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Pharmacology of AMD3465: A small molecule antagonist of the chemokine receptor CXCR4

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ABSTRACT

CXCR4 is widely expressed in multiple cell types, and is involved in neonatal development, hematopoiesis, and lymphocyte trafficking and homing. Disruption of the CXCL12/CXCR4 interaction has been implicated in stem cell mobilization. Additionally CXCR4 is a co-receptor for HIV. Selective small molecule antagonists of CXCR4 therefore have therapeutic potential. AMD3465 is an N-pyridinylmethylene monocyclam CXCR4 antagonist which can block infection of T-tropic, CXCR4-using HIV. Using the CCRF-CEM T-cell line which expresses CXCR4 we have demonstrated that AMD3465 is an antagonist of SDF-1 ligand binding (K_i of 41.7 ± 1.2 nM), and inhibits SDF-1 mediated signaling as shown by inhibition of GTP binding, calcium flux, and inhibition of chemotaxis. AMD3465 is selective for CXCR4 and does not inhibit chemokine-stimulated calcium flux in cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5 or CCR7, nor does it inhibit binding of LTB_4 to its receptor, BLT1. The pharmacokinetics of AMD3465 was investigated in mice and dogs. Absorption was rapid following subcutaneous administration. AMD3465 was cleared from dog plasma in a biphasic manner with a terminal half-life of 1.56–4.63 h. Comparison of exposure to the intravenous and subcutaneous doses indicated 100% bioavailability following subcutaneous administration. AMD3465 caused leukocytosis when administered subcutaneously in mice and dogs, with peak mobilization occurring between 0.5 and 1.5 h following subcutaneous dosing in mice and with maximum peak plasma concentration of compound preceding peak mobilization in dogs, indicating that AMD3465 has the potential to mobilize hematopoietic stem cells. These data demonstrate the therapeutic potential for the CXCR4 antagonist AMD3465.

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1. Introduction

Chemokines, chemoattractant cytokines, are integral components of the hematopoietic cascade and the inflammatory response, regulating lymphocyte development, homing and trafficking. They are 8–10 kDa proteins that are subdivided into four families 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). Two other families are CX_3C in which the cysteine residues are separated by three amino acid residues, and C in which one cysteine residue is lacking. To date there is only one member of each of these families, fractalkine, and lymphotactin, respectively [1,2].

In general, within the respective families, each chemokine has more than one receptor, and likewise each receptor generally has more than one ligand. One exception to this is the chemokine receptor CXCR4, which has only one reported ligand CXCL12 (SDF-1) [3]. The CXCR4/CXCL12 receptor/ligand pair has been shown to play a crucial role in neonatal development and hematopoiesis, and lymphocyte trafficking and homing [3–6]. Significantly CXCR4 is important for the homing and retention of hematopoietic stem cells within bone marrow niches, and hence disruption of the CXCL12/CXCR4 interaction has been implicated in stem cell mobilization [7,8]. Also CXCR4 is one of the two main co-receptors used by HIV for viral cell entry with CXCR4 being the principal co-receptor for T-cell line adapted HIV-1 isolates [9–11]. CXCR4 is therefore a validated target for drug development.

The first reported small molecule CXCR4 antagonist was the bicyclam, plerixafor (AMD3100, Mozobil™) [12]. Plerixafor is a

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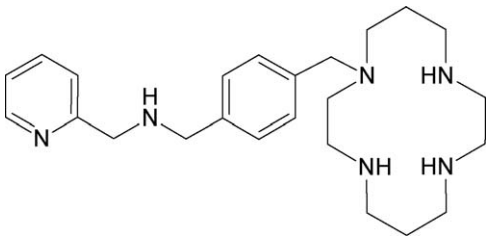

Fig. 1. Chemical structure of the N-pyridinylmethylene monocyclam CXCR4 antagonist AMD3465.

potent and selective inhibitor of CXCR4 and was shown to block HIV infection of T-tropic, X4-using, virus *in vitro* [13–16]. Clinical trials with plerixafor validated CXCR4 as a target for HIV [17] and subsequently an orally bioavailable CXCR4 antagonist, AMD070, entered Phase II clinical trials for treatment of HIV infection [18]. Plerixafor was found to be an effective mobilizer of human hematopoietic stem cells (HSC) [19–23] and has recently been approved in combination with G-CSF for stem cell mobilization for autologous HSC transplantation in non-Hodgkin's lymphoma and multiple myeloma.

CXCR4 is widely expressed on a number of different tissue types, and has been implicated in the pathology of other diseases [3]. CXCR4 is expressed on synovial T cells within the rheumatoid arthritis synovium [24,25] and treatment with plerixafor decreased the inflammatory response in models of collagen-induced arthritis [26]. Similarly CXCR4 inhibition with plerixafor reduced lung eosinophilia and airway hyperresponsiveness in mouse models of allergic airway disease [27]. There are a number of similarities between the biology of tumor progression and inflammatory disease, including increased cytokine and chemokine expression, and cell migration [28]. CXCR4 expression has been demonstrated on hematological cancers, and on a variety of solid tumors of different tissue origins [29,30]. Positive outcomes have been observed using CXCR4 blockade by antibodies in mouse cancer models using human tumor xenografts. Treatment with anti-CXCR4 antibodies has been shown to be curative in a model of non-Hodgkin's lymphoma [31], and to inhibit metastasis in a model of breast cancer [32].

