.0 ± 4.1 nM in the Eu-GTP-binding and [^{35}S]-GTPγS assays respectively.

Upon the activation of the G-protein coupled receptor, intracellular signaling pathways are triggered resulting in the release of calcium from intracellular stores. This calcium flux can be assayed using a calcium-chelating molecule, Fluo-4, which fluoresces upon binding calcium. AMD11070 was able to inhibit SDF-1α (2.5 nM SDF-1α) mediated calcium flux in CCRF-CEM cells with an IC_{50} of 9.0 ± 2.0 nM. A typical result is shown in Fig. 3c.

A key property of all chemokines is that they induce a chemotactic response to a chemokine concentration gradient. AMD11070 was able to inhibit SDF-1α mediated chemotaxis of CCRF-CEM cells with an IC_{50} of 19.0 ± 4.0 nM as shown in Fig. 3d. All results are summarized in Table 1.

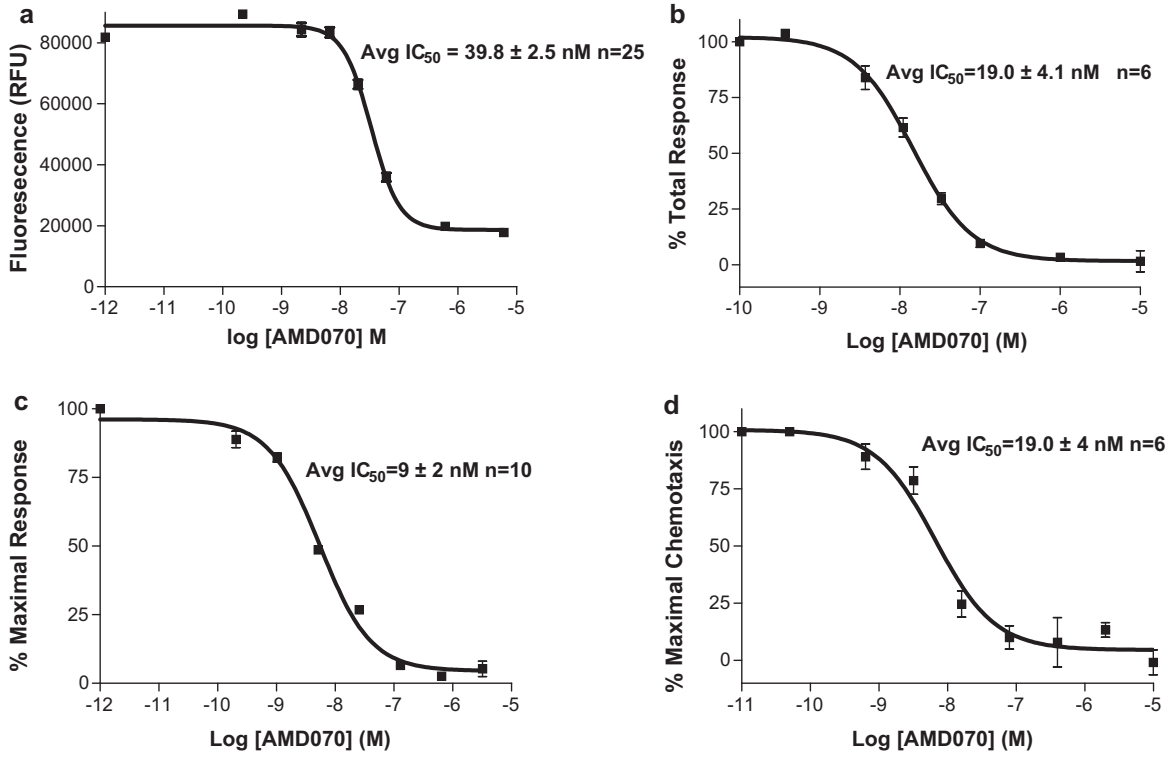

Fig. 3. Inhibition of SDF-1α mediated signaling by AMD11070: (a) inhibition of SDF-1α stimulated Eu-GTP binding; (b) inhibition of SDF-1stimulated [^{35}S]-GTPγS binding; (c) inhibition of SDF-1α-induced calcium flux and (d) inhibition of SDF-1α stimulated CCRF-CEM chemotaxis.

Table 2
AMD11070 is a selective inhibitor of CXCR4. Data is shown for calcium flux response for CCR1, CCR2b, CXCR3, CCR4, CCR5 and CCR7; and ligand binding for CXCR7 and LTB_4.

