Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4

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Abstract This study was undertaken to demonstrate the unique specificity of the chemokine receptor CXCR4 antagonist AMD3100. Calcium flux assays with selected chemokine/cell combinations, affording distinct chemokine receptor specificities, revealed no interaction of AMD3100 with any of the chemokine receptors CXCR1 through CXCR3, or CCR1 through CCR9. In contrast, AMD3100 potently inhibited CXCR4-mediated calcium signaling and chemotaxis in a concentration-dependent manner in different cell types. Also, AMD3100 inhibited stromal cell-derived factor (SDF)-1-induced endocytosis of CXCR4, but did not affect phorbol ester-induced receptor internalization. Importantly, AMD3100 by itself was unable to elicit intracellular calcium fluxes, to induce chemotaxis, or to trigger CXCR4 internalization, indicating that the compound does not act as a CXCR4 agonist. Specific small-molecule CXCR4 antagonists such as AMD3100 may play an important role in the treatment of human immunodeficiency virus infections and many other pathological processes that are dependent on SDF-1/CXCR4 interactions (e.g. rheumatoid arthritis, atherosclerosis, asthma and breast cancer metastasis). **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Bicyclam; Chemokine receptor antagonist; CXCR4; Calcium signaling; Chemotaxis

1. Introduction

In the last few years, chemokines have emerged as an important family of pro-inflammatory cytokines, which drive a variety of normal and pathological processes involving cell migration. Chemokines are soluble proteins of low molecular mass (5–15 kDa), and their nomenclature and classification is based on the arrangement of four conserved N-terminal cysteine residues [1]. The two major subfamilies are the CC- (or β-) and the CXC- (or α-) chemokines, in which the first two cysteines are adjacent or separated by one amino acid, respectively. Chemokines mediate their effects by binding to specific seven transmembrane G protein-coupled receptors. CC- and CXC-chemokines are recognized by distinct subsets of the chemokine receptor family, termed CCR and CXCR, respectively [1]. However, there exists a marked binding promiscuity between many ligands and receptors, in that several chemokines may bind to a single receptor and, vice versa, a single chemokine may bind to several receptors. For example, the CC-chemokine receptor 5 (CCR5) binds the CC-chemokine ligands RANTES (regulated on activation, normal T cell expressed and secreted), MIP (macrophage inflammatory protein)-1α and MIP-1β. On the other hand, RANTES binds to CCR1 and CCR3, in addition to CCR5 [1]. Upon ligand activation, chemokine receptors trigger an intracellular signal transduction cascade comprising a transient increase in cytosolic free calcium [2]. Also, chemokine binding results in phosphorylation of the receptor protein at serine and threonine residues in the C-terminal cytoplasmic tail, followed by rapid receptor internalization [2].

Chemokines and their receptors mediate the recruitment of immune cells to sites of inflammation or infection, and, consequently, are implicated in several disease states including infectious diseases, allergy, atherosclerosis, and autoimmune disorders such as rheumatoid arthritis, multiple sclerosis and psoriasis [3–6]. Besides their well-known function as inflammatory mediators, chemokines also play an important role in organogenesis, angiogenesis and tumor growth and metastasis [4,7,8]. Most particularly, the CXC-chemokine/receptor pair stromal cell-derived factor (SDF)-1/CXCR4 appears to play a central role in allergic airway disease [9], rheumatoid arthritis [10] and breast carcinoma metastasis [11]. In addition, the chemokine receptors CCR5 and CXCR4 were identified as the principal coreceptors, besides the CD4 receptor, for human immunodeficiency virus (HIV) particles to gain entry into target cells [12,13]. The so-called 'R5' virus strains that predominantly infect macrophages by the use of CCR5 with CD4 [12] are isolated from patients during the initial asymptomatic stage of HIV infection, and are largely responsible for virus transmission between individuals. The more pathogenic 'X4' viruses, which are typically T cell-tropic, use CXCR4 as their coreceptor [13]. They emerge at a later stage of the disease and cause a rapid $CD4^+$ T cell depletion and progression towards AIDS.

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Abbreviations: FBS, fetal bovine serum; GFP, green fluorescent protein; GROα, growth-related oncogene α; HIV, human immunodeficiency virus; IL, interleukin; IP-10, interferon-inducible protein-10 kDa; MCP-1, monocyte chemoattractant protein-1; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; RANTES, regulated on activation, normal T cell expressed and secreted; SDF-1, stromal cell-derived factor 1; TARC, thymus and activation-regulated chemokine; TECK, thymus-expressed chemokine

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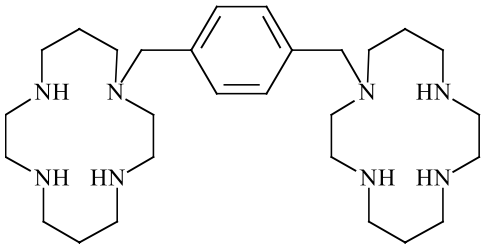


Fig. 1. Chemical structure of the bicyclam AMD3100, 1,1′-[1,4-phenylenebis(methylene)]-bis-1,4,8,11-azatetradecane.