As CXCR4 presents itself as a therapeutic target for a number of diseases, there is abundant potential for novel CXCR4 antagonists. The CXCR4 antagonist plerixafor is a symmetrical molecule consisting of two cyclam rings connected by an aromatic linker. In this paper we examine the pharmacology of AMD3465 [33], a novel, N-pyridinylmethylene monocyclam, CXCR4 antagonist (Fig. 1). Using a cell line that constitutively expresses CXCR4 [16] we have demonstrated that AMD3465 is a specific antagonist of CXCL12/CXCR4-mediated interactions including ligand binding and receptor activation. We also report preliminary pharmacokinetic data for AMD3465 showing that AMD3465 has 100% bioavailability in dogs when administered as a subcutaneous dose, and is eliminated from plasma in a bi-exponential manner. In addition we have shown that AMD3465 causes leukocytosis in mice and dogs, a pharmacodynamic property shown to be associated with CXCR4 blockade by plerixafor, and a surrogate marker for HSC mobilization. These data together indicate that AMD3465 possesses appropriate pharmacological properties for a therapeutic CXCR4 antagonist.

2. Materials and methods

2.1. Compounds and chemokines

AMD3465 was synthesized at AnorMED, Langley, BC. All chemokines were provided by the late Dr. I. Clark-Lewis (University of British Columbia, Vancouver, BC). All chemicals were obtained from Sigma–Aldrich, USA, unless otherwise stated.

2.2. Cell lines

All cell lines were obtained from the ATCC (Manassas, Virginia). CCRF-CEM cells, naturally express CXCR4, CCR4 and CCR7, HEK293F cells were transfected to express CCR1, CCR2b, CXCR3, CCR4, or CCR5. These receptors were cloned from commercial cDNA libraries by PCR using published gene sequences, and inserted into the TOPO-pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Cells expressing CXCR3 and CCR4 were also co-transfected with a chimeric $G\alpha_{qi5}$ protein to improve signaling [16]. CHO-S cells were transfected to express BLT1 (plasmid kindly provided by T. Schwartz, University of Copenhagen, Denmark). Cells were transfected using lipofectamine and stable clones isolated. 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 (Hyclone Inc., Logan, Utah) and 10% fetal bovine serum (Invitrogen Inc., Carlsbad, CA) and 800 μg/mL geneticin. Hygromycin B (Calbiochem, Gibbstown, NJ) was added to cultures expressing the chimeric G-protein.

2.3. Receptor binding assays

For the competition binding studies against CXCR4, a concentration range of AMD3465 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 (PerkinElmer Life Sciences, USA 2200 Ci/mmol) in Millipore Durapore™ filter plates (Millipore, Billerica, MA). 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, resuspended in 50 mm HEPES, 5 mM $MgCl_2$ buffer and flash frozen. The membrane preparation was incubated with AMD3465 for 1 h at room temperature in an assay mixture containing 50 mM Tris, pH7.4, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 4 nM LTB_4 mixed with 1 nM ^{3}H-LTB_4 (195.0 Ci/mmol, PerkinElmer Life Sciences, USA) and 8 μg membrane. The unbound ^{3}H-LTB_4 was separated by filtration on Millipore Type GF-C filter plates (Millipore, Billerica, MA). 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, Invitrogen, Carlsbad, CA). 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 preincubated for 15 min at 37 °C with a concentration range AMD3465. 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, Sunnyvale, CA). Chemokine concentrations used were 2.5 nM SDF-1α (CXCL12) for CXCR4, 10 nM MIP1α (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 AMD3465.

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 AcroWell (Pall Gelman, East Hills, NY) filter plates for 1 h at 30 °C with a concentration range of AMD3465 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 (PerkinElmer Life Sciences, USA), a non-hydrolysable, Europium-labeled analogue of GTP [34]. 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 (PerkinElmer).

2.6. Chemotaxis assay

For the chemotaxis studies, CCRF-CEM cells were loaded with 5 mM calcein-AM (Molecular Probes, Invitrogen, Carlsbad, CA). The dye-loaded cells were washed and resuspended in RPMI 1640 containing 10 mg/mL BSA. Cells were then preincubated for 10 min at 37 °C with indicated AMD3465 concentrations. Chemotaxis assays were performed in Corning Transwell plates (5 μM pore 24-well plate). AMD3465 in RPMI +BSA containing 10 nM SDF-1α was first placed below the porous membrane followed by addition of the cell-AMD3465 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 (PerkinElmer).

