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Characterization of the molecular pharmacology of AMD3100: A specific antagonist of the G-protein coupled chemokine receptor, CXCR4

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ABSTRACT

The chemokine receptor CXCR4 is widely expressed on different cell types, is involved in leukocyte chemotaxis, and is a co-receptor for HIV. AMD3100 has been shown to be a CXCR4 receptor antagonist, and to block HIV infection of T-tropic, X4-using, virus *in vitro* and *in vivo*. AMD3100 is an effective mobilizer of hematopoietic stem cells and is being investigated in clinical trials in multiple myeloma and non-Hodgkins lymphoma patients. Using the CCRF–CEM T-cell line that constitutively expresses CXCR4 we confirmed that AMD3100 was an antagonist of SDF-1/CXCL12 ligand binding (IC_{50} = 651 ± 37 nM). We have also shown that AMD3100 inhibits SDF-1 mediated GTP-binding (IC_{50} = 27 ± 2.2 nM), SDF-1 mediated calcium flux (IC_{50} = 572 ± 190 nM), and SDF-1 stimulated chemotaxis (IC_{50} = 51 ± 17 nM). AMD3100 did not inhibit calcium flux against cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5 or CCR7 when stimulated with their cognate ligands, nor did it inhibit receptor binding of LTB_4. AMD3100 did not, on its own, induce a calcium flux in the CCRF–CEM cells, which express multiple GPCRs including CXCR4, CCR4 and CCR7. Furthermore, AMD3100 neither stimulated GTP-binding, an assay for GPCR activation, in CEM cell membranes; nor chemotaxis of CCRF–CEM cells. These data therefore demonstrate that AMD3100 is a specific antagonist of CXCR4, is not cross-reactive with other chemokine receptors, and is not an agonist of CXCR4.

1. Introduction

Stromal cell-derived factor-1 (SDF-1, SDF-1α, CXCL12) is a member of the chemokine superfamily of chemotactic cytokines and is the sole ligand for its cognate receptor CXCR4 [1,2]. SDF-1/CXCL12 is constitutively expressed in a broad range of tissues and has been classified as a homeostatic chemokine. The receptor CXCR4, is widely expressed on both hematopoietic and non-hematopoietic cells [3]. The SDF-1/CXCR4 axis is involved in T-lymphocyte trafficking and homing to sites within the body including bone marrow; hematopoiesis, immunomodulation and development [4–7]. Both CXCR4 and SDF-1 knockout mice display defects in B cell hematopoiesis, vascularisation, and cerebellar development. The engraftment and repopulation of stem cells in the bone marrow of NOD/SCID mice is dependent upon CXCR4 expression and retention of stem cells, and hence mobilization, is in part regulated by SDF-1/CXCR4 [8,9]. In addition,

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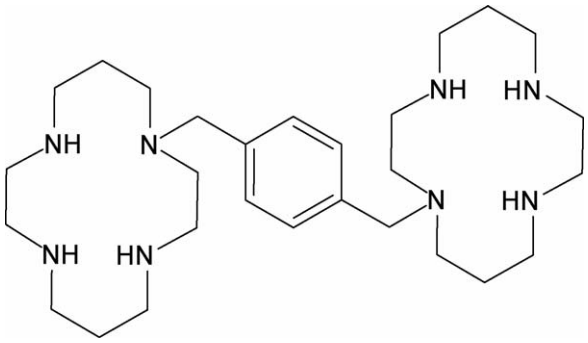

Fig. 1 – Chemical structure of the bicyclam AMD3100, 1,1′-[1,4-phenylene-bis(methylene)]-bis-1,4,8,11-azatetradecane.

CXCR4 is the principal co-receptor for T-cell line adapted HIV-1 isolates, which makes it an attractive disease model for further study[10–12].

AMD3100 (Fig. 1), a bicyclam molecule, has been identified as a specific inhibitor of CXCR4, and an inhibitor of T-tropic HIV infectivity [13–15], and has been used to validate CXCR4 as a target for HIV therapy in clinical trials [16]. AMD3100 has also been demonstrated to be an effective mobilizer of hematopoietic stem cells in both healthy volunteers and multiple myeloma and non-Hodgkins lymphoma patients [17–19]. Furthermore, AMD3100 has been shown to have beneficial effects in animal models of inflammatory disease, rheumatoid arthritis [20] and asthma [21]. These findings demonstrate that inhibiting SDF-1/CXCR4 interactions may have a variety of therapeutic benefits.

Chemokine receptors are seven-transmembrane, G-protein coupled receptors (GPCR) [22,23] and therefore SDF-1/CXCR4 interactions can therefore be studied using GPCR assays such as ligand binding, G-protein activation and downstream signaling processes such as intracellular calcium flux. Utilizing a cell line that naturally expresses CXCR4 the inhibitory effects of AMD3100 on SDF-1/CXCR4 mediated processes was further characterized with ^{125}I-SDF-1 ligand binding, calcium flux, GTP-binding and chemotaxis. Calcium flux or binding assays were also performed on cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5, CCR7 and BLT1 to demonstrate the specificity of AMD3100 for CXCR4. Furthermore, the mechanism of inhibition was investigated and it was shown that AMD3100 is a tight binding, slowly reversible antagonist of CXCR4. Further testing of AMD3100 in calcium flux, GTP and chemotaxis assays revealed that AMD3100 displayed no agonist activity.