The bicyclam AMD3100 (Fig. 1) was originally described as a highly potent and selective inhibitor of HIV-1 and HIV-2 replication [14,15]. Only after the elucidation of the cellular tropism of HIV by the discovery that CCR5 and CXCR4 function as HIV-1 coreceptors [12,13], the selective activity of AMD3100 could be attributed to a specific interaction of the compound with CXCR4 but not CCR5 [16,17]. AMD3100 inhibited the intracellular calcium signaling and chemotactic response elicited by the natural CXCR4 ligand SDF-1 in different cell types [16,17]. We have recently shown that AMD3100 also effectively inhibits autoimmune collagen-induced arthritis in mice, by specific interference with the SDF-1-mediated migration of Mac-1$^+$/CXCR4$^+$ leukocytes towards the inflamed joints [18].

Thus, AMD3100 is an attractive drug candidate for therapeutic intervention in several pathologies in which CXCR4 is critically involved. However, the specificity of AMD3100 for CXCR4 has never been extensively documented. Here, we report the inability of the compound to interfere with cell surface (chemokine) receptors other than CXCR4. We have also examined whether the compound on its own can generate intracellular calcium signaling events, induce cell migration or trigger internalization of CXCR4.

2. Materials and methods

2.1. Compounds and chemokines

The bicyclam AMD3100 was synthesized as described previously [19]. SDF-1 and RANTES were synthesized by Dr. I. Clark-Lewis. Interleukin (IL)-8, interferon-inducible protein-10 kDa (IP-10), eotaxin, thymus and activation-regulated chemokine (TARC), MIP-1α, MIP-1β, MIP-3α, MIP-3β, I309 and thymus-expressed chemokine (TECK) were all purchased from PeproTech (London, UK). Growth-related oncogene-α (GROα) and macrophage-derived chemokine (MDC) were obtained from R&D Systems (Minneapolis, MN, USA). Monocyte chemoattractant protein-1 (MCP-1) was obtained from Dr. Paul Proost.

2.2. Cell lines and cell culture

CXCR4-, CCR1-, CCR3- and CCR5-transfected human astroglioma U87.CD4 cells were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York, NY, USA) and were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) containing 10% heat-inactivated fetal bovine serum (FBS) (BioWhittaker Europe, Verviers, Belgium), 0.01 M HEPES buffer (Life Technologies), 0.2 mg/ml geneticin (G-418 sulfate) (Life Technologies) and 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). CCR4-transfected human osteosarcoma HOS.CD4 cells were a kind gift from Dr. N. Landau and were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 0.01 M HEPES buffer and 1 μg/ml puromycin. Human T-lymphoid SupT1, Molt-4 and HSB-2 cells, human myeloid THP-1 cells and murine B-lymphoblastic leukemia L1210 cells were all obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS (BioWhittaker Europe) and 2 mM glutamine (Life Technologies). All cell cultures were maintained at 37°C in a humidified, CO_2-controlled atmosphere and subcultivations were done every 2–3 days.

2.3. Isolation of primary lymphocytes and monocytes

Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over LymphoprepTM (d = 1.077 g/ml) (Nycomed, Oslo, Norway). The PBMCs were either directly used in calcium flux assays, or transferred to RPMI-1640 medium supplemented with 10% FBS (BioWhittaker Europe) and 2 mM glutamine, and stimulated for 7 days with phytohemagglutinin (PHA) (Murex Biotech Limited, Dartford, UK) at 2 μg/ml and IL-2 at 10 ng/ml to obtain 'PHA/IL-2 blasts'. Monocytes were enriched from freshly isolated PBMCs by adherence to Petri dishes at 37°C in RPMI-based growth medium. After 2 h, adherent cells were washed several times at room temperature with growth medium, harvested by scraping with a policeman and resuspended in RPMI-based growth medium.

2.4. Measurement of intracellular calcium mobilization

Adherent chemokine receptor-transfected U87.CD4 and HOS.CD4 cells and freshly isolated primary monocytes were seeded in 0.1% gelatin-coated 96-well black-wall microplates (Costar, Cambridge, MA, USA) at 2×10^4 cells per well for U87.CD4 and HOS.CD4 cells and at 2×10^5 cells per well for primary monocytes on the day prior to the experiment. Suspension tumor cell lines were used in stationary growth phase (2–3 days after 2/3 subcultivation). PBMCs were used either immediately after isolation or after a 7-day culture period in the presence of PHA and IL-2 (PHA/IL-2 blasts). On the day of the experiment, suspension cells and plated monolayers were loaded with the fluorescent calcium indicator Fluo-3 acetoxymethyl (Molecular Probes, Leiden, The Netherlands) at 4 μM for 45 min at room temperature (suspension cells) or at 37°C (adherent cells). After thorough washing with calcium flux assay buffer (Hanks' balanced salt solution with 20 mM HEPES buffer and 0.2% bovine serum albumin (BSA), pH 7.4), the cells were preincubated for 15 min at 37°C with AMD3100 at the indicated concentrations in the same buffer. Then, the intracellular calcium mobilization in response to the appropriate chemokine was measured at 37°C by monitoring the fluorescence as a function of time simultaneously in all the wells using a Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA).