2.7. Pharmacokinetic and toxicology studies

The maximum tolerated dose (MTD) of AMD3465 was determined in mice. AMD3465 was given as a single subcutaneous injection to five male Swiss Webster mice per dose group (5, 10, 20, 50 and 100 mg/kg). Mice were observed for 48 h following administration; evaluations were made of mortality and morbidity, clinical observations, body weight and gross pathology. The pharmacokinetics of AMD3465 in male Swiss Webster mice were determined for a single 25 mg/kg subcutaneous dose. Blood was collected by cardiocentisis from three mice per time point at 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, and 24 h post-administration. Plasma was obtained following centrifugation (3000 rpm, 10 min) and concentrations of AMD3465 in plasma were determined by LC–MS.

White blood cell mobilization was determined in DBA/2, BALB/c and C57Bl/6 strains of mice. AMD3465 was given as a single 5 mg/kg subcutaneous injection and blood collected for hematological analysis at 0.5, 1, 1.5, 2, 3, and 4 h post-dosing or from saline-treated control animals at 2 h.

In dogs, AMD3465 was administered as a single subcutaneous injection at 0.41 and 2.05 mg/kg (1 and 5 μmol/kg, respectively) and as an intravenous injection at 0.41 mg/kg (n = 1/dose level). Blood samples were taken by direct venipuncture of a jugular vein for both pharmacokinetic assessment and hematological analysis. Samples were obtained at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 9, 12, 15, and 24 h post subcutaneous administration, and at 0 (pre-dose), 0.083, 0.25, 0.5, 1, 2, 3, 5, 7, 10, and 24 h post intravenous administration. Plasma samples were obtained following centrifugation (3000 rpm, 10 min) and concentrations of AMD3465 in plasma were determined by LC–MS.

Animals were housed and maintained in accordance with the Guide for Care and Use of Laboratory Animals and under AAALAC-I accreditation and all animal protocols used in these studies were approved by the institutional IACUC review committee. White blood cell counts (manual or Vicell, Beckman Coulter) and differentials (manual or Advia120, Bayer) were determined for blood samples collected for hematological analysis. Plasma AMD3465 concentrations were determined by LC–MS. Following addition of a structural analog as an internal standard and pH adjustment, samples were prepared by liquid–liquid extraction with t-butyl methyl ether, followed by back extraction in 0.5% TFA-water. Calibration standards were prepared in an identical manner using blank plasma across a concentration range of 0.01–12.5 μM.

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. Pharmacokinetic analysis was performed using WinNonlin V.4.0.1 (Pharsight Corp., Mountain View, CA).

3. Results

3.1. AMD3465 inhibits SDF-1 binding to cells expressing CXCR4

The CCRF-CEM T lymphoblastoid cell line has been reported to naturally express CXCR4, and to exhibit very low non-specific binding of SDF-1 in ligand binding assays. We have previously shown using a homologous competition binding assay that SDF-1α binds to CCRF-CEM cells with a K_D of 24 ± 3 nM [16]. This cell line was therefore chosen and used throughout to demonstrate the interactions of AMD3465 with the CXCR4 receptor. AMD3465 was shown to inhibit ^{125}I-SDF-1α ligand binding to CCRF-CEM cells in a heterologous competition binding assay. A typical result is shown in Fig. 2a. The data was fitted to a single site binding model and gave a K_i of 41.7 ± 1.2 nM (n = 3).

3.2. AMD3465 inhibits SDF-1α mediated cell signaling

Chemokine receptors are G-protein coupled receptors 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 can be assayed using a non-hydrolysable analogue of GTP, thus trapping the formation of the GTP/G protein. We have used a Europium-labeled analogue of GTP [34]. A typical result is shown in Fig. 2b. AMD3465 inhibited CXCR4 activation as measured by GTP binding with an IC_{50} of 10.38 ± 1.99 nM (n = 5).

Upon the activation of 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. AMD3465 was able to inhibit SDF-1α mediated calcium flux with an IC_{50} of 12.07 ± 2.42 nM (n = 5). A typical result is shown in Fig. 2c.

A key property of all chemokines is that they induce a chemotactic response to a chemokine concentration gradient. AMD3465 was able to inhibit SDF-1α mediated chemotaxis of CCRF-CEM cells with an IC_{50} of 8.7 ± 1.2 nM (n = 3). A typical result is shown in Fig. 2d.

3.3. AMD3465 is a specific inhibitor of the CXCR4 chemokine receptor

In order to demonstrate the specificity of AMD3465 for the CXCR4 over other chemokine receptors 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).