2. Materials and methods

2.1. Cell lines

CCRF–CEM cells, which naturally express CXCR4, CCR4 and CCR7, were obtained from the ATCC (Manassas, Virginia). HEK293F cells were transfected to express CCR1, CCR2b, CXCR3, CCR4, or CCR5. Cells expressing CXCR3 and CCR4 were also co-transfected with a chimeric $G\alpha_{qi5}$ protein to improve signaling [24]. CHO-S cells were transfected to express BLT1. CCRF–CEM cells were cultured in RPMI 1640 containing 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum. 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.

2.2. Compounds and chemokines

The bicyclam AMD3100 was synthesized as described previously [25]. All chemokines were provided by the late Dr. I. Clark-Lewis (University of British Columbia, Vancouver, BC).

2.3. Receptor binding assays

For the competition binding studies against CXCR4, a concentration range of AMD3100 was 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 5×10^5 CCRF–CEM cells and 100 pM ^{125}I-SDF-1α (Perkin-Elmer, 2200 Ci/mmol) in Milipore Durapore™ filter plates. 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 BLT1 was performed on membranes from CHO-S cells expressing recombinant BLT1. 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 AMD3100 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.

2.4. Calcium flux assays

For the calcium flux studies, CCRF–CEM cells or HEK293F cells expressing CCR1, CCR2b, CXCR3, CCR4 or CCR5 were loaded with the calcium-indicator Fluo-4-AM (Molecular Probes Inc.). The loaded cells were then incubated 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 a concentration range AMD3100. 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). Chemokine concentrations used were 2.5 nM SDF-1 (CXCL12) 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. Results were normalized with respect to a control without AMD3100. Agonist assays were set up in the same way except that AMD3100 was added to the assay plate instead of chemokine.

2.5. GTP-binding assays

GTP-binding studies were performed with membranes prepared from CCRF–CEM cells. CCRF–CEM membranes (10 mg) were incubated on Pall Gelman AcroWell filter plates for 1 h at 30 °C with a concentration range AMD3100 in an assay mixture containing 5 mM GDP, 10 mM NaCl, 5 mM $MgCl_2$, 5 nM SDF-1α, 0.1 mg/mL saponin and 5 nM Eu-GTP (Perkin-Elmer), a non-hydrolyzable, europium-labelled 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).

2.6. Chemotaxis assay

For the chemotaxis studies, CCRF–CEM cells were loaded with 5 mM calcein-AM (Molecular Probes). The dye-loaded cells were washed and re-suspended in RPMI 1640 containing 10 mg/mL BSA. Cells were then pre-incubated for 10 min at 37 °C with indicated AMD3100 concentrations. Chemotaxis assays were performed in Corning Transwell plates (5 μM per 24-well plate). AMD3100 in RPMI + BSA containing 10 nM SDF was first placed below the porous membrane followed by addition of the cell-AMD3100 mixture above the membrane. Plates were incubated for 2.5 h at 37 °C, 5% CO_2. Migration of calcein-loaded cells to the lower chamber wells was evaluated by fluorescence, E_{ex} 485 nm, E_{em} 525 nm using a Victor 2 fluorescent plate reader (Perkin-Elmer). Agonist assays were set up in the same way except that AMD3100 was only added to the lower wells in the absence of SDF.

2.7. Kinetics of association and dissociation of AMD3100

Association and dissociation of AMD3100 was measured indirectly by flow cytomety using a FITC-labelled anti-CXCR4 antibody, 12G5. CCRF–CEM cells were incubated with 10, 1 or 0.1 μM AMD3100 for 0, 15, 30, 45, or 60 min at 37 °C, 5% CO_2 and then labelled with an anti-CXCR4 FITC antibody and binding assessed by flow cytometry. For assessment of dissociation CCRF–CEM cells were incubated with 10, 1 or 0.1 μM AMD3100 for 1 h at 37 °C, 5% CO_2, washed by centrifugation and re-suspended in RPMI with 10% FBS. Cells were then either directly stained with FITC-labelled 12G5, or incubated for a further 24 or 48 h and then stained with 12G5-FITC antibody.