2.5. Antibody staining and flow cytometry

To demonstrate the long-lasting inhibitory effect of AMD3100 on the binding of the CXCR4-specific antibody 12G5 at the cell surface, SupT1 cells were preincubated with AMD3100 at 0 or 5 μg/ml for 30 min at room temperature in RPMI-based growth medium. Then, the drug was washed out and the cells were further incubated at 37°C in drug-free growth medium. At each time point (i.e. 0, 1, 6, 24 and 48 h after drug removal), 0.5×10^6 cells were washed once in cold phosphate-buffered saline (PBS) containing 2% FBS, and were then incubated for 30 min on ice with phycoerythrin-conjugated anti-CXCR4 mAb clone 12G5 (BD Pharmingen) in PBS containing 2% FBS. Thereafter, the cells were thoroughly washed twice with PBS, fixed in 1% paraformaldehyde in PBS and analyzed on a FACScalibur flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA, USA). As a negative control for aspecific background staining, the cells were stained in parallel with Simultest Isotype Control mAb (Becton Dickinson).

2.6. Receptor internalization assay

U87.CD4 cells stably transfected with green fluorescent protein (GFP)-coupled CXCR4 (U87.CD4.CXCR4–GFP) were seeded in 0.001% poly-D-lysine-coated 8-well Lab-Tek® chamber slides (Nalge Nunc International, Naperville, IL, USA) at 4×10^4 cells per well. The next day, the cells were preincubated in cell culture medium with or without 5 μg/ml AMD3100 for 15 min at room temperature. Then, SDF-1 or the phorbol ester, phorbol 12-myristate 13-acetate (PMA), were added at final concentrations of 1 μg/ml and 100 ng/ml, respectively. After incubation at 37°C for 45 min, the chamber slides were placed on ice, the cells were washed once with ice-cold PBS, fixed with 1% paraformaldehyde in PBS for 5 min on ice and washed three times with ice-cold PBS. The chambers were removed from the glass slides

and a cover slip was placed on the cells. Cell-associated fluorescence was examined by a Nikon fluorescence microscope.

2.7. Chemotaxis assay

SDF-1-induced cell migration was assessed using 5-μm pore Transwell filter membranes (Costar, Boston, MA, USA). The membrane inserts were placed in the wells of a 24-well plate, containing 600 μl buffer (HBSS/20 mM HEPES/0.2% BSA; pH 7.4) with either no chemokine, 200 ng/ml SDF-1 or 25 μg/ml AMD3100. After preincubation with AMD3100 at different concentrations (i.e. 0, 0.04, 0.2, 1, 5 or 25 μg/ml), 1×10^6 freshly isolated PBMCs in 100 μl buffer were loaded into each Transwell filter. The plate was then incubated at 37°C for 2.5 h, whereafter the filter inserts were carefully removed. The migrated cells were collected from the wells and counted on a flow cytometer (FACScalibur, Becton Dickinson).

3. Results and discussion

3.1. AMD3100 exclusively inhibits SDF-1/CXCR4-mediated intracellular calcium signaling

In a first set of calcium flux experiments, we have demonstrated the potent antagonistic activity of AMD3100 against SDF-1/CXCR4 in different cell types. After preincubation with AMD3100 at different concentrations, the cells were stimulated with SDF-1 at the appropriate concentration (see Table 1) and the changes in intracellular calcium concentration were recorded by the use of the advanced FLIPR system.

Fig. 2 clearly shows the concentration-dependent effect of AMD3100 on SDF-1-induced calcium signaling in CXCR4-transfected human astroglioma U87.CD4 cells, freshly isolated human PBMCs, $CXCR4^+$ human T-lymphoid HSB-2 cells and murine B-lymphoblastic leukemia L1210 cells. As CXCR4 is the sole receptor for SDF-1 [20,21], all four assay systems allow specific examination of CXCR4-mediated signaling events. Table 1 displays the 50% inhibitory concentration (IC_{50}) values of AMD3100 for inhibition of SDF-1-induced calcium flux in these and various other cell types; the values ranged between 0.01 and 0.13 μg/ml. Note that AMD3100 also potently antagonized CXCR4 in the murine L1210 cell line, the IC_{50} value being 0.08 μg/ml (Fig. 2 and Table 1). Also, intracellular calcium signaling elicited in SupT1 cells by either the α- or the β-isoform of SDF-1 were inhibited to the same extent by AMD3100 (data not shown).

To address the receptor specificity of AMD3100, we evaluated its ability to inhibit chemokine-induced intracellular calcium signaling mediated by CXCR1 through CXCR3 and CCR1 through CCR9. For each of these chemokine receptors, we performed calcium flux experiments with one or several selected combinations of receptor-specific chemokine ligands and cells expressing the corresponding chemokine receptor. Appropriate chemokine receptor expression on the particular cell types chosen was ascertained by reverse transcription-polymerase chain reaction and/or antibody staining and flow cytometry (data not shown). The different ligand/cell assay systems affording the desired receptor specificities, and the chemokine concentrations that were applied to obtain adequate and reliable calcium responses, are listed in Table 1. After preincubation with or without AMD3100 at 25 μg/ml, the cells were stimulated with the chemokine and the changes in intracellular calcium concentration were recorded by the use of the FLIPR system.