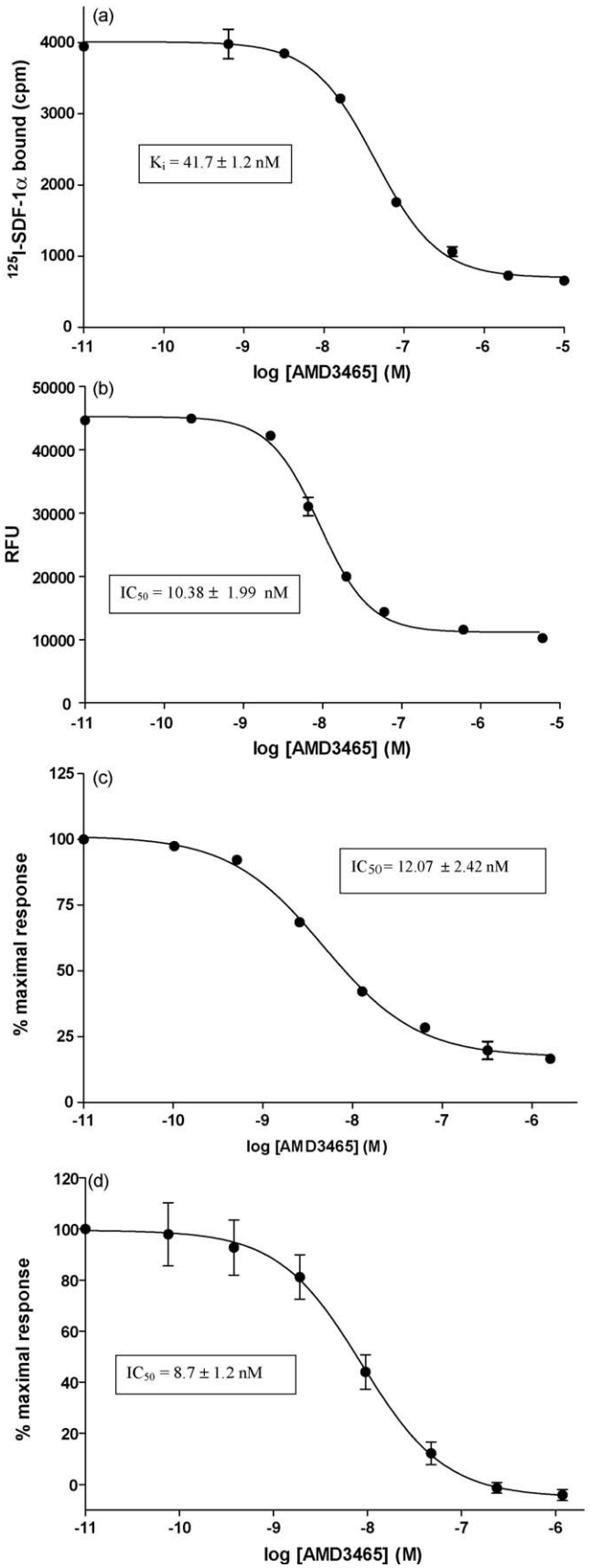

Fig. 2. Inhibition of SDF-1α/CXCR4 interactions by AMD3465. All assays were conducted using CCRF-CEM cells or membrane preparations (see Section 2 for details). The graphs are typical results. Insets show mean values ± SEM from a series of repeat experiments, n = 3–5 (see text for details). (a) Inhibition of SDF-1α ligand binding, (b) inhibition of SDF-1α-stimulated Eu-labeled GTP binding, (c) inhibition of SDF-1α-stimulated calcium flux, and (d) Inhibition of SDF-1α-stimulated chemotaxis.

Table 1
Cross-reactivity of AMD3465 with selected chemokine receptors and BLT1. The potential inhibitory effect of AMD3465 on the chemokine receptors was determined using ligand-mediated calcium flux, and radiolabeled ligand binding for BLT1. Each experiment was repeated twice in duplicate for the calcium flux assays and triplicate for the ligand binding assay. All results were within ±15% of control values at the concentration of AMD3465 shown.

Receptor	Cell line	Chemokine	IC_{50} (nM)
CCR1	HEK293F.CCR1	MIP1-α/CCL3	>5,000
CCR2b	HEK293F.CCR2b	MCP-1/CCL2	>5,000
CCR4	HEK293F.CCR4	TARC/CCL17	>5,000
CCR5	HEK293F.CCR5	RANTES/CCL5	>10,000
CCR7	CCRF-CEM	ELC/CCL19	>5,000
CXCR3	HEK293F.CXCR3	IP-10/CXCL10	>5,000
BLT1	CHO.LTB_4	LTB_4	>50,000

Though not a chemokine receptor LTB_4 is a potent chemoattractant and its receptor is a GPCR. The results in Table 1 show that the IC_{50} of AMD3465 against CCR1, CCR2b, CCR4, CCR5, CCR7, CXCR3 and LTB_4 was at least >5 μM in all cases, i.e. approximately 400-fold less than comparable inhibition of CXCR4.