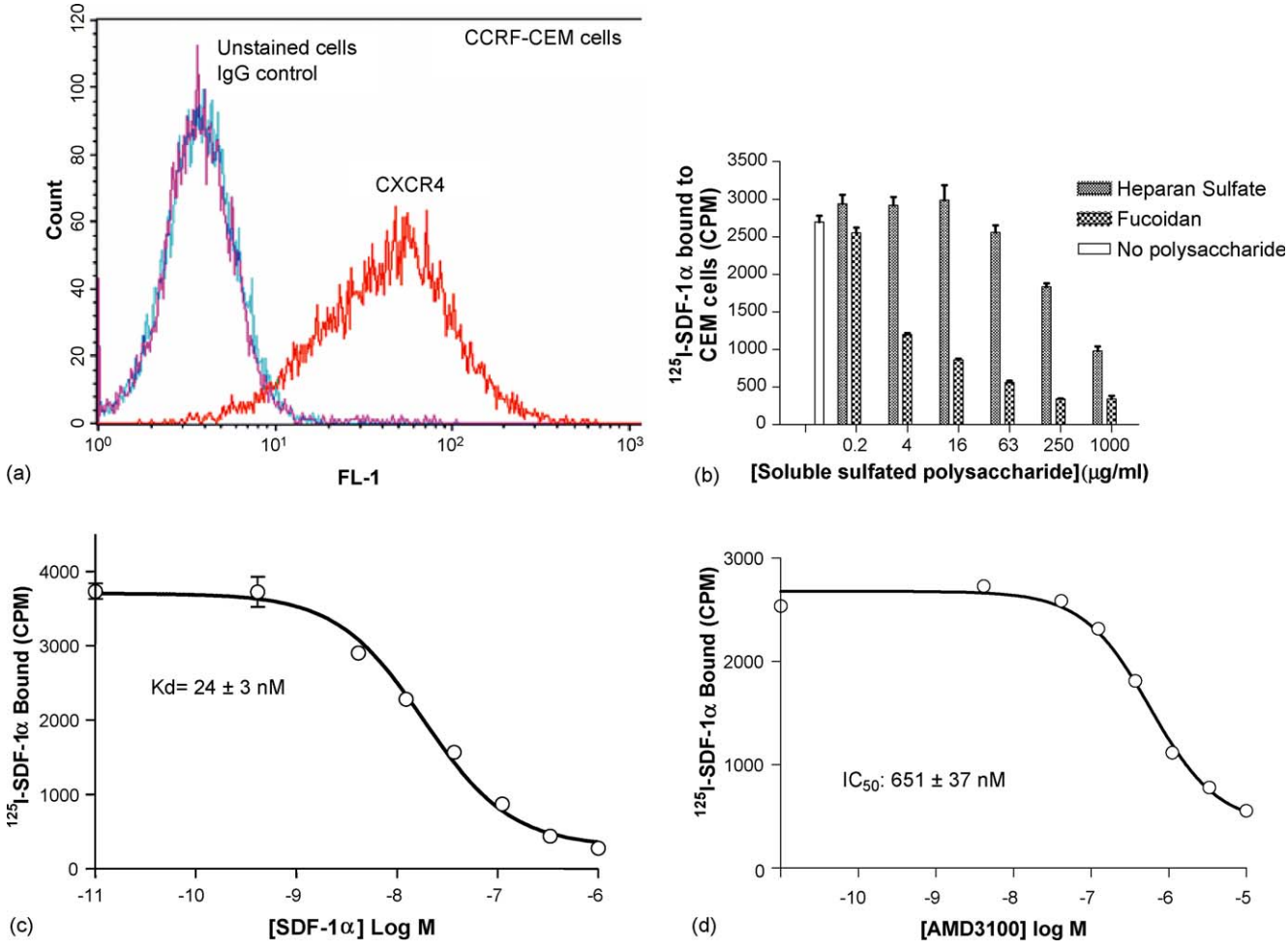

Fig. 2 – AMD3100 inhibition of SDF-1 binding to CEM–CCRF cells expressing CXCR4: (a) cell surface expression of CXCR4 by CCRF–CEM cells as determined by FACS using the CXCR4 specific mAb 12G5; (b) inhibition of SDF-1 binding to CXCR4 on CCRF–CEM cells by soluble sulfated polysaccharides; (c) homologous competition binding of SDF-1 against ^{125}I-SDF-1; (d) inhibition of ^{125}I-SDF-1 binding to CCRF–CEM cells by AMD3100.

The percent antibody bound was calculated from the ratio of 12G5 binding observed in the presence of AMD3100 to 12G5 binding observed in the absence of AMD3100.

2.8. Data analysis

Ligand binding and concentration/response curves were analyzed by nonlinear regression using PRISM® 3.0 (GraphPAD Software, San Diego, CA). Results are expressed as mean ± S.E.

3. Results

3.1. AMD3100 inhibits SDF-1 binding to cells expressing CXCR4

The CCRF–CEM T-lymphoblastoid cell line has been reported to naturally express CXCR4 [26]. CXCR4 expression was confirmed by flow cytometry as described in Section 2 (Fig. 2a). In addition, SDF-1 is a very basic protein such that sulfated polysaccharides such as heparin sulfate, and fucoidan sulfate can inhibit SDF-1 binding (Fig. 2b). SDF-1 binds avidly to cell surface glycosoaminoglycans (GAG) [27] which can give problems with high non-specific binding. The CCRF–CEM cells were chosen as they have been shown to exhibit very low non-specific binding to of SDF-1 in ligand binding assays, presumably in part due to low expression of surface GAGs [26]. This cell line was therefore chosen and used throughout to demonstrate the interactions of AMD3100 with the CXCR4 receptor. In a homologous competition binding assay SDF-1 was found to bind to CCRF–CEM cells with a K_D of 24 ± 3 nM (Fig. 2c). The binding curve was fitted to a one site binding model. AMD3100 was shown to inhibit ^{125}I-SDF-1 ligand binding to CCRF–CEM cells in a heterologous competition binding assay. A typical result is shown in Fig. 2d. The data was fitted to a single site binding model and gave a K_i of 651 ± 37 nM (n = 3).