Receptor	Cell line	Ligand	IC_{50} AMD11070 (μM)
CCR1	HEK293F-CCR1	MIP-1α/CCL3	>50
CCR2b	HEK293F-CCR2b	MCP-1/CCL2	>50
CXCR3	HEK293F-CXCR3-$G\alpha_{qi5}$	IP-10/CXCL10	>50
CXCR7	Cf2Th.CXCR7	SDF-1α/CXCL12	>10
CCR4	HEK293F-CCR4-$G\alpha_{qi5}$	TARC/CCL17	>50
CCR5	HEK293F-CCR5	RANTES/CCL5	>50
CCR7	CCRF-CEM	MIP-3β/CCL19	>50
BLT_1	CHO-S-LTB_4	LTB_4	>50

3.4. AMD11070 activity is selective for CXCR4

In order to demonstrate the specificity of AMD11070 for CXCR4 it was tested in calcium signaling assays against a panel of chemokine receptors, and in ligand binding assays for BLT_1, the receptor for leukotriene B4 (LTB_4), and CXCR7. LTB_4 is a potent chemoattractant and its receptor is a GPCR. The results in Table 2 show that the IC_{50} of AMD11070 against CCR1, CCR2b, CCR4, CCR5, CCR7, CXCR3, and LTB_4 was >50 μM in all cases. AMD11070 did not inhibit SDF-1α binding to CXCR7 at a concentration of 10 μM, the maximum concentration tested in this assay. Together these data show that AMD11070 is a selective inhibitor of CXCR4.

3.5. AMD11070 inhibits SDF-1 signaling of C-terminal variants of CXCR4

From an HIV therapeutic perspective it is important that CXCR4 antagonists can act on CXCR4 variants. C-terminal truncated variants of CXCR4 have been reported associated with WHIM syndrome; nonsense mutations resulting in a 19 amino acid truncation (R334X), and a 10 amino acid truncation (E343X) and a frameshift mutation resulting in a 13 amino acid truncation (S339fs342X) [35,36]. In order to address this we cloned the R334X and E343X CXCR4 variants and transiently expressed them in the canine thymus cell line Cf2Th. This cell line was chosen due to its lack of expression of CXCR4 [31]. Wild type CXCR4 was similarly sub-cloned into this cell line for control studies. In control studies it was demonstrated that both these C-terminal truncated variants were able to respond to SDF-1α in the calcium flux assay with a similar potency to wild type CXCR4. The EC_{50} values for SDF-1α were 13.6, 11.3 and 15.3 nM against the wild type, R334X and E343X variants of CXCR4 respectively (Fig. 4a). The inhibitory effect of AMD11070 on SDF-1α-mediated calcium flux was assessed for the two CXCR4 variants. Both variants were inhibited to a similar extent as the wild type CXCR4 with IC_{50} values of 3.1, 8.5 and 4.6 nM for the wild type, R334X and E343X variants respectively (Fig. 4b).

3.6. AMD11070 is an allosteric inhibitor of CXCR4

In order to further analyze the mechanism of AMD11070 inhibition of the SDF-1/CXCR4 interaction we investigated the effect of increasing concentrations of inhibitor on the SDF-1α dose/response in the calcium flux assay. The data in Fig. 5a shows a concentration dependent depression of the SDF-1α dose/response curves as high concentrations of the ligand are unable to overcome the inhibitory response of high concentrations of AMD11070. A more detailed analysis of these data provides further insight into the mechanism of inhibition. Whereas a linear correlation of a logarithmic plot of SDF-1α EC_{50} versus AMD11070 concentration would be expected for a competitive inhibitor the plot clearly shows a non-linear relationship which is indicative of a non-competitive, or mixed inhibitor (Fig. 5b). Assuming EC_{50} is directly related to ligand K_D it is apparent that ligand binding affinity is modified by increasing inhibitor concentration. Furthermore in Fig. 5c the maximal SDF-1α-induced calcium response (E_{max}) is decreased by increasing concentrations of inhibitor. The effect on both EC_{50} and E_{max} indicates that AMD11070 inhibits the SDF-1/CXCR4 response with a mixed-type inhibition mechanism, indicative of allosteric inhibition.