3.4. Toxicology and pharmacokinetics

In the MTD study all mice (5 males/dose level) appeared normal through 48 h after receiving single subcutaneous injections of AMD3465 at 5, 10, or 20 mg/kg. Following a 50 mg/kg subcutaneous injection, two of five males appeared normal at 1-h post-dose, while the remaining three had ruffled fur and decreased activity. By 48 h all five males appeared normal. For animals receiving 5, 10, 20, and 50 mg/kg doses of AMD3465, there were no adverse effects on body weight, and there were also no visible lesions observed at necropsy. At 1 h following a single subcutaneous injection of AMD3465 at 100 mg/kg, one mouse was dead, one was prostrated, and three had ruffled fur, decreased activity and were cool to the touch. As the signs did not change at 4 h post-dose, the four remaining male mice were euthanized due to their moribund conditions. The abdominal cavities of all animals in the 100 mg/kg group were filled with red fluid; no other findings were noted at necropsy. Based on these observations the MTD of AMD3465 was determined to be 50 mg/kg.

The plasma concentration profiles of AMD3465 in mice and dogs are shown in Fig. 3a and b, respectively. The calculated pharmacokinetic parameters following a single i.v. or s.c. dose are shown in Table 2. As the $AUC_{0-24\ h}$ values were nearly identical following s.c. and i.v. dosing of 0.41 mg/kg AMD3465 to dogs, bioavailability was considered to be complete following s.c. administration. Dose normalized exposures in dogs ($AUC_{0-24\ h}$/D) were similar between the 0.41 and 2.05 mg/kg s.c. doses indicating that, over this dose range, exposure to AMD3465 in dogs was roughly dose proportional. Both plasma clearance and volume of distribution values were higher in mice than in dogs, calculated values for elimination half-life were 3.23 h for mice and 1.56–4.63 h for dogs.

3.5. AMD3465 causes transient leukocytosis

The CXCR4 inhibitor plerixafor was shown to cause rapid and transient leukocytosis in a Phase I clinical trial. Subsequently it was shown that plerixafor was a potent stem cell mobilizer, acting via inhibition of the SDF-1/CXCR4 interactions, which play a fundamental role in homing and trafficking of stem cells to the bone marrow. Similarly AMD3465 mobilized white blood cells in three

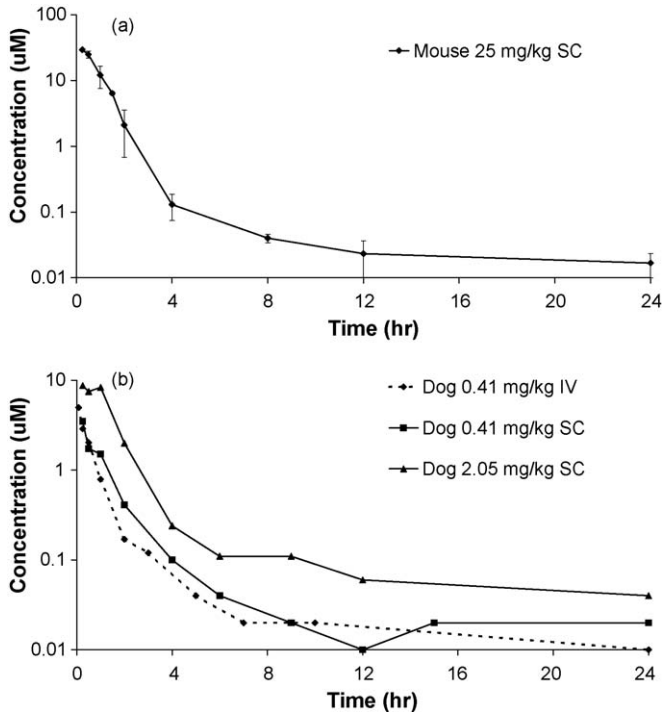

Fig. 3. Pharmacokinetics of AMD3465. (a) Plasma clearance in mice dosed with AMD3465 at 25 mg/kg s.c., $n = 3$ mice per time point. (b) Plasma clearance in dogs dosed with AMD3465 either i.v. or p.o. at 0.41 mg/kg (1 μmol/kg), or with 2.05 mg/kg (5 μmol/kg) s.c., $n = 1$ per dosing regimen. Blood samples were taken at the time points shown and the concentrations of AMD3465 in plasma were determined by LC–MS.

