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AMD3465, a monomacrocyclic CXCR4 antagonist and potent HIV entry inhibitor

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Abstract

The chemokine receptors CCR5 and CXCR4 function as coreceptors for human immunodeficiency virus (HIV) and are attractive targets for the development of anti-HIV drugs. The most potent CXCR4 antagonists described until today are the bicyclams. The prototype compound, AMD3100, exhibits potent and selective anti-HIV activity against CXCR4-using (X4) viruses and showed antiviral efficacy in X4 HIV-1-infected persons in a phase II clinical trial. However, AMD3100 lacks oral bioavailability due to its high overall positive charge. Initial structure-activity relationship studies with bicyclam analogues suggested that the bis-macrocyclic structure was a prerequisite for anti-HIV activity. Now, we report that the *N*-pyridinylmethylene cyclam AMD3465, which lacks the structural constraints mentioned above, fully conserves all the biological properties of AMD3100. Like AMD3100, AMD3465 blocked the cell surface binding of both CXCL12 (the natural CXCR4 ligand), and the specific anti-CXCR4 monoclonal antibody 12G5. AMD3465 dose-dependently inhibited intracellular calcium signaling, chemotaxis, CXCR4 endocytosis and mitogen-activated protein kinase phosphorylation induced by CXCL12. Compared to the bicyclam AMD3100, AMD3465 was even 10-fold more effective as a CXCR4 antagonist, while showing no interaction whatsoever with CCR5. As expected, AMD3465 proved highly potent against X4 HIV strains (IC_{50}: 1–10 nM), but completely failed to inhibit the replication of CCR5-using (R5) viruses. In conclusion, AMD3465 is a novel, monomacrocyclic anti-HIV agent that specifically blocks the interaction of HIV gp120 with CXCR4. Although oral bioavailability is not yet achieved, the monocyclams, with their decreased molecular charge as compared to the bicyclams, embody an important step forward in the design of oral CXCR4 antagonists that can be clinically used as anti-HIV drugs.

Keywords: HIV entry; CXCR4 antagonist; Chemokine binding; Calcium signaling; MAP kinase; Chemotaxis

1. Introduction

Because of the increasing problems of drug resistance associated with the use of currently approved reverse transcriptase and protease inhibitors, there is an urgent need for new anti-HIV[1] drugs that specifically target alternative events in the HIV replicative cycle. The discovery of chemokine receptors as cofactors involved in the entry of HIV in the host cells has highlighted the early steps of virus replication as an attractive intervention site for antiviral therapy. The chemokine receptor CCR5 is used by M-tropic (macrophage-tropic) viruses – now termed R5

Abbreviations: HIV, human immunodeficiency virus; CCR5, CC-chemokine receptor 5; CXCR4, CXC-chemokine receptor 4; AIDS, acquired immune deficiency syndrome; IC_{50}, 50% inhibitory concentration; CXCL12, CXC-chemokine ligand 12 (formerly 'stromal cell-derived factor-1'); $CXCL12^{AF647}$, alexa fluor 647-conjugated CXCL12; PE, phycoerythrin; (m)Ab, (monoclonal) antibody; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; PBMC, peripheral blood mononuclear cell; IL-2, interleukin-2; PHA, phytohemagglutinin; MFI, mean fluorescence intensity; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; ELISA, enzyme-linked immuno-sorbent assay; CC_{50}, 50% cytotoxic concentration

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viruses – which are typically associated with the early asymptomatic phase of infection and replication in HIV-infected patients [1,2]. At later stages, more pathogenic T cell-tropic viruses become predominant, which use CXCR4 as their chemokine coreceptor and are therefore termed X4 viruses [3,4]. The transition from R5 to X4, or mixed R5/X4, virus during the course of HIV infection accelerates $CD4^+$ T cell decline and disease progression towards immune deficiency [5]. Thus, treatment of HIV-infected persons with CXCR4 antagonists might delay the onset of AIDS in some individuals by preventing the CCR5 to CXCR4 coreceptor switch.

The first class of specific CXCR4 inhibitors that were identified are the bicyclams [6], which consist of two monocyclam (1, 4, 8, 11-tetraazacyclotetradecane) units connected by an aliphatic or aromatic linker. The prototype of this series of bis-macrocycles, AMD3100 (Fig. 1), is a highly potent CXCR4 antagonist that selectively inhibits X4, but not R5, HIV strains and clinical isolates with a 50% inhibitory concentration of 1–10 nM and a selectivity index of at least 100,000 [7,8]. Moreover, a phase II clinical study demonstrated the in vivo efficacy of AMD3100 against X4 virus variants [9]. Likewise, the CCR5 antagonist SCH-C effectively suppressed R5 virus replication in vivo in a separate phase II clinical trial [10]. Thus, there is a proof-of-principle for chemokine receptor antagonists as effective anti-HIV drugs, and both CCR5 and CXCR4 are now validated as valuable targets for the treatment of HIV infections.

However, AMD3100 lacks oral bioavailability and has to be administered by intraveneous infusion, which imposes severe limitations to its clinical applicability for long-term anti-HIV therapy. In order to improve oral absorption, aza-macrocyclic analogs must be re-designed to reduce the number of basic amine groups and the overall charge at physiological pH. Here, we report on AMD3465, a monomacrocyclic *N*-pyridinylmethylene cyclam structure (Fig. 1), with comparable or even slightly improved anti-HIV activity and CXCR4 antagonism as compared to AMD3100. Although still lacking oral bioavailability, AMD3465 provides evidence that monocyclams can also be effective against HIV-1 and HIV-2.