Several splice variants of SDF-1 have been reported [37–40]. The two most studied are SDF-1α and SDF-1β, with SDF-1α being the predominant isoform. The gene for SDF-1 encodes an 89 amino acid protein with a 21 amino acid signal peptide cleavage site giving rise to a 68 amino acid protein which is SDF-1α. The splice variant SDF-1β is a 72 amino acid protein with an additional 4 residues at the C-terminus [38]. As shown in Table 3 both isoforms were able to stimulate a calcium flux in CCRF-CEM cells with EC_{50} values of 10 ± 0.5 nM for SDF-1α and of 2.9 ± 0.7 nM for SDF-1β. A concentration of 10 nM SDF-1α or β was used for subsequent inhibition studies with AMD11070. The data in Table 3 show that AMD11070 was able to inhibit CXCR4-mediated calcium flux initiated by both isoforms of SDF-1 with IC_{50} values of 18.2 ± 2.0 nM for SDF-1α, and 45.8 ± 2.5 nM for SDF-1β. If we hypothesize that AMD11070 acts via a mixed-inhibition mechanism as indicated above then the IC_{50} is a good approximation of the K_i [41]. As the K_i values are different for the two ligand isoforms this further supports the hypothesis that AMD11070 is acting as an allosteric inhibitor of the SDF-1/CXCR4 interaction.

4. Discussion

AMD11070 is an orally bioavailable CXCR4 antagonist with potent activity against T-tropic X4 HIV [27,29]. AMD11070 was

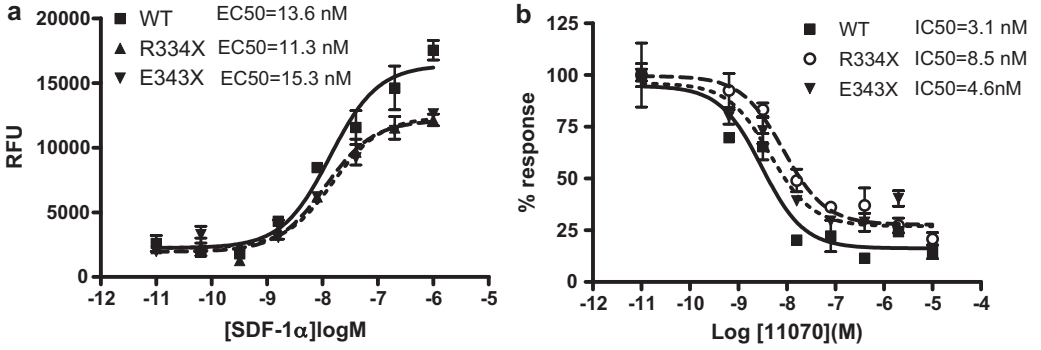

Fig. 4. AMD11070 inhibition of CXCR4 N-terminus truncated variants. (a) SDF1-α stimulation of calcium flux in wild type and R334X and E343X CXCR4 variants, and (b) inhibition of SDF-1α stimulated calcium flux in wild type and R334X and E343X CXCR4 variants.