3.2. AMD3100 inhibits SDF-mediated cell signaling

Chemokine receptors are G-protein coupled receptors [28], 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 non-hydrolysable analogue of GTP, thus trapping the formation of the GTP/G-protein. We have used a europium-labelled analogue of GTP [29]. The europium fluoresces with a long fluorescence decay (microseconds), which allows for time-

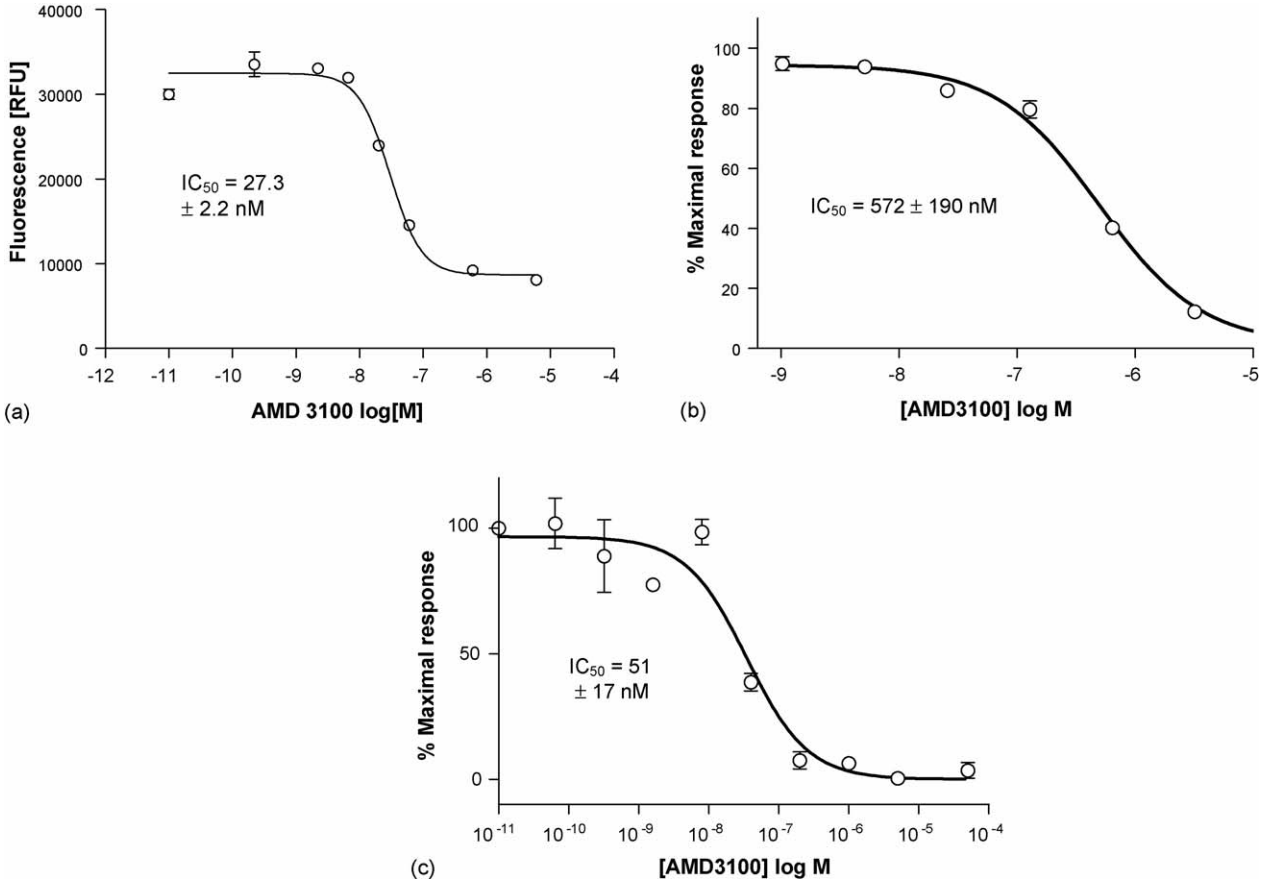

Fig. 3 – Inhibition of SDF-1-mediated signaling by AMD3100: (a) inhibition of SDF-1α stimulated GTP-binding; (b) inhibition of SDF-1α stimulated calcium flux; (c) inhibition of SDF-1α stimulated CCRF–CEM chemotaxis.

Table 1 – Cross screening of different receptors for AMD3100 activity

Receptor	Cell line	Ligand	IC_{50} AMD3100 (μM)
CCR1	HEK293F–CCR1	MIP-1α/CCL3	>100
CCR2b	HEK293F–CCR2b	MCP-1/CCL2	>100
CXCR3	HEK293F–CXCR3–$G\alpha_{qi5}$	IP-10/CXCL10	>100
CCR4	HEK293F–CCR4–$G\alpha_{qi5}$	TARC/CCL17	>100
CCR5	HEK293F–CCR5	RANTES/CCL5	>100
CCR7	CCRF–CEM	MIP-3β/CCL19	>100
LTB_4	CHO-S–LTB_4	LTB_4	>100

delayed signal detection (time resolved fluorescence), and in addition has a large Stokes shift. Together these properties eliminate most background interference. A typical result is shown in Fig. 3a. AMD3100 inhibited CXCR4 activation as measured by GTP-binding with an IC_{50} of 27.3 ± 2.2 nM (n = 3).