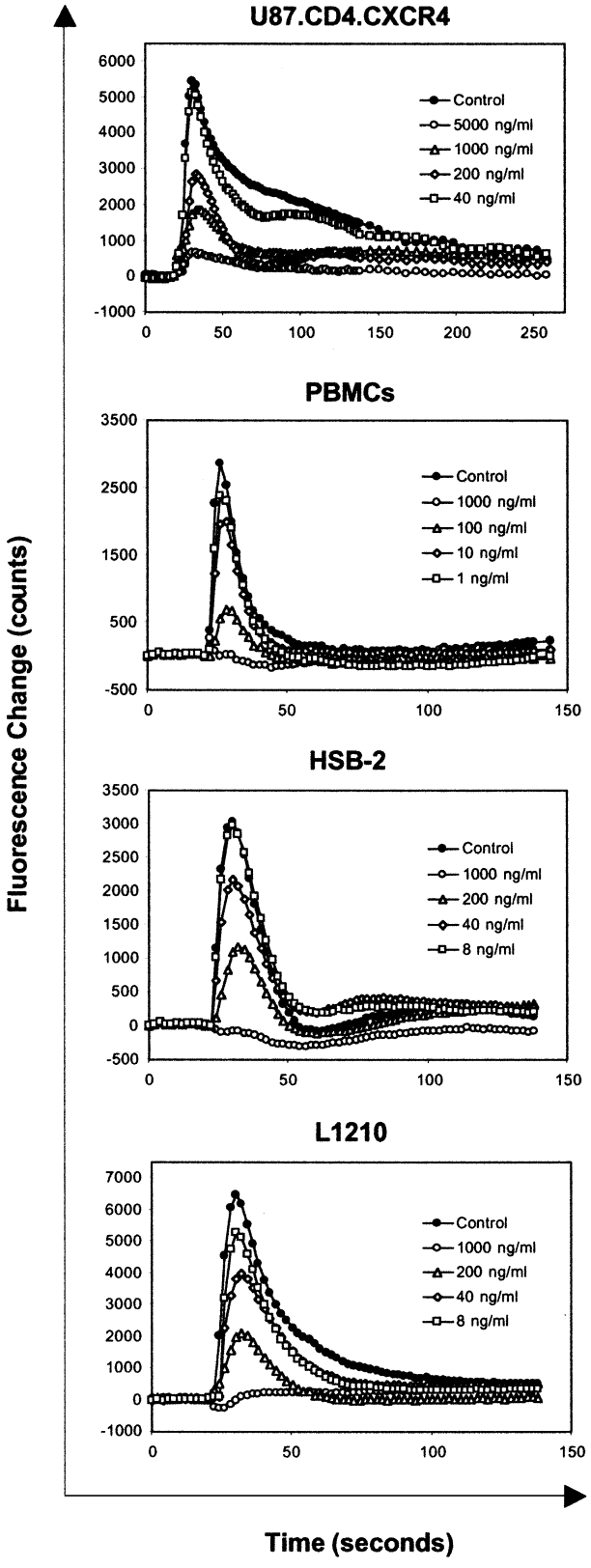
In sharp contrast to its strong antagonistic activity against CXCR4, AMD3100 at 25 μg/ml completely failed to block chemokine-induced calcium signaling through CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8 and CCR9 (Table 1). For each of these chemokine receptors, a representative calcium flux experiment, marked in the last column of Table 1, is shown in Fig. 3. Thus, it is clear that AMD3100 specifically antagonizes CXCR4-mediated intracellular signaling without interacting with any of the chemokine receptors CXCR1 through CXCR3 or CCR1 through CCR9. In addition, it has been

Table 1
Overview of calcium flux experiments performed to demonstrate the CXCR4-specific antagonism by AMD3100

Chemokine receptor	Ligand	Concentration (ng/ml)	Cell type	IC_{50} of AMD3100 (μg/ml)	Figure
CXCR1	IL-8	500	freshly isolated PBMCs	>25	3
CXCR2	IL-8	500	freshly isolated PBMCs	>25	not shown
	GROα	500	freshly isolated PBMCs	>25	3
CXCR3	IP-10	50	U87.CXCR3	>25	not shown
		200	PHA/IL-2-stimulated PBMCs	>25	3
CXCR4	SDF-1	10	U87.CD4.CXCR4	0.13	2
		100	freshly isolated PBMCs	0.03	2
		20	SupT1	0.04	not shown
		10	THP-1	0.01	not shown
		20	HSB-2	0.10	2
		100	Molt-4	0.02	not shown
		2.5	L1210	0.08	2
CCR1	MIP-1α	50	U87.CD4.CCR1	>25	3
		50	purified monocytes	>25	not shown
CCR2	MCP-1	50	purified monocytes	>25	3
CCR3	eotaxin	50	U87.CD4.CCR3	>25	3
CCR4	MDC	20	HOS.CD4.CCR4	>25	3
	TARC	20	HOS.CD4.CCR4	>25	not shown
CCR5	RANTES	5	U87.CD4.CCR5	>25	not shown
		50	purified monocytes	>25	not shown
	MIP-1β	10	U87.CD4.CCR5	>25	3
		50	purified monocytes	>25	not shown
CCR6	MIP-3α	200	PHA/IL-2-stimulated PBMCs	>25	3
CCR7	MIP-3β	200	HSB-2	>25	3
CCR8	I309	500	freshly isolated PBMCs	>25	3
CCR9	TECK	100	Molt-4	>25	3

previously shown that AMD3100 has no effect whatsoever on ligand activation of NK1 tachykinin (substance P) receptor, fMLP receptor or C5a receptor [18].

To examine if AMD3100 by itself can induce calcium signaling events, we triggered several cell types, including T-lymphoid SupT1 cells and primary human monocytes, with the compound in the absence of any chemokine. Even at a concentration as high as 25 μg/ml, AMD3100 completely failed to elicit a detectable intracellular calcium flux in these cells, while SDF-1 at 500- to 1000-fold lower concentrations induced strong calcium responses under the same experimental conditions (Fig. 4).