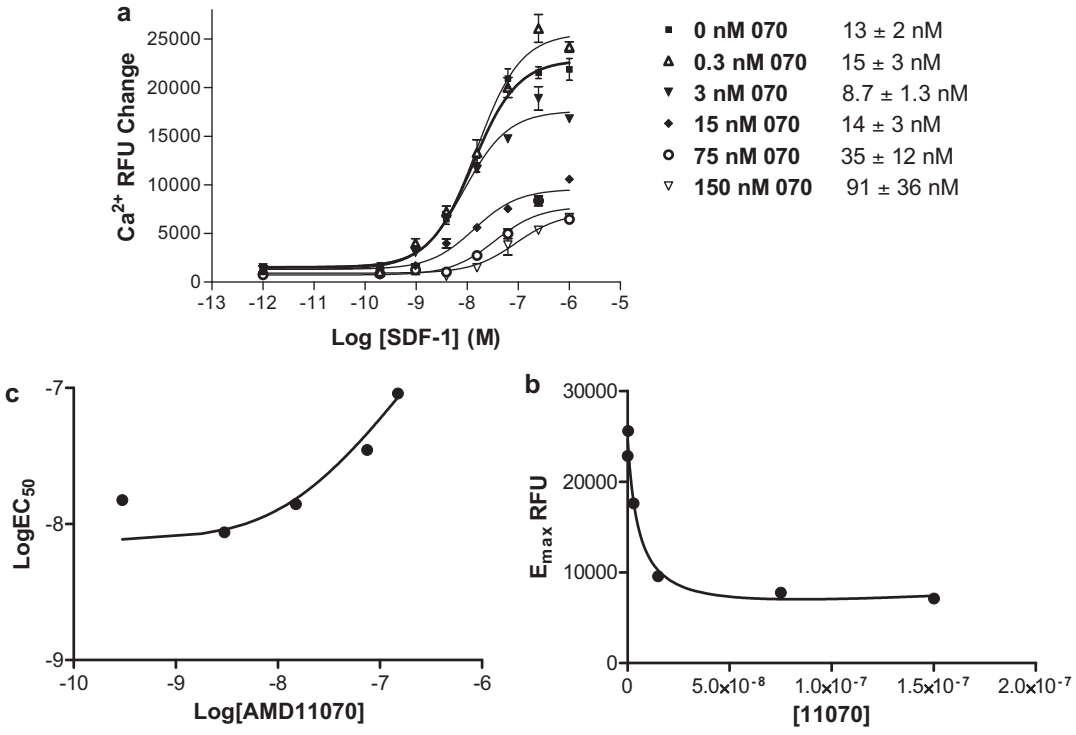

Fig. 5. Allosteric inhibition of SDF-1α-induced calcium flux in CCRF-CEM cells. (a) The effect of increasing concentrations of AMD11070 on the SDF-1α dose/response curve, (b) the effect of increasing concentrations of AMD11070 on the SDF-1α EC_{50}, and (c) the effect of increasing concentrations of AMD11070 on the maximum SDF-1α response (E_{max}).

able to inhibit replication of the HIV-1 T-tropic NL4.3 strain in the low nanomolar range *in vitro*. Confirmation that this was due to the inhibition of CXCR4 was provided by the demonstration of inhibition of the viral envelope/CXCR4 interaction in a cell/cell fusion assay using a cell line expressing viral envelope from the X4 virus pNL4.3. In this paper we further investigate the molecular pharmacology of CXCR4 inhibition by AMD11070.

Using the CCRF-CEM cell line, which naturally expresses CXCR4 [13,32] we have shown that AMD11070 inhibits SDF-1α ligand binding to CXCR4 with an IC_{50} of 12.5 ± 1.3 nM. AMD11070 also inhibits CXCR4 activation and signaling as shown by inhibition of SDF-1α mediated G-protein activation of the CXCR4 receptor in two assays using either the fluorescent Eu-GTP or the radiolabeled [^{35}S]-GTPγS binding assays with IC_{50} values of IC_{50} of 39.8 ± 2.5 nM and 19.0 ± 4.1 nM respectively, and inhibition of SDF-1α mediated calcium flux with an IC_{50} of 9.0 ± 2.0 nM. AMD11070 also inhibited SDF-1α-mediated chemotaxis, a CXCR4-mediated physiological response, with an IC_{50} of 19.0 ± 4.0 nM. In addition, AMD11070 had little or no inhibitory effect on either MIP1α, MCP-1, TARC, RANTES, MIP-3β, or IP10 mediated calcium flux, ligands for CCR1, CCR2b, CCR4, CCR5, CCR7 and CXCR3 respectively, or SDF-1α binding to CXCR7, or LTB_4 binding to BLT_1, an alternative GPCR that mediates chemotaxis. These data indicate that AMD11070 is a selective inhibitor of CXCR4 over these chemokine receptors.