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 (see Section 2). AMD3100 was able to inhibit SDF-1 mediated calcium flux with an IC_{50} of 572 ± 190 nM (n = 3). A typical result is shown in Fig. 3b.

A key property of all chemokines is that they induce a chemotactic respsonse to a chemokine concentration gradient. AMD3100 was able to inhibit SDF-1 mediated chemotaxis of CCRF–CEM cells with an IC_{50} of 51 ± 17 nM (n = 3). A typical result is shown in Fig. 3c.

3.3. *AMD3100 activity is selective for CXCR4*

In order to demonstrate the specificity of AMD3100 for CXCR4 it was tested in calcium flux assays against a panel of chemokine receptors, and in ligand binding assay for BLT1, the receptor for leukotriene B_4 (LTB_4). LTB_4 is a potent chemoattractant and its receptor is a GPCR. The results in Table 1 show that the IC_{50} of AMD3100 against CCR1, CCR2b, CCR4, CCR5, CCR7, CXCR3 and LTB_4 was >100 μM in all cases, i.e. over 100-fold less than comparable inhibition of CXCR4.

3.4. *AMD3100 is a tight binding, slowly reversible antagonist of CXCR4*

AMD3100 binding to CXCR4 was assessed indirectly by flow cytometry using the FITC-labelled anti-CXCR4 antibody 12G5. In order to obtain a qualitative measure of the rate of AMD3100 binding to CXCR4, CEM cells were incubated with AMD3100 over 60 min and then stained with 12G5-FITC to assess available CXCR4 receptor. After 15 min at an AMD3100 concentration of 10 μM only 11% of the potential binding sites were occupied by the 12G5 antibody implying that 89% of available CXCR4 receptors were occupied by AMD3100, with almost complete inhibition of antibody binding at 60 min implying almost complete receptor occupancy by AMD3100. Inhibition of antibody binding was lower at AMD3100 concentrations of 1 and 0.1 μM. At 1.0 μM AMD3100 42% of the potential binding sites were occupied by the 12G5 antibody after 15 min, and 16% after 60 min compared with 0.1 μM AMD3100 where 92% and 40% receptors were still available for 12G5 binding respectively, indicating that compound binding is both concentration and time dependent (Fig. 4a). A qualitative measure of AMD3100 dissociation was obtained by incubating CCRF–CEM cells with AMD3100 for 1 h and then labeling with 12G5-FITC immediately post-incubation or at 24 or 48 h post-incubation. Antibody labeling increased with time indicating time dependent release of AMD3100. There was still significant labeling of CCRF–CEM cells with 12G5-FITC at 48 h post-incubation with all concentrations of AMD3100, with 80%, 69%, and 26% antibody labelling at 10, 1, 0.1 μM AMD3100, respectively indicating that AMD3100 binding is slowly reversible (Fig. 4b).

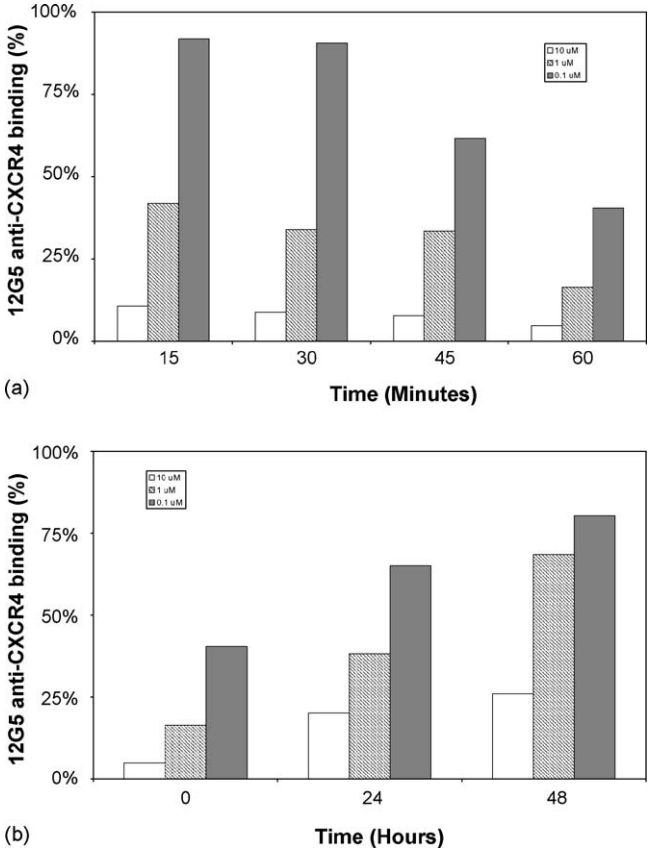

Fig. 4 – AMD3100 is a tight binding, slowly reversible antagonist of CXCR4: (a) assessment of AMD3100 binding to CXCR4; (b) dissociation of AMD3100 from CXCR4. Experiments were conducted using CCRF–CEM cells. The amount of AMD3100 bound was assessed indirectly by flow cytometry using the FITC-labelled anti-CXCR4 antibody 12G5. The results are expressed as the percent 12G5 antibody bound in the presence of varying concentrations of AMD3100.