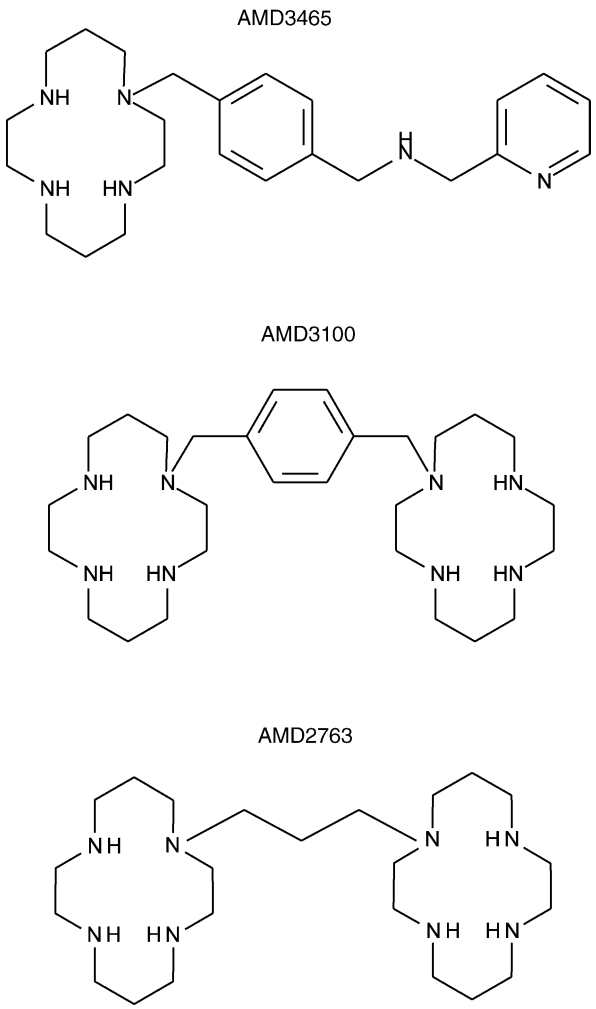

Fig. 1. Chemical structures of the monocyclam AMD3465 {*N*-[1,4,8,11-tetraazacyclotetradecanyl-1,4-phenylenebis(methylene)]-2-(aminomethyl)-pyridine}, and the bicyclams AMD3100 {1,1′-[1,4-phenylenebis(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane} and AMD2763 {1,1′-propylene-bis-1,4,8,11-tetraazacyclo-tetradecane}.

2. Materials and methods

2.1. Compounds, chemokines and antibodies

The bicyclams AMD3100 and AMD2763 [6] and the monocyclam AMD3465 (Fig. 1) were chemically synthesized at AnorMED, Langley, Canada. Zidovudine and dextran sulphate MW 5000 (DS-5000) were puchased from Sigma–Aldrich (St. Louis, MO). $CXCL12^{AF647}$ (i.e., human CXCL12 carrying an Alexa Fluor 647® moiety at its second last amino acid position) [11] was obtained from RMF Dictagene (Lausanne, Switzerland). Unlabeled CXCL12 was synthesized by Dr. I. Clark-Lewis (University of British Columbia, Vancouver, BC, Canada). IL-2 and PHA were respectively purchased from R&D Systems Europe, Abingdon, Oxon, UK and Sigma–Aldrich, St. Louis, MO. The antibodies used in this study were PE-conjugated anti-human CXCR4 mAb clone 12G5 (BD Pharmingen, San Diego, CA); mouse anti-human phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) mAb clone E10 and rabbit anti-p44/42 MAPK Ab (Cell Signaling Technology, Beverly, MA); PE-conjugated goat-anti-mouse and goat-anti-rabbit IgG Ab (Caltag Laboratories, San Francisco, CA).

2.2. Cell cultures and viruses

Human T-lymphoid SupT1 and MT-4 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI-1640 medium

(Invitrogen, Paisley, UK) supplemented with 10% FBS (BioWhittaker Europe, Verviers, Belgium) and 2 mM glutamine (Invitrogen, Paisley, UK). All cell cultures were maintained at 37 °C in a humidified, CO_2-controlled atmosphere and subcultivations were done every 2–3 days. Human astroglioma U87 cells expressing human CD4 (U87.CD4) were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, NY) and were cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% FBS (BioWhittaker Europe), 0.01 M HEPES buffer (Invitrogen) and 0.2 mg/ml geneticin (G-418 sulphate) (Invitrogen). Subcultivations were done every 2–3 days by digestion of the monolayers with trypsin (Invitrogen). U87.CD4.CXCR4-GFP cells were constructed in our laboratory by transfection of U87.CD4 cells with GFP-coupled CXCR4 using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) with puromycin as a selection marker. The stably transfected cells were maintained in the presence of 1 μg/ml puromycin (Sigma–Aldrich, St. Louis, MO). Buffy coat preparations from healthy donors were obtained from the Leuven Blood Bank. PBMCs were isolated by density gradient centrifugation over Lymphoprep™ (d = 1.077 g/ml) (Nycomed, Oslo, Norway) and were stimulated with 10 ng/ml IL-2 and 2 μg/ml PHA during 3–4 days prior to HIV infection.

The III_B, RF and NL4.3 strains of HIV-1 and the ROD and EHO strains of HIV-2 were obtained from the National Institute of Allergy and Infectious Disease AIDS Reagent Program (Bethesda, MD). HIV-1 NL4.3 resistant to the prototype bicyclam AMD3100 has previously been established and was characterized in our laboratory [12,13]. The R5 HIV-1 strain BaL was obtained through the MRC (Centralised Facility for AIDS Reagents, Hertfordshire, UK). The dual-tropic (R5/X4) HIV-1 HE strain was initially isolated from a patient at the University Hospital in Leuven, and had been routinely cultured in MT-4 cells [14].