Given the relative size of the cognate ligand for CXCR4 with a molecular weight of approximately 10 kDa, and that of AMD11070 with a molecular weight of 350 Da, it is unlikely that the small molecule inhibitor would act as a direct competitive antagonist of CXCR4. In fact it has been reported that other small molecule antagonists of chemokine receptors such as the CXCR1/2 inhibitors repertaxin and Sch527123 [42,43], and the CCR5 inhibitors TAK-779, SCH-C and 873140 [7,44–46], are allosteric inhibitors of their respective chemokine receptor. We therefore decided to ascertain if AMD11070 was an allosteric inhibitor using classical pharmacology. We compared the dose/response of SDF-1α in the calcium flux assay in the presence of increasing concentrations of inhibitor. The dose/response curves were depressed with increasing AMD11070 concentration, and the inhibitory effect of AMD11070 could not be overcome by high ligand concentrations. A similar observation was reported for the allosteric inhibitor of CXCR2, Sch527123 [43]. These data show that AMD11070 is not a competitive inhibitor of the SDF-1α/CXCR4 interaction, but is most likely an allosteric inhibitor. Interestingly the SDF-1α EC_{50} was not significantly affected at low inhibitor concentrations suggesting that the affinity of the agonist for the receptor was not changed by the inhibitor at these concentrations. At higher AMD11070 concentrations there was an apparent increase in the EC_{50} suggesting a change in agonist affinity. Additionally the maximum response, E_{max}, was depressed by increasing concentrations of AMD11070. The effects on both EC_{50} and E_{max} are indicative of a mixed-type mechanism of inhibition and are typical of an allosteric modulator [47]. Further support for allosteric inhibition comes from a comparison of the effect of different SDF-1 isoforms on CXCR4-mediated calcium flux, and inhibition by AMD11070. SDF-1α and SDF-1β stimulate calcium flux with different EC_{50} values, 10 ± 0.5 nM and of 2.9 ± 0.7 nM respectively, and AMD11070 inhibits the respective response with IC_{50} values of 18.2 ± 2.0 nM and 45.8 ± 2.5 nM. These differential effects suggest that different isoforms of the ligand may influence binding of the inhibitor to an allosteric site.

In previously reported studies we have mapped the binding site of AMD11070 on the CXCR4 receptor to the extracellular

Table 3
A comparison of the inhibitory effects of AMD11070 on CXCR4-mediated calcium flux stimulated by different isoforms of SDF-1.

Chemokine isoform	SDF-1α	SDF-1β
EC_{50} SDF-1 (nM)	10 ± 0.5	2.9 ± 0.7
IC_{50} AMD11070 (nM)	18.2 ± 2.0	45.8 ± 2.5

transmembrane region of the receptor [31]. CXCR4 mutagenesis and molecular modeling studies have shown that binding of the bicyclam CXCR4 antagonist plerixafor to the CXCR4 receptor is dependent upon three positively charged amino acids in transmembrane regions TMIV and TMVI, Asp^{171}, Asp^{262} and Glu^{288} [48,49]. Similar studies have shown that these interactions are important for AMD11070 binding. However, significant additional interactions with Tyr^{45} (TMI), Trp^{94} and Asp^{97} (TMII) were also identified for AMD11070. As one single binding mode could not explain all these interactions we proposed three alternative binding modes for AMD11070 [31]. Though the precise binding mode of SDF-1 has not been elucidated it is thought that binding is a two-stage process with initial binding to the N-terminus of CXCR4 with subsequent presentation of the ligand to the extracellular loops and the transmembrane region [34]. Key amino acids involved in receptor signaling have been identified in ECL2 and 3, and Asp^{97} and Glu^{288} have been identified as important interaction sites in the transmembrane region [50–52]. AMD11070 therefore can potentially interact with residues involved in SDF-1 interactions, but can also occupy unique binding sites which supports an allosteric, mixed-inhibition model. Interestingly Asp^{97} is required for HIV gp120 interaction with CXCR4 [50,53], thus indicating that binding to this residue can contribute to the anti-HIV activity of AMD11070.

We have extended this mutational analysis. It has been reported that mutations of the intracellular regions of CXCR4, including the intracellular loops and the C terminal cytoplasmic tail, have no effect on HIV infectivity [4,50]. Mutations in CXCR4 resulting in truncation of the intracellular C-terminus of the receptor have been linked to the rare autoimmune disease, WHIM syndrome [35,36]. We cloned and expressed two of these CXCR4 variants and demonstrated that AMD11070 was able to inhibit CXCL12-mediated calcium flux in these C-terminal truncated variants. These data further indicate that AMD11070 acts via interaction with the extracellular region of CXCR4. Furthermore it is significant from the perspective of AMD11070 as a potential therapeutic option for HIV-1 that it can inhibit multiple variants of CXCR4.

In conclusion we have shown that AMD11070 is a selective inhibitor of the chemokine receptor CXCR4. It can inhibit binding of different isoforms of SDF-1 to CXCR4, and inhibit SDF-1 stimulation of different variants of CXCR4. Furthermore, we present evidence that AMD11070 is an allosteric inhibitor of the SDF-1/CXCR4 interaction, though further studies are required to fully define the precise mechanism of inhibition.

A proof-of-concept clinical trial has shown that AMD11070 can reduce the viral load of X4 HIV [29]. Together these data add further support to the potential beneficial role of an orally bioavailable CXCR4 inhibitor as a therapeutic option for HIV/AIDS.

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