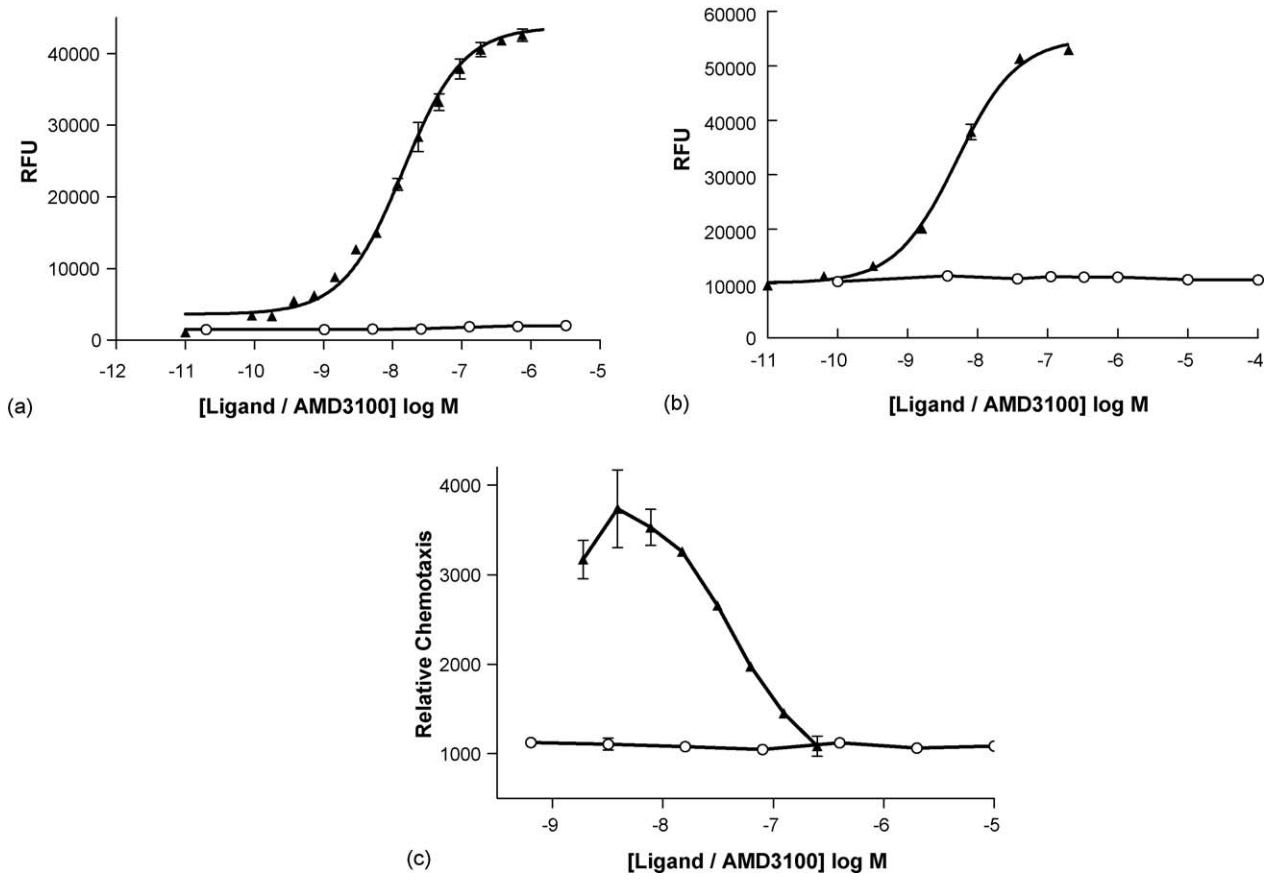

Fig. 5 – Analysis of AMD3100 (○) for agonist activity in (a) the calcium flux assay in CCRF–CEM cells, (b) GTP-binding assay on CCRF–CEM cell membranes and (c) chemotaxis assay on CCRF–CEM cells. SDF-1 (▲) is shown for comparison.

3.5. *AMD3100 displays no agonist activity*

CCRF–CEM cells express a number of GPCRs including CXCR4, CCR4 and CCR7. AMD3100 alone did not induce a calcium flux in CCRF–CEM cells, whereas SDF-1, the cognate ligand for CXCR4, induced calcium flux with an EC_{50} of 14.0 nM (Fig. 5a). Furthermore, AMD3100 neither stimulated GTP-binding on CCRF–CEM cell membranes (Fig. 5b), nor chemotaxis of CCRF–CEM cells (Fig. 5c), compared with SDF-1, which has an EC_{50} of 8.2 nM in the GTP-binding assay and gives maximal CCRF–CEM cell chemotaxis at 10 nM.