2.3. Anti-CXCR4 mAb binding assay and flow cytometry

Human T-lymphoid SupT1 cells (0.5×10^6 cells/sample) were washed once with PBS containing 2% FBS and preincubated with AMD3465 or AMD3100 at different concentrations for 15 min at room temperature. After centrifugation and washing, the cells were incubated for 30 min on ice with PE-conjugated anti-CXCR4 mAb clone 12G5 in PBS containing 2% FBS. Thereafter, the cells were 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). As a negative control for aspecific background staining, the cells were stained in parallel with Simultest Isotype Control mAb (Becton Dickinson).

2.4. $CXCL12^{AF647}$ binding assay

Chemokine binding was measured by a novel fluorescent assay as described recently [11]. Briefly, human T-lymphoid SupT1 cells were washed once with assay buffer [Hanks' balanced salt solution with 20 mM HEPES buffer and 0.2% BSA, pH 7.4] and were then incubated for 30 min at room temperature with 25 ng/ml $CXCL12^{AF647}$ in the presence or absence of AMD3465 or AMD3100 at different concentrations in assay buffer. Thereafter, the cells were washed twice in assay buffer, fixed in 1% paraformaldehyde in PBS and analyzed on the FL4 channel of a FACSCalibur™ flow cytometer equipped with a 635 nm red diode laser (Becton Dickinson). The percentages of inhibition of $CXCL12^{AF647}$ binding were calculated according to the formula $[1 - (MFI - MFI_{NC})/(MFI_{PC} - MFI_{NC})] \times 100$, where MFI is the mean fluorescence intensity of the cells incubated with $CXCL12^{AF647}$ in the presence of the inhibitor, MFI_{NC} is the mean fluorescence intensity measured in the negative control (i.e., autofluorescence of unlabeled cells) and MFI_{PC} is the mean fluorescence intensity of the positive control (i.e., cells exposed to $CXCL12^{AF647}$ alone).

2.5. Measurement of intracellular calcium mobilization

Human T-lymphoid SupT1 cells were loaded with the fluorescent calcium indicator Fluo-3 acetoxymethyl (Molecular Probes, Leiden, The Netherlands) at 4 μM in RPMI-based growth medium for 45 min at room temperature. After thorough washing with assay buffer (see above), the cells were preincubated for 15 min at 37 °C in the same buffer with AMD3465 or AMD3100 at different concentrations. Then, the intracellular calcium mobilization in response to CXCL12 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).

2.6. Flow cytometric measurement of chemokine-induced p44/42 MAPK phosphorylation

This flow cytometric MAPK phosphorylation assay has been previously described by Chow et al. [15]. Briefly, SupT1 cells were serum-starved during 24 h prior to the experiment. The cells were then washed once in assay buffer (see above), preincubated for 10 min at 37 °C with or without 6250 nM AMD3465 or AMD3100 and subsequently incubated at 37 °C in the presence of 200 ng/ml CXCL12. At different time points (i.e., 0 and 30 s, and 2, 5, 15 and 45 min), cell samples ($\sim 3 \times 10^6$ cells each) were fixed by the addition of paraformaldehyde to a final concentration of 2% and incubation at 37 °C for 10 min. Then the fixative was removed by centrifugation and the cells were washed once with PBS and permeabilized with

ice-cold 90% methanol for 30 min on ice. After washing with PBS containing 2% FBS, cells from each sample were incubated for 30 min at room temperature in parallel with anti-p44/42 MAP kinase antibody (diluted 1/50 in PBS containing 2% FBS) and with phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) mAb (diluted 1/500 in PBS containing 2% FBS). Next the cells were washed once with PBS containing 2% FBS and incubated for 30 min at room temperature with the appropriate PE-conjugated secondary antibody (respectively goat-anti-rabbit IgG Ab and goat-anti-mouse IgG Ab), diluted 1/200 in PBS containing 2% FBS. Thereafter, the cells were washed twice with PBS, resuspended in PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

2.7. Chemotaxis assay

Chemokine-induced cell migration was assessed using 5-μm pore transwell filter membranes (Costar, Boston, MA). The membrane inserts were placed in the wells of a 24-well plate, containing 600 μl assay buffer (see above) with either no chemokine (negative control) or CXCL12 at 100 ng/ml. Human T-lymphoid SupT1 cells (1×10^6 cells in 100 μl buffer) that had been preincubated with AMD3465 or AMD3100 at different concentrations were loaded into each Transwell filter. The plates were 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 by a 2-min acquisition on the flow cytometer (FACScalibur, Becton Dickinson).

2.8. Receptor internalization assay

U87.CD4 cells stably transfected with 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) at 4×10^4 cells per well. The next day, the cells were preincubated in cell culture medium with or without 6250 nM AMD3465 for 15 min at room temperature. Then, CXCL12 was added at a final concentration of 500 ng/ml. 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 (Nikon, Tokyo, Japan).