← Fig. 2. Concentration-dependent inhibition of SDF-1/CXCR4-mediated intracellular calcium flux by AMD3100 in CXCR4-transfected human U87.CD4 cells, freshly isolated human PBMCs, human T-lymphoid HSB-2 cells and murine B-lymphoblastic leukemia L1210 cells. After loading with the fluorescent calcium indicator Fluo-3, the cells were preincubated for 15 min with AMD3100 at the indicated concentrations, and were then stimulated with SDF-1 at the appropriate concentration (see Table 1). The transient increase in the intracellular calcium concentration was recorded by monitoring the change in fluorescence of the cells as a function of time using the FLIPR. Each data point represents the mean fluorescence of quadruplicate microplate wells. The data of one representative experiment are shown.

3.2. Long-term interaction of AMD3100 with CXCR4

It has previously been reported that AMD3100 potently blocks the binding of the anti-CXCR4 mAb 12G5 at the cell membrane [16]. Using this feature, we have now demonstrated that the blocking of CXCR4 by AMD3100 is a long-lasting effect. Immediately after preincubation of SupT1 cells with AMD3100 at 5 μg/ml, the binding of the mAb 12G5 at the cell surface was inhibited by 85–90%, as compared to untreated SupT1 cells. The inhibitory effect of AMD3100 remained at exactly the same high level for at least 24 h after transfer of the cells to drug-free medium (Fig. 5). At 48 h after drug removal, the percentage of inhibition of 12G5 mAb binding in drug-treated compared to untreated SupT1 cells had declined to approximately 60% (Fig. 5). Such long-term effect of AMD3100 on CXCR4 points to a high-affinity interaction between the compound and the receptor protein.

3.3. AMD3100 exclusively inhibits CXCR4-mediated host cell entry of HIV

The highly potent and selective anti-HIV activity of AMD3100 (IC_{50}: 1–10 ng/ml) against virus strains that use CXCR4, but not CCR5, to enter their target cells, has been described in our previous reports [14–17]. On the other hand, Labrosse et al. demonstrated that U373MG-CD4 cells transfected with the orphan receptor BOB/gpr15, the CXC-chemokine receptor Bonzo/STRL33 (now designated CXCR6) or the human cytomegalovirus-encoded CC-chemokine receptor US28 are not protected by AMD3100 against infection with the HIV-1 strains LAI, NDK, 89.6 and ADA and the HIV-2 strain ROD, which all can use these three chemokine receptors as alternative coreceptors for cell entry [22]. Zhang et al. also reported that AMD3100 failed to inhibit the entry of SIV into GHOST-CD4 cells expressing BOB/gpr15, Bonzo/STRL33, US28, GPR1, APJ or V28, indicating that AMD3100 does not interact with any of these receptors [23].

3.4. AMD3100 specifically blocks SDF-1- but not phorbol ester-induced internalization of CXCR4

SDF-1-induced endocytosis of CXCR4, and the inhibitory effect of AMD3100 on the internalization process was visual-