4. Discussion

AMD3100 was initially identified as a potent and selective inhibitor of HIV-1 and HIV-2 replication. Upon the discovery of the essential role of chemokine receptors in the viral infectivity process the molecular target of AMD3100 was shown to be the chemokine receptor CXCR4 [13–15]. A clinical trial with AMD3100 in HIV patients has subsequently validated CXCR4 as a therapeutic target for HIV. Safety and pharmacokinetic considerations precluded further development of AMD3100 for HIV infection. This trial was terminated because of the perceived lack of efficacy and the occurrence of unexplained premature ventricular contractions (PVCs) in two patients, though the PVCs were not dose-related and in one instance was likely due to a pre-existing condition. Furthermore, a retrospective analysis of the data indicated a decrease in X4 viral load with AMD3100 treatment. The most common and, only dose-related, side effects were gastrointestinal symptoms and paresthesias. However, AMD3100 is not orally bioavailable, and was administered by continuous infusion for 10 days, which poses limitations on development of a drug for chronic use [16].

This receptor, and its cognate ligand SDF-1, have an essential role in lymphopoiesis, lymphocyte and stem cell homing and trafficking, immunomodulation and development [3]. The pivotal role of the SDF-1/CXCR4 interaction in stem cell homing to the bone marrow has led to the development of AMD3100 as a potential stem cell mobilizer for hematopoietic stem cell transplantation in patients with non-Hodgkins lymphoma and multiple myeloma. Additionally, CXCR4 has also been implicated in a number of other disease states including leukemias and lymphomas [30–33], solid tumours [34–38], and inflammatory diseases such as rheumatoid arthritis [39,40] and asthma [41]. Selective small molecule inhibitors of CXCR4 therefore have great therapeutic potential.

We have further investigated the interactions of AMD3100 with the CXCR4 receptor using the CCRF–CEM cell line, which naturally expresses CXCR4. SDF-1 binding to the CCRF–CEM

cells was shown to fit a one site binding model with a K_D of 24 nM. Reported K_D values range from 16 to 0.5 nM [26,42–46], and in one case a biphasic binding mode has been observed with K_D values of 0.005 and 24 nM [46]. This range can be attributed to the use of cells naturally expressing CXCR4 versus transfected cell lines, and/or different experimental conditions. A K_D of 0.49 nM was found using transiently transfected COS-7 cells and a ligand concentration of 0.012 nM [43], whereas a K_D of 3.6 nM was reported for CCRF–CEM cells with endogenous CXCR4 expression using a ligand concentration of 4 nM [26], compared with our conditions using CCRF–CEM cells and a ligand concentration of 0.1 nM. In addition, non-specific ligand binding is a confounding feature of SDF-1 ligand binding assays. SDF-1 is a very basic protein and binds avidly to cell surface glycosoaminoglycans (GAG) [27] and we have found that sulfated polysaccharides such as heparin sulfate, chondroitin sulfate and fucoidan sulfate can inhibit SDF-1 binding to CCRF–CEM cells. The CCRF–CEM cells were chosen as they have been shown to exhibit very low non-specific binding of SDF-1 in ligand binding assays [26], presumably in part due to low expression of surface GAGs. It has been suggested that SDF-1 binding may exhibit a biphasic binding mode [46]. It is possible that the reported apparent low affinity binding site found on HL-60 cells for SDF-1 is provided by binding to GAGs, and hence is not seen in the CCRF–CEM cells.

AMD3100 was shown to inhibit SDF-1 ligand binding with a K_i of 651 ± 37 nM. Inhibition of CXCR4 signaling pathways was demonstrated by inhibition of SDF-1 stimulated calcium flux and GTP-binding. Furthermore, AMD3100 was able to inhibit a CXCR4 mediated physiological response, cell chemotaxis. As SDF-1 is the only known ligand for CXCR4 it is likely that these processes are mediated by the CXCR4 receptor. However, it has recently been reported that SDF-1 is also a ligand for the orphan receptor RDC1, now renamed CXCR7 [47]. There is, therefore a possibility for RDC1 involvement, this is currently being investigated. The IC_{50} for calcium flux of 572 ± 190 nM is comparable to the K_i for inhibition of ligand binding, whereas the IC_{50} values for GTP-binding and chemotaxis are 27.3 ± 2.2 and 51 ± 17 nM, respectively. A similar disparity was observed in HL60 cells [46] with IC_{50} values of 4.7 and 7.4 nM for chemotaxis and GTP-binding respectively compared to an IC_{50} of 15.2 μM in SDF-1 ligand binding. The authors proposed that this was due to a two-site binding model for SDF-1 which would allow AMD3100 to act as a functional antagonist by perturbing ligand interaction by binding at one site, but without displacing the ligand SDF-1. However, the reported IC_{50} for AMD3100 inhibition of ligand binding is significantly greater than that reported here, or that found in transiently transfected COS-7 cells of 74 nM [43]. As discussed above this may be due to differences in the cells that are used including differences in receptor expression and non-specific binding, or different experimental conditions. Also the GTP assay is performed on cell membranes rather than whole cells and may be in part responsible for this observation. On the other hand the chemotaxis assay is performed with whole cells.