2.9. Anti-HIV activity assay

The antiviral activity of the drugs was determined using a tetrazolium-based colorimetric assay in MT-4 cells [16]. Briefly, five-fold dilutions of the test compounds in 100 μl of medium were added to duplicate wells of 96-well flat bottom plates (Iwaki, Japan). Then 6×10^4 MT-4 cells were added in 50 μl of medium, and finally 50 μl of diluted HIV-1 stocks (strains III_B, RF, NL4.3, $NL4.3^{AMD3100}$, HE, ROD and EHO) was added to each well. Cytopathic effect induced by the virus was monitored by daily microscopic evaluation of the virus-infected cell cultures. At day 4–5 after infection, when strong cytopathic effect was observed in the positive control (i.e., untreated HIV-infected cells), the cell viability was assessed via the in situ reduction of the tetrazolium compound MTS, using the CellTiter 96® AQ_{ueous} one solution cell proliferation assay (Promega, Madison, WI). The absorbance was then measured spectrophotometrically at 490 nm with a 96-well plate reader and compared with four cell control replicates (cells without virus and drugs) and four virus control wells (virus-infected cells without drugs). The IC_{50}, i.e., the drug concentration that inhibits HIV-induced cell death by 50%, was calculated for each compound from the dose-response curve.

For HIV-1 BaL, five-fold dilutions of the compound (in 250 μl of medium) were added to each well of 48-well flat bottom plates (Iwaki, Japan). Then PBMCs were seeded in the tissue culture plates (5×10^5 cells in 200 μl of medium) together with IL-2 (1 ng/ml) (R&D Systems Europe, Abingdon, Oxon, UK) and 50 μl of the diluted virus stock, yielding a final concentration of 1000 pg/ml. After 3 days, 100 μl of fresh medium with IL-2 was added. The supernatant of each sample was collected after 8–10 days of incubation, stored at −20 °C, and analysed for HIV-1 core antigen by p24 antigen ELISA (Perkin-Elmer, Boston, MA).

The CC_{50} or 50% cytotoxic concentration of each of the compounds was determined from the reduction of viability of uninfected MT-4 cells exposed to the compounds, as measured by the MTS method described above.

3. Results

3.1. Inhibition of anti-CXCR4 mAb 12G5 binding in SupT1 cells by AMD3465

We investigated the ability of AMD3465 to block the binding at the cell surface of the CXCR4-specific mAb 12G5, which recognizes a conformational epitope located in the second extracellular loop of the 7-transmembrane receptor protein [17]. After preincubation with AMD3465 at different concentrations, human T-lymphoid SupT1 cells were stained with the PE-conjugated mAb 12G5 and analysed by flow cytometry. The MFI values, reflecting the amount of fluorescent antibody bound to the cells, were 30 (relative units) for untreated SupT1 cells versus 19, 11, 5.0, 4.1, 3.8 and 2.9 for SupT1 cells pretreated with AMD3465 at 0.4, 2, 10, 50, 250 and 1250 nM, respectively (Fig. 2). For comparison, the respective values for SupT1 cells pretreated with AMD3100 were 27, 27, 24, 14, 7.9

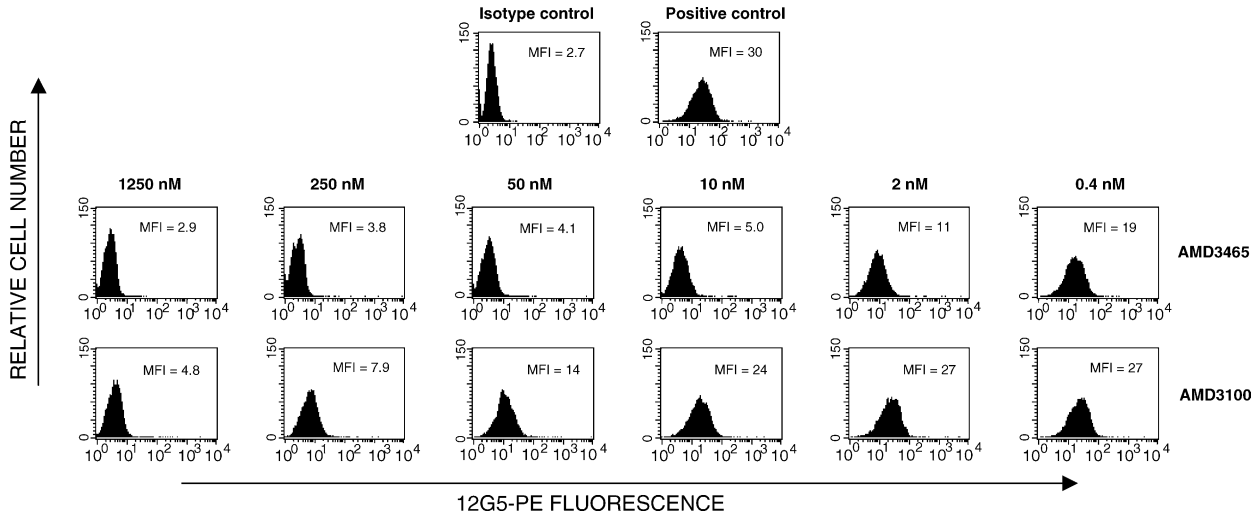

Fig. 2. Concentration-dependent inhibitory effect of AMD3465 and AMD3100 on binding of 12G5 mAb to CXCR4 in SupT1 cells. The cells were preincubated with the compounds at the indicated concentrations and then stained with PE-conjugated 12G5 mAb. The mean fluorescence intensity (MFI) values are indicated in each histogram. The aspecific background fluorescence of the cell population was measured by an isotype control mAb. Cell debris was excluded from the analysis by dot plot gating. These are the data from one representative experiment, which was repeated several times with similar results.

and 4.8. As a control for aspecific antibody binding, the cells were stained with an isotype control mAb, yielding an MFI of 2.7. Thus, the compound concentration yielding 50% inhibition of 12G5 mAb binding was 0.75 nM for AMD3465 versus 37.5 nM for AMD3100 (Fig. 2).