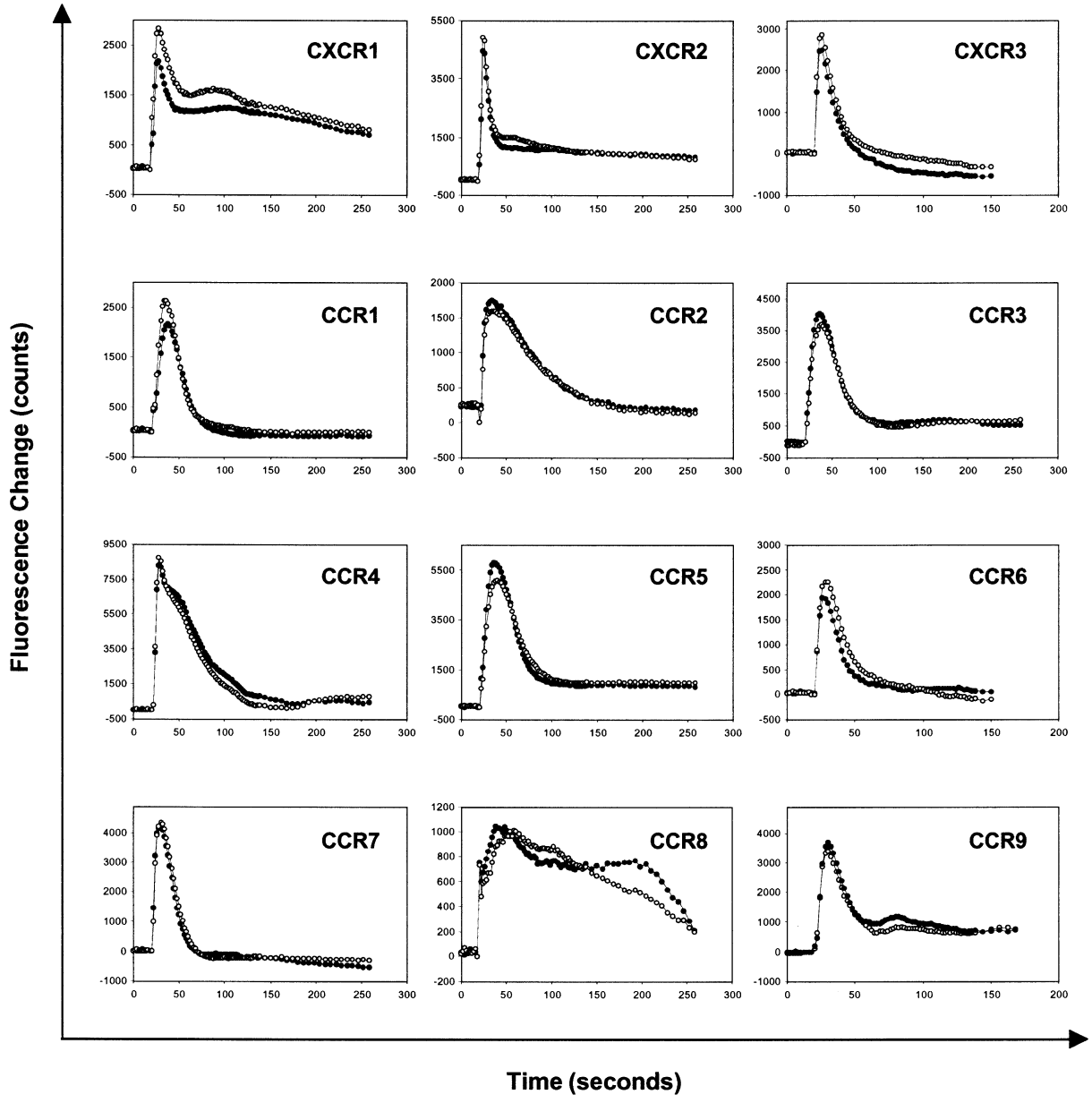


Fig. 3. Lack of inhibitory effect of AMD3100 on chemokine-induced signaling mediated by CXCR1 through CXCR3 and CCR1 through CCR9. Refer to Table 1 for details concerning the distinct combinations of cell types and chemokine ligands that were used to specifically evaluate the individual receptors. After loading with the fluorescent calcium indicator Fluo-3, the cells were incubated in the presence (open circles) or absence (closed circles) of AMD3100 at 25 μg/ml, and were then stimulated with the appropriate chemokine (see Table 1). The transient increase in the intracellular calcium concentration was recorded by monitoring the change in fluorescence of the cells as a function of time using the FLIPR. Each data point represents the mean fluorescence of quadruplicate microplate wells. The data of one representative experiment are shown.

ized by fluorescence microscopy in stably transfected U87.CD4 cells expressing GFP-coupled CXCR4. As shown in Fig. 6A, the fluorescent chemokine receptor was mainly concentrated on the cell membrane in unstimulated U87.CD4. CXCR4–GFP cells. Upon stimulation with 1 μg/ml SDF-1, the receptor was sequestered in endosomal vesicles which were visible as bright spots accumulating near the nucleus (Fig. 6B). Similar observations were made when the cells were exposed to the phorbol ester PMA at 100 ng/ml (Fig. 6C). Signoret et al. have demonstrated that SDF-1 and phorbol esters trigger CXCR4 internalization through entirely different mechanistic pathways [24]. Unlike phorbol ester-induced CXCR4 down-modulation, SDF-1-mediated receptor internalization appears to be independent of protein kinase C activation [24]. Moreover, while SDF-1 exclusively induces CXCR4 internalization, receptor endocytosis triggered by phorbol esters is a rather aspecific process, which also implies other cell surface receptors, such as CD4 [24]. In our experiments, we found that AMD3100 prevents SDF-1- but not PMA-induced CXCR4 internalization (Fig. 6E,F), which further attests as to the selective antagonization of CXCR4 by AMD3100. When the U87.CD4.CXCR4–GFP were incubated with AMD3100 at 5 μg/ml in the absence of SDF-1 or PMA, the membrane localization of the CXCR4–GFP fusion protein

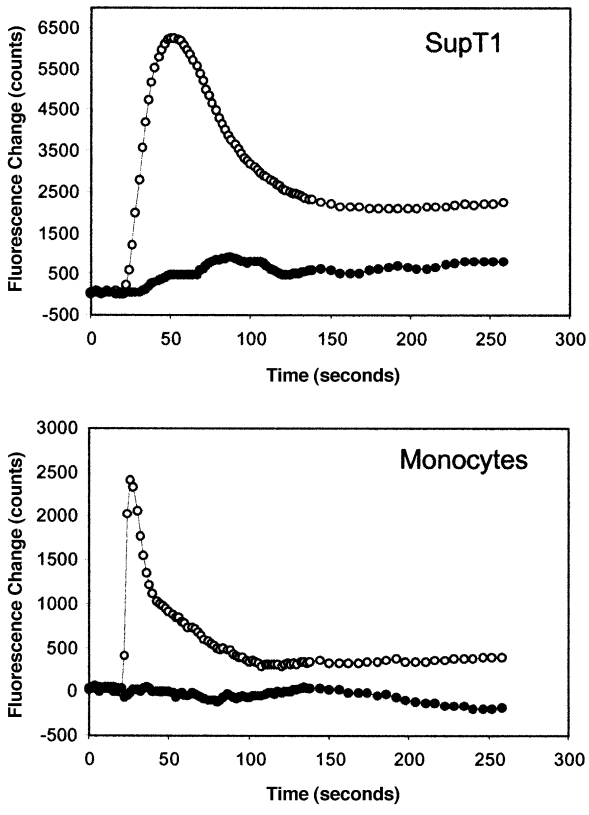


Fig. 4. Inability of AMD3100 to trigger intracellular calcium fluxes in human T-lymphoid SupT1 cells and purified monocytes. The cells were stimulated with AMD3100 at 25 μg/ml in the absence of any chemokine (closed circles). As a positive control, SupT1 cells and monocytes were triggered in the same experiment with SDF-1 at 20 ng/ml and 50 ng/ml, respectively, in the absence of AMD3100 (open circles). Each data point represents the mean fluorescence of quadruplicate microplate wells.

was not altered (Fig. 6D), indicating that AMD3100 by itself cannot induce CXCR4 internalization.