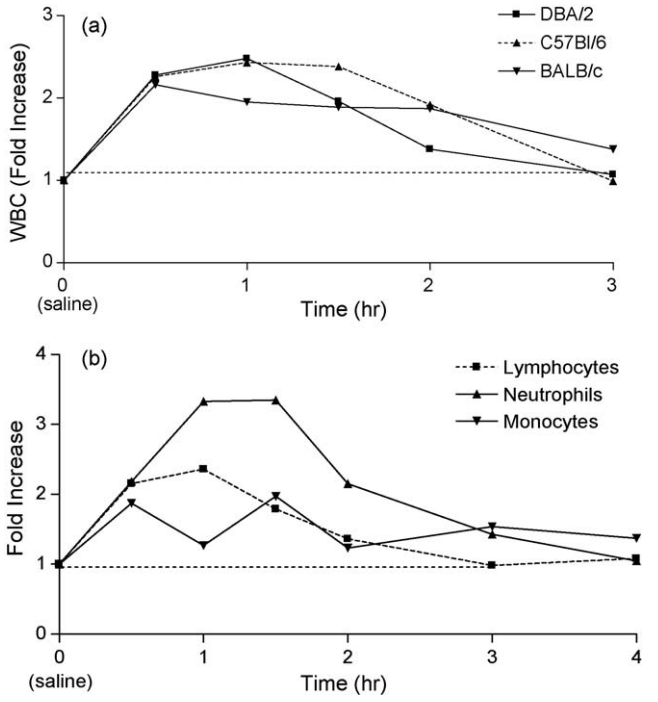

Fig. 4. Elevation of peripheral blood white blood cell counts, neutrophils, lymphocytes, and monocytes by AMD3465 in mice after dosing with 5 mg/kg s.c. Complete blood counts were performed on blood obtained from the indicated strains of mice at 0.5, 1, 1.5, 2 and 3 h following AMD3465 administration as described in Section 2. Fold increases in (a) total white blood cells in the indicated strains of mice (b) neutrophils, lymphocytes and monocytes in DBA/2 mice are shown. Fold increases in cell populations were calculated by dividing the average of AMD3465-treated groups at each time point by the average of the saline-treated group ($n = 3$ mice/group).

different strains of mice and in dogs. In mice, total white blood cells were significantly elevated in DBA/2, C57Bl/6 and BALB/c mice between 0.5 and 2 h following a single subcutaneous dose of 5 mg/kg of AMD3465 with values returning to baseline by 3 h (Fig. 4a). Peak mobilization reached 2.1- to 2.5-fold increases between 0.5 and 1 h, depending on the strain (Fig. 4a). A dose response study was performed at one time point, 45 min with 2.5, 5 and 10 mg/kg. No marked difference in white blood cell mobilization was seen at these doses. The specific cell populations significantly increased by AMD3465 in all three strains of mice included neutrophils, lymphocytes, and monocytes. The mobilization of these populations showed similar kinetics to the total white blood cells and at peak times reached 3.4-, 2.4- and 2.0-fold increases, respectively, in DBA/2 mice between 0.5 and 1.5 h post-dosing (Fig. 4b). Similar results were obtained for C57Bl/6 (2.2-fold increases for all populations) and BALB/c mice (3.2-, 1.4- and 2.7-fold increases, respectively). In dogs, there was a maximum increase of total white blood cells approximately 1.7-fold over baseline at 4 h following a single subcutaneous dose of 2.05 mg/kg. Increases in total white blood cells, lymphocytes, neutrophils, and monocytes were observed at all three dose levels with peaks occurring 2–4 h post-dose and values returning to baseline by the 24-h time point (Fig. 5).

4. Discussion

Selective small molecule inhibitors of CXCR4 have great therapeutic potential. In this paper we examine the pharmacology of the N-pyridinylmethylene monocyclam CXCR4 antagonist, AMD3465. We present *in vitro* data demonstrating the selective inhibitory effect of AMD3465 towards CXCR4, and present preliminary *in vivo* pharmacokinetic and pharmacodynamic data.

We have used the CCRF-CEM cell line, which naturally expresses CXCR4, to investigate the receptor pharmacology of AMD3465. We have previously shown that these cells express CXCR4, bind SDF-1 with a K_D of 24 nM, and upon stimulation with SDF-1α activate receptor-mediated intracellular signaling resulting in GTP binding to intracellular G-proteins, intracellular calcium flux, and chemotaxis [16]. Using this cell line we have shown that AMD3465 was able to inhibit SDF-1α ligand binding with a K_i of 41.7 ± 1.2 nM. Inhibition of CXCR4 signaling pathways was demonstrated by inhibition of SDF-1α stimulated calcium flux and GTP binding with IC_{50} values of 12.07 and 10.38 nM, respectively. Furthermore AMD3465 was able to inhibit a CXCR4-mediated physiological response, cell chemotaxis, with an IC_{50} value of

Table 2
Pharmacokinetic parameters of AMD3465 following single administration to mice and dogs.

Species	Route	Dose	C_{max} (μM)	T_{max} (min)	$AUC_{0-24\ h}$ (h-μM)	$AUC_{0-24\ h}$ (h-μM)/D	Cl/F (L/(h kg))	Vz/F (L/kg)	$t^{1/2}$ (h)
Mouse	SC	25 mg/kg	29.34	15	29.35	1.17	0.85	3.95	3.23
Dog	IV	0.41 mg/kg	4.96	5	3.56	8.68	0.11	0.26	1.56
	SC	0.41 mg/kg	3.50	15	3.87	9.44	0.10	0.33	2.21
	SC	2.05 mg/kg	8.72	15	15.65	7.63	0.13	0.86	4.63

Abbreviations. SC: subcutaneous, IV: intravenous, C_{max}: peak plasma concentration, T_{max}: time of C_{max}, AUC: area under the curve, AUC/D: area under the curve normalized to dose D, Cl: clearance, Vz: volume of distribution, $t^{1/2}$: elimination half-life. For Vz and Cl following SC administration, bioavailability (F) was taken to be 1.0.