3.2. AMD3465 dose-dependently inhibits CXCL12 binding in SupT1 cells

Chemokine receptor antagonists impede the binding of the natural chemokine ligand(s) to the receptor. To examine whether AMD3465 blocks the binding of the only known CXCR4 ligand, CXCL12, SupT1 cells were exposed to an alexa fluor 647-conjugate of CXCL12 ($CXCL12^{AF647}$) [11] in the presence of increasing concentrations of AMD3465. Subsequent flow cytometric analysis of the cells revealed a gradual decrease of fluorescent CXCL12 binding with increasing compound concentrations (Fig. 3). At 250 nM, AMD3465 inhibited chemokine binding by >90%. Also, AMD3465 proved slightly more potent than AMD3100: the IC_{50} values for inhibition of $CXCL12^{AF647}$ binding were 18 nM for AMD3465 versus 33 nM for AMD3100 (Fig. 3).

3.3. Antagonization of CXCL12-induced intracellular signaling by AMD3465

A transient increase in the cytosolic calcium concentration is a key component of the intracellular signaling cascade that is activated upon binding of a chemokine to its receptor. The intracellular calcium flux can be monitored by measuring the fluorescence of cells loaded with a fluorescent calcium indicator and subsequently stimulated with the chemokine. Fig. 4 shows the calcium fluxes induced by CXCL12 in human T-lymphoid SupT1 cells that had been preincubated with varying concentrations of AMD3465 or AMD3100. The calcium response was completely abrogated in SupT1 cells exposed to either compound at 250 nM. At 50 nM, AMD3465 still totally blocked CXCL12-induced calcium mobilization, whereas AMD3100 could only afford partial inhibition of the signaling response (Fig. 4). The IC_{50} for inhibition of CXCL12-induced calcium signaling in SupT1 cells was 17 nM for AMD3465 and 61 nM for AMD3100. In human astroglioma U87.CD4.CXCR4 cells, AMD3465 was even >10-fold more potent than AMD3100 in inhibiting calcium mobilization, the respective IC_{50} values being 4 and 58 nM. Like AMD3100, AMD3465 completely failed to

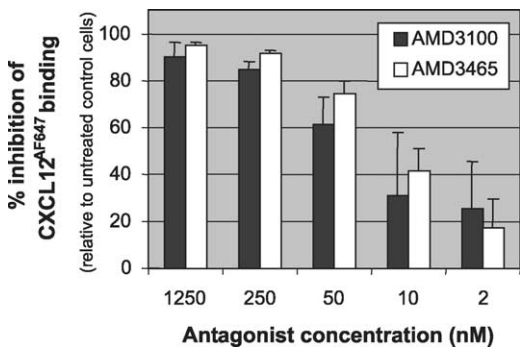

Fig. 3. Inhibitory effect of AMD3465 and AMD3100 on the binding of $CXCL12^{AF647}$ in human T-lymphoid SupT1 cells. The cells were incubated with 25 ng/ml $CXCL12^{AF647}$ in the presence of increasing concentrations of either compound. The bars represent the percentages of inhibition of $CXCL12^{AF647}$ binding in the presence of the inhibitor, relative to the untreated positive control where the cells were exposed to 25 ng/ml $CXCL12^{AF647}$ alone. Each value represents the mean ± standard deviation of three independent experiments.

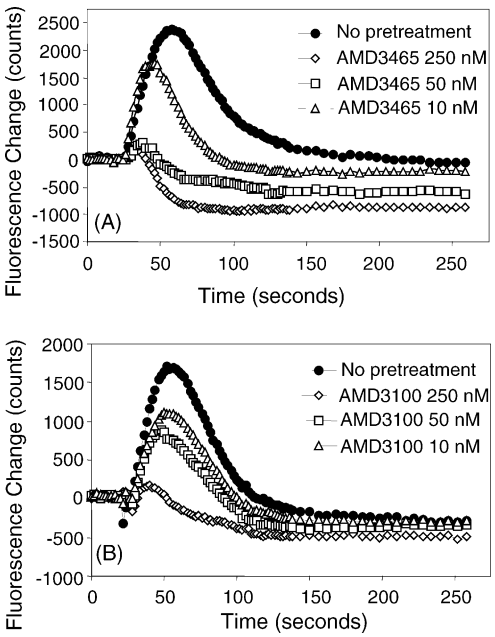

Fig. 4. Inhibitory effect of AMD3465 (A) and AMD3100 (B) on CXCL12-induced intracellular calcium signaling in SupT1 cells. After loading with the fluorescent calcium indicator Fluo-3, SupT1 cells were pretreated with the compounds at the indicated concentrations (open symbols) prior to stimulation with CXCL12 (20 ng/ml). The closed symbols represent the positive control where untreated cells were stimulated with the chemokine. The transient increase in cytosolic calcium concentration upon addition of the chemokine was monitored by measuring the fluorescence in function of time using the Fluorometric Imaging Plate Reader (FLIPR). Each data point represents the mean fluorescence of quadruplicate microplate wells. These data are from one representative experiment out of three.