3.5. Dose-dependent inhibition of SDF-1-induced chemotaxis by AMD3100

We also examined the ability of AMD3100 to inhibit chemokine-induced migration of freshly isolated human PBMCs by the use of the Transwell membrane system. SDF-1 at 200 ng/ml elicited a strong chemotactic response in PBMCs: 18.5×10^3 cells transmigrated across the membrane, compared to only 2.2×10^3 cells in the negative control (no chemokine added), resulting in a chemotactic index of 8.4. As shown in Fig. 7, AMD3100 effectively blocked SDF-1-induced chemotaxis in a dose-dependent manner. Upon pretreatment of the cells with AMD3100 at 0.04, 0.2, 1 or 5 μg/ml, the number of cells that migrated in response to the chemokine was reduced by 26, 58, 80 and 100%, respectively, yielding an IC_{50} value of 0.13 μg/ml for AMD3100. In contrast, AMD3100 had no significant effect on cell migration induced by the CXCR3 ligand IP-10 (data not shown). As expected, the compound on its own, in the absence of SDF-1, did not exhibit any chemotactic activity. When AMD3100 was added to the lower compartment at a concentration as high as 25 μg/ml, the number of migrated cells remained at the background level (i.e. 2.6×10^3 cells).

3.6. Molecular interactions between AMD3100 and CXCR4

Receptor mutagenesis identified Asp^{171} and Asp^{262}, located in transmembrane domain-IV and transmembrane domain-VI, respectively, at each end of the main ligand-binding crevice of CXCR4, as essential interaction sites for AMD3100 [25,26]. It is assumed that one cyclam moiety of AMD3100 (see Fig. 1) directly interacts with the carboxylic acid group of Asp^{171}, and the other with Asp^{262}. In a three-dimensional molecular model of CXCR4, based on the crystal structure of the prototype seven-transmembrane G protein-coupled receptor rhodopsin, AMD3100 exactly fits between these two proposed cyclam-binding sites in the binding pocket of CXCR4 [25]. Such high-affinity molecular interactions between AMD3100 and the aspartate residues at these specific positions of CXCR4 may in part explain the remarkable receptor specificity of the compound. Indeed, this particular pair of aspartic acids, located at the interphase between the transmembrane domains and the extracellular regions of the receptor protein, appears to be highly conserved among species. Murine, rat, feline and chimpanzee CXCR4 all have an identical pair of aspartic acid residues at the positions corresponding to 171 and 262 in human CXCR4 (Swiss-Prot li-

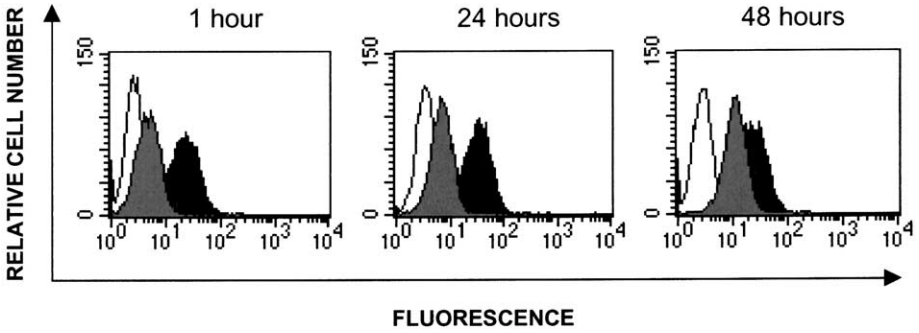


Fig. 5. Long-lasting inhibitory effect of AMD3100 on the binding of the CXCR4-specific mAb 12G5 at the cell surface. SupT1 cells were preincubated with or without AMD3100 at 5 μg/ml and were then transferred to drug-free growth medium. At 0, 1, 6, 24 and 48 h after drug removal, the cells were stained with phycoerythrin-conjugated 12G5 mAb and analyzed by flow cytometry. An isotype control mAb was used to correct for aspecific background staining. At 1 h after drug removal, the mean fluorescence intensities of the stained cell samples were 22 (relative units) for 12G5 mAb staining of the untreated control SupT1 cells (black histogram), 5 for 12G5 mAb staining of the SupT1 cell population that had been pretreated with AMD3100 at 5 μg/ml (gray histogram), and 3 for aspecific background staining (white histogram). The respective mean fluorescence intensity values were 35, 7 and 4 at 24 h and 25, 12 and 3 at 48 h after drug removal. The results from one representative experiment are shown.