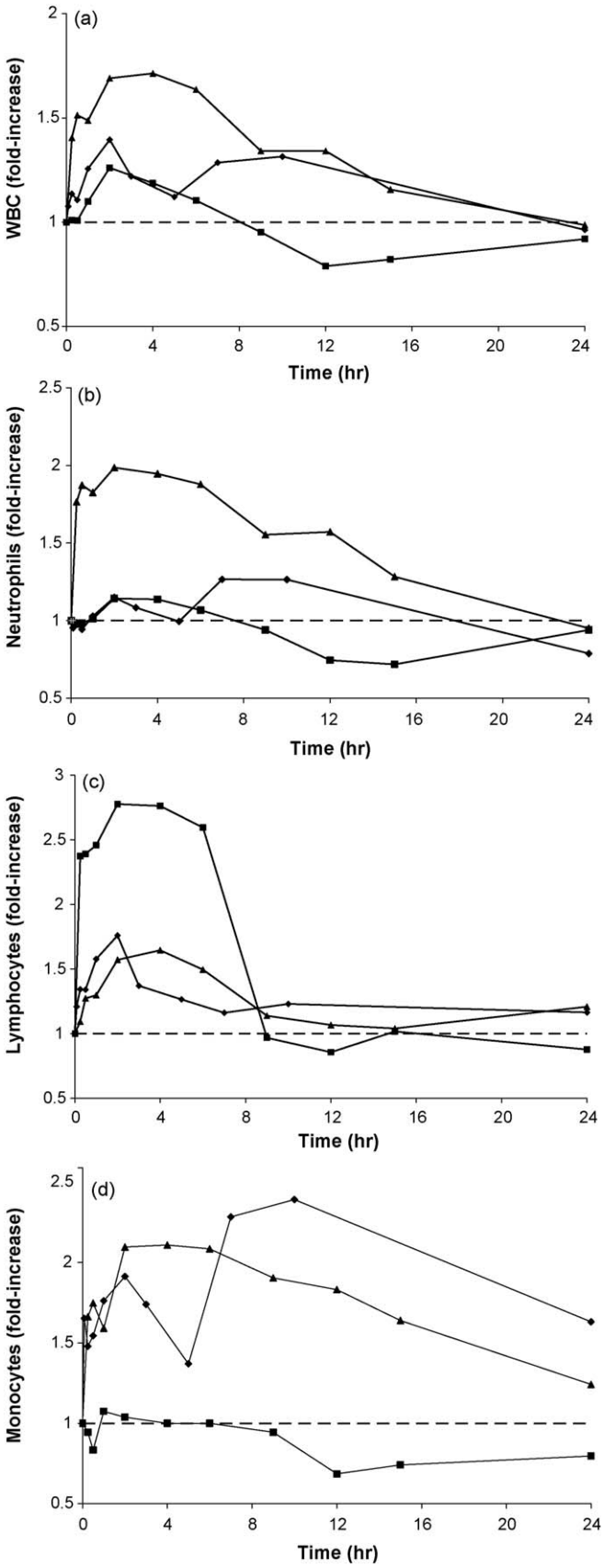

Fig. 5. Observed leukocytosis in dogs after dosing with AMD3465 at either 0.41 mg/kg (1 μmol/kg) i.v. (◆), 0.41 mg/kg (1 μmol/kg) s.c. (■), or 2.05 mg/kg (5 μmol/kg) s.c. (▲), $n = 1$ per dosing regimen. The four graphs show the fold increase in elevation of (a) peripheral blood white blood cell, (b) neutrophils, (c) lymphocytes and (d) monocytes. Fold increases were calculated with respect to baseline values before dosing.

8.7 nM. In these respects AMD3465 appears to be a more potent antagonist of CXCR4 than the parent molecule plerixafor which gave IC_{50} values 651 ± 37 nM (ligand binding), 27 ± 2.2 nM (GTP binding), 572 ± 190 nM (calcium flux), 51 ± 17 nM (chemotaxis) in the same assays using the CCRF-CEM cell line [16]. In addition, AMD3465 had 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 LTB_4 binding to BLT1, an alternative GPCR that mediates chemotaxis, thus indicating that AMD3465 is a selective inhibitor of CXCR4 over other chemokine receptors. In a separate study AMD3465 was shown to inhibit SDF-1-mediated CXCR4 internalization in cells expressing GFP-linked CXCR4, and did not cause receptor internalization when incubated with the cells alone [33].

A maximum tolerated dose (MTD) study was conducted with AMD3465 in mice prior to the commencement of *in vivo* pharmacokinetic and pharmacodynamic studies. The MTD in mice after subcutaneous injection was determined to be 50 mg/kg therefore a dose of 25 mg/kg was selected to determine the pharmacokinetics of AMD3465 in mice. Following a 25 mg/kg single subcutaneous injection of AMD3465, absorption was rapid with a T_{max} of 15 min and a C_{max} of 29.3 μM. It was rapidly cleared from the plasma in a biphasic manner with a terminal half-life of 3.23 h.