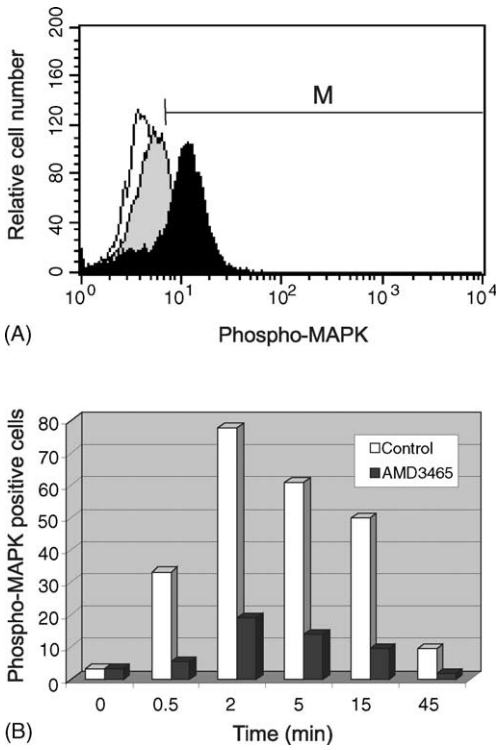

Fig. 5. Effect of AMD3465 on phosphorylation of p44/42 MAPK by CXCL12. SupT1 cells were stimulated with CXCL12 (200 ng/ml) after the cells had been incubated with or without 6250 nM AMD3465. At different time points (i.e., 0 and 30 s, and 2, 5, 15 and 45 min) cell samples were stained for phosphorylated MAPK by a phospho-specific primary antibody and a PE-conjugated secondary antibody and the cells were analyzed by flow cytometry. (A) Phospho-p44/42 MAPK staining of SupT1 cell populations that had not been stimulated with CXCL12 (white histogram), that had been stimulated with CXCL12 for 2 min (black histogram), and that had been stimulated with CXCL12 for 2 min after preincubation with AMD3465 at 6250 nM (grey histogram). The percentages of phospho-p44/42 MAPK-positive cells (i.e., cells residing in the marker 'M'-region) were 5.3, 78 and 19%, respectively. (B) The numbers of phospho-p44/42 MAPK-positive cells in AMD3465-pretreated versus untreated SupT1 cells in function of time after CXCL12 stimulation. The results from one out of three independent experiments are shown.

block the intracellular calcium fluxes elicited by the CCR5 ligands RANTES, LD78β and MIP-1β in U87.CD4.CCR5 cells (data not shown).

A downstream event in chemokine-induced intracellular signaling is the activation of the MAP kinase pathway. Phosphorylation of MAPK in response to CXCL12 and inhibition by AMD3465 was assessed by a flow cytometric assay [15], using a phospho-specific mAb recognizing p44/42 MAP kinase phosphorylated at Thr^{202}/Tyr^{204} (Fig. 5A). This technique allows to monitor the percentage of cells containing phosphorylated MAP kinase in function of time upon exposure of the cells to exogenous stimuli (e.g. chemokines, growth factors, phorbol ester) (Fig. 5B). In an unstimulated SupT1 cell population, 3.4% of the cells stained positive for phospho-MAPK. The proportion of phospho-MAPK positive cells rapidly increased after CXCL12 stimulation, reached a maximum after 2 min (78%) and then declined back to 9% after 45 min. When the cells had been pretreated with AMD3465, the percentage of phospho-MAPK positive cells did not exceed 19% during the 45-min follow-up period after the addition of CXCL12 (Fig. 5B). Thus, AMD3465 clearly inhibits CXCL12-induced intracellular signaling, both at the level of calcium mobilization and MAPK phosphorylation.

3.4. Inhibition of CXCL12-induced cell migration by AMD3465

We also examined the ability of AMD3465 to inhibit CXCL12-induced chemotaxis of human T-lymphoid SupT1 cells by the use of the Transwell membrane system. As shown in Fig. 6, CXCL12 at 100 ng/ml elicited a strong chemotactic response in SupT1 cells: 28% of the cells transmigrated across the membrane, compared to only 3% cells in the negative control (no chemokine added), resulting in a chemotactic index (i.e. ratio of migrated cells in the positive versus the negative control) of 9.3. When the cells were pre-exposed to AMD3465, the chemotactic response to CXCL12 was effectively inhibited in a dose-dependent fashion (Fig. 6). At 6250 nM, AMD3465 totally blocked the chemotaxis; the percentages of cells that migrated towards CXCL12 after preincubation with AMD3465 at

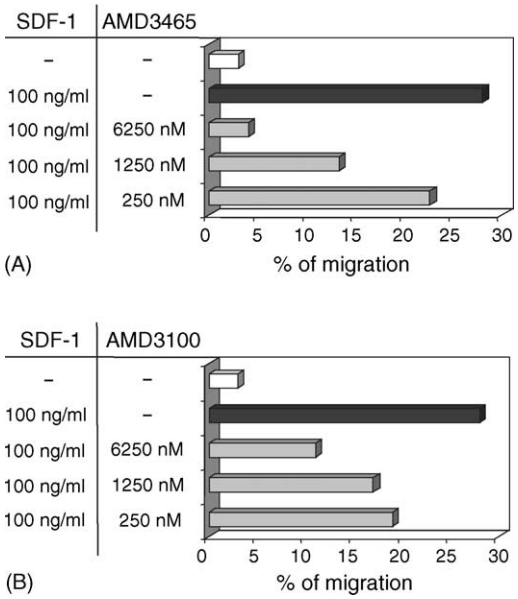

Fig. 6. Inhibitory effect of AMD3465 and AMD3100 on CXCL12-induced chemotaxis of SupT1 cells. Chemotaxis was assessed with the Transwell® cell migration system and subsequent flow cytometric counting of migrated cells by a 2-min acquisition. The cells were preincubated with the compounds at the indicated concentrations and were then loaded into 5-μm filter inserts which were placed in a 24-well plate containing buffer (negative control) or 100 ng/ml CXCL12 in buffer. The bars represent the percentages of cells having crossed the Transwell® membrane after 2.5 h of incubation in the absence of any chemokine (negative control: white bars) or in the presence of 100 ng/ml CXCL12 (positive control: black bars and drug-treated cells: grey bars).