One possible explanation for the difference between the inhibitory response of calcium flux and chemotaxis could be due to divergent signaling pathways. An alternative explanation for the differences in activity observed between these assays may also be in part due to the tight binding of AMD3100 to CXCR4, and the differences in the relative time course of the calcium flux and chemotaxis studies. The calcium flux involves a 15 min pre-incubation followed by challenge with SDF-1 resulting in a calcium flux that is over in seconds. The chemotaxis study also involves a pre-incubation however this assay takes place over a 2.5 h period. In order to address this possibility we have assessed the kinetics of AMD3100 receptor association and dissociation using flow cytometry to monitor CXCR4 receptor available for labeling with the anti-CXCR4 antibody 12G5-FITC. It should be noted that the 12G5 antibody may not recognize all forms of the CXCR4 receptor and therefore this is only a qualitative, and indirect, assessment of compound association and dissociation [48]. At high concentrations of AMD3100 available CXCR4 receptors are blocked within 15 min. At lower concentrations closer to the inhibitory concentrations observed in the activity assays binding is slower demonstrating that rate of CXCR4 receptor occupancy is concentration dependent. The apparent rate of dissociation from the receptor is also concentration dependent with 20%, 31%, and 74% receptor unavailable to anti-CXCR4 antibody at AMD3100 concentrations of 0.1, 1, and 10 μM, respectively (T = 48 h). More significantly AMD3100 blocking of anti-CXCR4 antibody binding persists for up to 48 h at all drug concentrations indicative of slow dissociation of the compound from the receptor. Together these data indicate that AMD3100 is a tight-binding, slowly reversible inhibitor of CXCR4. Furthermore, the long incubation time in the chemotaxis assay therefore favors inhibition of CXCR4, thus offering one explanation for the low IC_{50} for chemotaxis inhibition.

AMD3100 had no inhibitory effect on either MIP-1α, MCP-1, TARC, RANTES, MIP-3β, or IP10 mediated calcium flux, ligands for CCR1, CCR2b, CCR4, CCR5, CCR7 and CXCR3, respectively, or LTB_4 binding to BLT1, an alternative GPCR that mediates chemotaxis. It has also been shown that AMD3100 is unable to inhibit CXCR1, CXCR2, CCR3, CCR6, CCR8, and CCR9 [49]. Together these data demonstrate that AMD3100 is a specific inhibitor of CXCR4.

Using three different assays, calcium flux, GTP-binding, and chemotaxis no agonist effect was observed with AMD3100 over a concentration range of 10^{-9} to 10^{-5} M AMD3100. Furthermore, stimulation of calcium flux was not observed in the CCRF–CEM cell line which expresses more than one GPCR including, the chemokine receptors CCR4 and CCR7 indicating that AMD3100 is not only not an agonist for CXCR4, but is also not an agonist for other GPCRs including CCR4 and CCR7. Similarly AMD3100 was unable to induce calcium flux in a human T-lymphoid cell line, Sup-T1, or purified monocytes [49]. It has been reported that AMD3100 at high concentrations 10^{-7} to 10^{-5} M can be a weak agonist against wild-type CXCR4, and also to stimulate a constitutively active CXCR4 mutant (N119S) [50]. We have been unable to reproduce the effect on wild-type CXCR4, which suggests this may be a cell line dependent phenomenon, or a function of the constitutively active mutant.

In conclusion, we have demonstrated that AMD3100 is a specific inhibitor of CXCR4, inhibiting SDF-1-mediated responses including ligand binding, GTP-binding and calcium flux. Indirect assessment of AMD3100 interactions with the CXCR4 receptor showed that AMD3100 was a slow,

tight-binding, reversible inhibitor. Specificity for CXCR4 over other G-protein coupled receptors, CXCR3, CCR1, CCR2b, CCR4, CCR5, CCR7 and BLT1 was demonstrated using calcium flux and ligand binding assays. Significantly, AMD3100 did not display agonist activity in several assays for GPCR activation. Both the receptor specificity and inability to stimulate GPCR activity are important attributes for a potential therapeutic agent. The CXCR4/SDF-1 axis has been shown to be central to the homing and retention of stem cells in bone marrow niches and AMD3100 is in development as a stem cell mobilizing agent for non-Hodgkin's lymphoma and multiple myeloma patients. The most common approach used to mobilize hematopoietic stem cells is to administer granulocyte colony-stimulating factor (G-CSF) for 5 days. This causes expansion of myeloid cells within the bone marrow resulting in release of stem cells into the circulation. However, 10–20% of patients fail to achieve adequate mobilization with G-CSF. In a clinical trial comparing G-CSF with a G-CSF + AMD3100 regimen, mobilization with AMD3100 + G-CSF was consistently superior to G-CSF mobilization, and successful mobilization was seen with the combination in patients who failed to mobilize with G-CSF alone [51]. Clinical data has shown AMD3100 to be both safe and effective, whilst offering improved mobilization over current modalities, thus demonstrating the potential for AMD3100 as a specific, potent inhibitor of CXCR4.

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