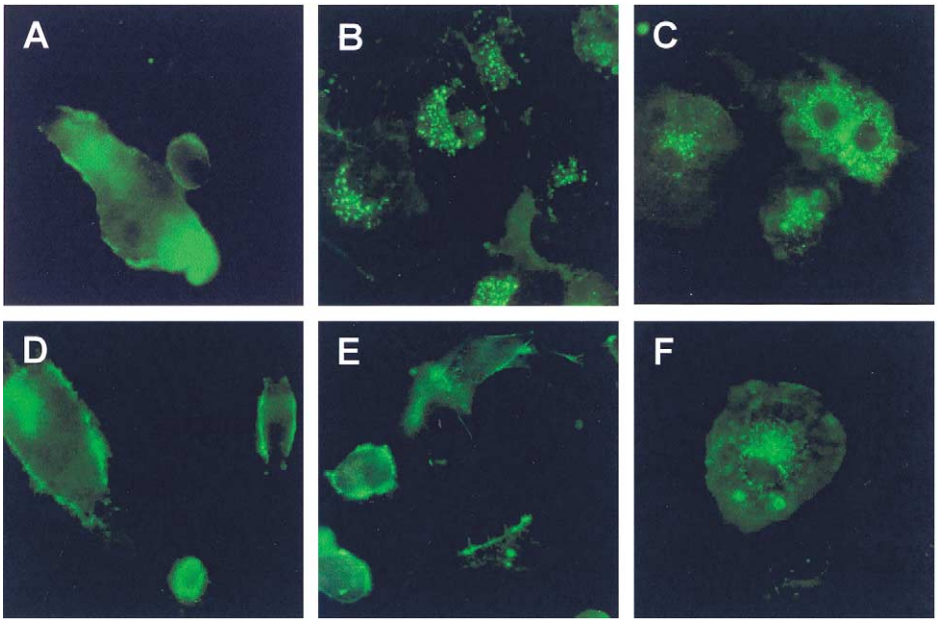


Fig. 6. Selective inhibitory effect of AMD3100 on SDF-1- but not phorbol ester-induced endocytosis of CXCR4. U87.CD4.CXCR4–GFP cells were grown in chamber slides for 24 h and were then preincubated for 15 min in the absence (A–C) or presence (D–F) of 5 μg/ml AMD3100. Then, the appropriate stimulus was added to the different samples, i.e. no stimulus in A and D, 1 μg/ml SDF-1 in B and E, and 100 ng/ml PMA in C and F. After incubation for 45 min at 37°C, the cells were washed, fixed, and the subcellular localization of the fluorescently labeled CXCR4 protein was examined in the different cell samples by fluorescence microscopy. The pictures were obtained from one representative experiment, which was repeated with similar results.

brary accession numbers P70658, O08565, P56498, P56438 and P30991, respectively). This explains why AMD3100 also inhibits SDF-1/CXCR4 interactions in the mouse (see Fig. 2), rat and cat [18,22,27].

3.7. Conclusion

The crucial involvement of chemokine receptors, particularly CXCR4, in several pathologies warrants the intense search for potent and specific receptor antagonists. In this paper we have addressed the receptor specificity of the bicyclam AMD3100. We have shown that AMD3100 potently inhibits CXCR4-mediated events, while showing no interaction at all with any of the other (chemokine) receptors that were evaluated. Moreover, the failure of AMD3100 to induce intracellular calcium mobilization and cell migration, and to trigger CXCR4 receptor internalization points to the lack of any agonistic effect of the compound. This is important with regard to the clinical applicability of the compound, because drug-induced chemokine receptor activation could produce side effects that may preclude its use in patients. The safety of AMD3100 in vivo was attested in a phase I dose escalation study with healthy volunteers [28]. Moreover, the compound clearly showed activity against CXCR4-using HIV-1 in a phase II clinical trial with HIV-infected persons [29]. Besides their therapeutic potential for the treatment of HIV infections, such highly specific, small-molecule CXCR4 antagonists may also be valuable drug candidates for the treatment of other pathological processes driven by the SDF-1/CXCR4 interplay, such as rheumatoid arthritis [10,18], breast cancer metastasis [11], atherosclerosis [30] and asthma [9,31].

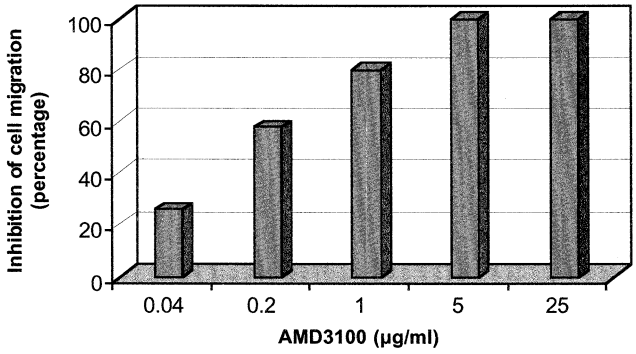


Fig. 7. Dose-dependent inhibition of SDF-1-induced chemotaxis of human PBMCs by AMD3100. Freshly isolated human PBMCs were preincubated with AMD3100 at 0, 0.04, 0.2, 1, 5 or 25 μg/ml and were then loaded into 5-μm filter inserts which were placed in a 24-well plate containing 200 ng/ml SDF-1 in 600 μl buffer. Transmigration of the PBMCs was followed microscopically, and after 2.5 h of incubation, the membrane inserts were removed and the cells in the wells were collected and counted by flow cytometry. The numbers of migrated cells in the AMD3100-exposed cell samples were compared with the number of migrated cells in the untreated control, and the percentages of inhibition were calculated. The data represent the mean of two independent experiments.

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