1250 and 250 nM were 13 and 23%, respectively. In contrast, AMD3100 was unable to completely block the CXCL12-induced chemotaxis at 6250 nM (Fig. 6), which again confirms the superior antagonistic activity of AMD3465 as compared to AMD3100.

3.5. AMD3465 prevents chemokine-induced internalization of CXCR4

CXCL12-induced endocytosis of CXCR4, and the inhibitory effect of AMD3465 on the internalization process were visualized by fluorescence microscopy in stably transfected U87.CD4 cells expressing GFP-coupled CXCR4. As shown in the upper left panel of Fig. 7, the fluorescent chemokine receptor was mainly concentrated on the cell membrane in unstimulated U87.CD4.CXCR4-GFP cells. Upon stimulation with 1 μg/ml CXCL12, the receptor was sequestered in endosomal vesicles, which were visible as bright spots accumulating near the nucleus (Fig. 7, upper right panel). Chemokine-induced receptor endocytosis was clearly blocked by preincubation of the cells with 6250 nM AMD3465 (Fig. 7, lower right panel). When the U87.CD4.CXCR4-GFP were incubated with AMD3465 in the absence of CXCL12 (Fig. 7, lower left panel), the membrane localization of the CXCR4-GFP fusion protein was not altered as compared to the unstimulated control, indicating that AMD3465 by itself cannot induce CXCR4 internalization.

3.6. AMD3465 is endowed with potent and selective anti-HIV activity against CXCR4-using viruses

AMD3465 proved active against the X4 HIV-1 strains III_B, NL4.3, RF and HE with an IC_{50} ranging from 6 to 12 nM. The IC_{50} for suppression of the HIV-2 strains ROD and EHO was 12.3 nM (Table 1). In contrast, AMD3465 was not active against the R5 HIV-1 strain BaL at concentrations up to 6250 nM. For comparison, the bicyclams AMD3100 and AMD2763 were also evaluated in these experiments (Table 1). From the data it can be concluded

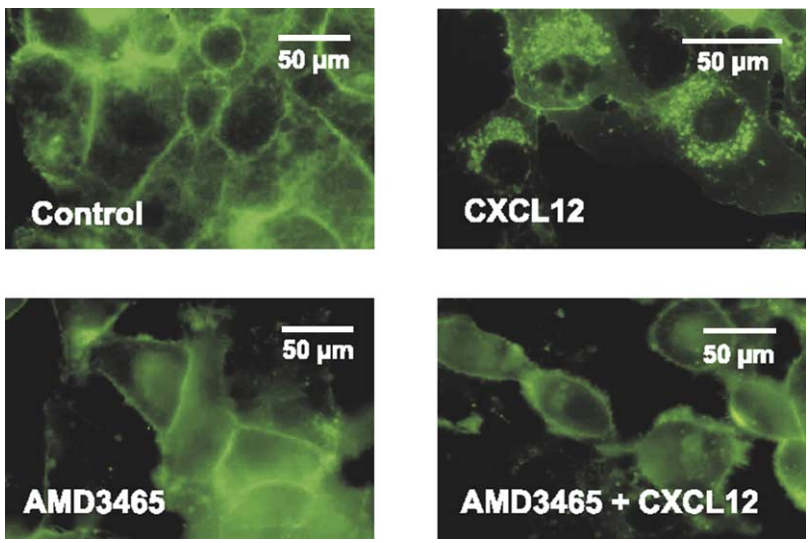

Fig. 7. Inhibitory effect of AMD3465 on CXCL12-induced endocytosis of CXCR4. U87.CD4.CXCR4-GFP cells were grown in chamber slides for 24 h and were then preincubated for 15 min in the absence (upper panels) or presence (lower panels) of 6250 nM AMD3465. Then, 1 μg/ml CXCL12 was added to the cells in both right panels, but not in the left panels. After incubation for 1 h 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. Magnification is indicated by the scale bar on each picture. The pictures were obtained from one representative experiment, which was repeated with similar results.

Table 1
Antiviral activity of different compounds against diverse HIV strains

Compound	IC_{50} (nM)[a,d]								CC_{50} (μM)[c]
	III_B	NL4.3	NL4.3^{AMD3100}	RF	HE	BaL[b]	ROD	EHO	
AMD3465	12.3	6.1	2822	7.4	9.8	>6250	12.3	12.3	>300
AMD3100	12.4	7.4	859	9.8	12.3	>6250	49.1	12.3	>300
AMD2763	994	490	67122	497	495	>6250	8950	1740	>300
Zidovudine	0.4	5.2	5.2	1.9	1.1	22.5	0.4	0.4	>7500
DS-5000[d]	0.3	0.1	>25	0.06	0.5	13	0.08	3.8	>250

[a] 50% Inhibitory concentration based on the inhibition of HIV-1-induced cytopathicity in MT-4 cells, as assessed by the MTS method.
[b] 50% Inhibitory concentration based on the replication of HIV-1 BaL in PBMC.
[c] 50% Cytotoxic concentration based on the reduction of viability of mock-infected MT-4 cells.
[d] Concentrations of dextran sulphate are expressed in μg/ml.