The pharmacokinetics of AMD3465 was also investigated in an initial study in dogs, using a single animal per dose group, with the aim of providing preliminary data for later toxicokinetic studies. AMD3465 was rapidly absorbed following single intravenous (0.41 mg/kg) and subcutaneous (0.41 and 2.05 mg/kg) doses. As observed in mice, AMD3465 was rapidly absorbed, with a T_{max} of 15 min, the first sampling time point. AMD3465 was cleared from dog plasma in a biphasic manner with a terminal half-life of 1.56–4.63 h. Comparison of exposure to an intravenous dose (0.41 mg/kg) indicated that bioavailability was essentially complete (100%) following subcutaneous administration to dogs. Comparison of exposure to the two subcutaneous doses indicated that exposure was dose proportional in dogs over the range evaluated (0.41–2.05 mg/kg). The pharmacokinetics of AMD3465 and plerixafor are similar in the mouse and dog with respect to their rapid and complete absorption following subcutaneous administration. AMD3465 may therefore be suitable for further development using a subcutaneous route of administration. AMD3465 exhibits a somewhat greater volume of distribution than plerixafor that may reflect its greater lipophilicity. The relevance of this difference with respect to its *in vivo* activity in unknown.

Leukocytosis was observed in a Phase I clinical trial in healthy volunteers with the bicyclam CXCR4 antagonist, plerixafor. It was hypothesized that this was a result of cell mobilization and subsequently, plerixafor was shown to be a potent mobilizer of hematopoietic stem cells in multiple animal species including mice, dog and monkey [21,35,36], and in clinical trials in both healthy volunteers, and patients with non-Hodgkin's lymphoma, and multiple myeloma [19–23]. As leukocytosis appears to correlate with stem cell mobilization after CXCR4 blockade, hematological changes were monitored in mice and dogs in the current study. Increases in total white blood cells, lymphocytes, neutrophils and monocytes were observed following subcutaneous dosing in three different strains of mice with peaks between 0.5 and 1.5 h and a return to baseline by 3 h. In dogs, increases in total white blood cells, lymphocytes, neutrophils, basophils and monocytes were observed after both i.v. and s.c. dosing, with peaks occurring 2–4 h post-dose and values returning to baseline by 24 h post-dosing. In dogs, a dose dependent elevation in total white blood cell count was observed after s.c. dosing (0.41–2.05 mg/kg), with a 1.7-fold increase over baseline observed at 4 h following the high dose (2.05 mg/kg). These data suggest that AMD3465 also has

the potential to mobilize hematopoietic stem cells. The observed lag between maximum plasma AMD3465 concentrations and peak increases in white blood cells in the dog are consistent with the observed kinetics of mobilization of white blood cells and CD34+ stem cells following plerixafor administration in both dogs and humans [36–38]. A pharmacokinetic–pharmacodynamic model for plerixafor CD34+ stem cell mobilization was produced based on data from healthy volunteers, which described the relationship between plerixafor pharmacokinetics and the mobilization response [38]. The exposure response relationship was best described by an indirect effect model that stimulates the entry process of CD34+ stem cells from the bone marrow to the peripheral blood in the form of a sigmoid maximum effect model. The observed similarity seen in the pharmacokinetic–pharmacodynamic relationship for WBC mobilization in dogs by AMD3465 and plerixafor is consistent with their common mechanism of action.

Plerixafor is symmetrical with two monocyclam (1,4,8,11-tetraazacyclotetradecane) rings connected by an aromatic linker, whereas AMD3465 is asymmetrical with one monocyclam replaced by an aminomethylpyridine moiety. The retention of potent CXCR4 inhibition after removal of one cyclam ring demonstrates that CXCR4 inhibition can be achieved with a molecule containing a single cyclam ring. CXCR4 mutagenesis and molecular modeling studies have shown that plerixafor binding to the CXCR4 receptor is dependent upon three positively charged amino acids in transmembrane regions TMIV and TMVI, D171, D262 and E283 [39,40]. Similar studies have shown that these interactions are important for AMD3465 binding. However, a significant additional interaction with H281 was identified for AMD3465 [41,42]. Together these data indicate that not only can a moncyclam compound bind to CXCR4, but may also interact with the receptor via an alternative binding mode to that of plerixafor, possibly explaining the enhanced interaction of AMD3465 with the CXCR4 receptor compared with plerixafor.

It is noteworthy that AMD3465, with its enhanced potency against the CXCR4 receptor compared with plerixafor, has been shown to be a potent HIV entry inhibitor [33], and to abrogate the Th2-cell-mediated inflammatory response in a schistosomal antigen-elicited model of pulmonary granuloma formation [43]. Together these data provide further support for the therapeutic potential for CXCR4 antagonists.

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