that AMD3465 is at least as potent as AMD3100 and 50- to 700-fold more potent than AMD2763 in inhibiting the replication of the X4 viruses III_B, NL4.3, RF, HE, ROD and EHO. The reverse transcriptase inhibitor zidovudine and the adsorption inhibitor dextran sulphate MW 5000 (DS-5000) were active against all these X4 HIV-1 and HIV-2 strains with IC_{50} values of 0.4 to 5.2 nM and 0.06 to 3.8 μg/ml, respectively. Unlike the cyclam analogues AMD3100, AMD2763 and AMD3465, zidovudine and dextran sulphate also inhibited the R5 HIV-1 strain BaL. In addition, AMD3465 remained equally active against HIV-1 III_B strains that were resistant to zidovudine (data not shown), but was >400-fold less active against an AMD3100-resistant NL4.3 strain, as compared to wild-type NL4.3 (Table 1). Like the bicyclams AMD3100 and AMD2763, the monocyclam AMD3465 was not toxic to MT-4 cells at a concentration as high as 300 μM.

4. Discussion

The observations that chemokine receptors act as entry cofactors for HIV infection [1–4], that certain natural chemokines have HIV-suppressive activity [18–20] and that mutations in the CCR5 chemokine receptor, which serves as coreceptor for M-tropic strains of HIV, afford natural resistance against HIV infection [21,22], have prompted the development of new therapeutic strategies targeting the chemokine receptors.

The bicyclams were identified in our laboratory as the first specific small-molecule CXCR4 antagonists. AMD3100, the prototype of this class of compounds, is the most potent and specific X4 HIV entry inhibitor that has been described to date [8,13,23,24]. AMD3100 (Fig. 1) has a center of symmetry and contains eight amino groups that are responsible for the high overall positive charge (+4) of the molecule and, consequently, the lack of oral bioavailability. As a reduction of this high overall charge is a prerequisite to improve oral absorption, studies were undertaken to identify the structural determinants essential for the potent antagonistic and anti-HIV activity of the molecule. The quantitative structure-activity relationship (QSAR) of bicyclam analogs is well documented [6,23,25–28], and the chemical requirements for maximal anti-HIV activity and minimal toxicity appeared to be (i) two metal-chelating macrocyclic rings, not necessarily identical, with an optimum ring size of 14 atoms, and (ii) a distance of 9.5–11.5 Å between the metal binding centers [6,28]. Moreover, it was found that compounds with an aromatic linker, such as 1,4-phenylenebis(methylene) in the case of AMD3100, exhibit superior antagonistic and anti-HIV activity as compared to compounds like AMD2763, which contain an aliphatic bridge between the two cyclam rings (Fig. 1) [6,23,27].

In the present study, we demonstrated that there is considerable structural redundancy in AMD3100: all eight amino groups are not required for potent activity. The overall charge at physiological pH can be significantly reduced by the elimination of basic amino groups without compromising antagonistic and anti-HIV potency. Indeed, we successfully designed monomacrocyclic (14-membered) ring structures, with the *N*-pyridinylmethylene cyclam AMD3465 as a lead molecule, showing comparable or even improved CXCR4 antagonism and X4 HIV inhibitory potency. AMD3465 effectively blocked the cell surface binding of anti-CXCR4 mAb 12G5 and the natural CXCR4 ligand CXCL12, and potently inhibited CXCL12-induced intracellular calcium mobilization, MAP kinase phosphorylation, receptor internalization and chemotaxis. Notably, AMD3465 proved 50-fold more potent than AMD3100 in inhibiting 12G5 mAb binding and up to 15-fold more potent than AMD3100 in antagonizing CXCL12-induced calcium signaling. Like AMD3100 [24], AMD3465 exclusively inhibited CXCR4 and did not interact with any of the chemokine receptors CXCR1 through CXCR3, or CCR1 through CCR9 (data not shown). Furthermore, AMD3465 was found to be a highly effective anti-HIV agent with a selectivity index greater than 100,000 in $CD4^+CXCR4^+$ cells. Thus, AMD3465 opposes the previous assumption that two cyclam rings are essential for anti-HIV activity.

We have shown that AMD3465 is able to inhibit the replication of virus strains that were made resistant to other anti-HIV agents like the reverse transcriptase inhibitor zidovudine. Hence, like the bicyclams, this new chemical class of monomacrocyclic CXCR4 inhibitors might also

provide a therapeutic alternative for patients carrying multi-resistant viruses that have become insensitive to the currently available reverse transcriptase and/or protease inhibitors. On the other hand, AMD3100-resistant HIV-1 NL4.3 [12,13] was cross-resistant to AMD3465, indicating that AMD3100 and AMD3465 share a similar mode of interaction with the CXCR4 receptor protein and/or overlapping interaction sites. Two aspartate residues at positions 171 and 262, and a glutamate at position 288 of the CXCR4 protein have recently been identified by mutational analysis as the essential binding sites for AMD3100 [29–31]. The molecular interaction profile of AMD3465 with CXCR4 is the subject of another study by Rosenkilde et al., who indeed found that AMD3465 binds to the same acidic anchor-point residues as AMD3100, but has an additional interaction with His^{281} (manuscript in preparation).

In summary, the monocyclam AMD3465 is a superior antagonist of the chemokine receptor/HIV coreceptor CXCR4 as compared to the bicyclam AMD3100 and is at least as potent as AMD3100 in inhibiting X4 HIV-1 and HIV-2 replication. Although AMD3465 still lacks oral bioavailability, this molecule supports the new chemical concept of non-bicyclam compounds with strong and specific affinity for CXCR4. These findings have had a crucial impact on the currently ongoing design of non-macrocyclic and orally available CXCR4 inhibitors, which will be described in due course.

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