Molecular Mechanism of Action of Monocyclam Versus Bicyclam Non-peptide Antagonists in the CXCR4 Chemokine Receptor*

Mette M. Rosenkilde†, Lars-Ole Gerlach†§, Sigrid Hatse‡, Renato T. Skerlj¶, Dominique Schols†, Gary J. Bridger¶, and Thue W. Schwartz†§

From the †Laboratory for Molecular Pharmacology, Department of Neuroscience and Pharmacology, University of Copenhagen, The Panum Institute, Copenhagen, DK-2200, Denmark, the AnorMED, Langley, British Columbia V2Y 1N5, Canada, §7TM Pharma A/S, Hørsholm, DK-2970, Denmark, and the ¶Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, 300, Belgium

AMD3465 is a novel, nonpeptide CXCR4 antagonist and a potent inhibitor of HIV cell entry in that one of the four-nitro- gen cyclam rings of the symmetrical, prototype bicyclam antag- onist AMD3100 has been replaced by a two-nitrogen N-pyridi- nylmethylen moiety. This substitution induced an 8-fold higher affinity as determined against 125I-12G5 monoclonal CXCR4 antibody binding, and a 22-fold higher potency in inhibi- tion of CXCL12-induced signaling through phosphatidylinositol accumulation. Mutational mapping of AMD3465 and a series of analogs of this in a library of 23 mutants covering the main ligand binding pocket of the CXCR4 receptor demonstrated that the single cyclam ring of AMD3465 binds in the pocket around AspIV:20 (Asp171), in analogy with AMD3100, whereas the N-pyridinylmethylen moiety mimics the other cyclam ring through interactions with the two acidic anchor- point residues in transmembrane (TM)-VI (AspVI:23/Asp262) and TM-VII (GluVII:06/Glu288). Importantly, AMD3465 has picked up novel interaction sites, for example, His281 located at the interface of extracellular loop 3 and TM-VII and HisIII:05 (His113) in the middle of the binding pocket. It is concluded that the simple N-pyridinylmethylen moiety of AMD3465 substitu- tes for one of the complex cyclam moieties of AMD3100 through an improved and in fact expanded interaction pattern mainly with residues located in the extracellular segments of TM-VI and -VII of the CXCR4 receptor. It is suggested that the remaining cyclam ring of AMD3465, which ensures the effica- cious blocking of the receptor, in a similar manner can be replaced by chemical moieties allowing for, for example, oral bioavailability.

The CXCR4 receptor is a broadly expressed chemokine receptor, which in contrast to chemokine receptors in general, is found not only on cells within the immune system, but also, for example, in the central nervous system and gastrointestinal system (1, 2). The CXCR4 receptor is activated by a single che- mokine, CXCL12 (previously called stromal-derived factor-1) in contrast to the promiscuous binding of several chemokines by other chemokine receptors. CXCR4 is involved in the migra- tion and homing of leukocytes, and importantly it plays a cen- tral role for the anchorage of CD34+ stem cells in bone mar- row. Yet, in contrast to most other 7TM2 receptors, targeted deletion of either the gene for CXCR4 or for CXCL12 leads to embryologic lethality (3–5) and thereby emphasize the impor- tance of proper CXCR4 function. In addition, CXCR4 is ex- pressed on many different types of cancer cells where it func- tions as a survival factor in addition to directing cancer cell migration, for example, metastasis to the bone marrow, where its ligand CXCL12 is produced in large quantities (6).

The CXCR4 receptor acts as the main co-receptor for cell entry by so-called CXCR4 using (X4) strains of HIV (human immunodeficiency virus). The prototype non-peptide antag- onist for CXCR4, AMD3100, was discovered as an anti-HIV agent long before the action through CXCR4 was described (7). AMD3100 is composed of two 1,4,8,11-tetraazacyclotetra- cane (cyclam) moieties connected by a conformationally con- strain dualaminobenzophenone linking structure (Fig. 1). AMD3100 is highly specific for CXCR4 and inhibits the binding and function of CXCL12 and the HIV cell entry with high affinity and potency (8,9) through an interaction with three acidic residues, Asp171 (AspIV:20), Asp262 (AspVI:23), and Glu288 (GluVII:06) located in the main ligand binding pocket of CXCR4 (10–12) (Fig. 1). Based on the knowledge of the strong preference of the cyclam moiety for interactions with carboxylic acid groups (21) and on molecular modeling, we have previously suggested that one cyclam ring of AMD3100 interacts with Asp171 in TM-IV, whereas the other ring is sand- wished between the carboxylic acid groups of Asp262 and Glu288 from TM-VI and -VII, respectively (11). Importantly, we were able to successfully transfer the essential components of this rather simple tridentate binding mode of the prototype CXCR4 non-peptide antagonist AMD3100 into the otherwise rather distinct CXCR3 receptor (11). Despite the fact that

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† To whom correspondence should be addressed. Tel.: 45-61364871; Fax: 45-35327610; E-mail: Rosenkilde@molpharm.dk.

‡ The abbreviations used are: 7TM, seven transmembrane domain; HIV, human immunodeficiency virus; PI turnover, phosphatidylinositol turn- over; EC, extracellular; CI, clinical isolate; WT, wild type.
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AMD3100 is a highly potent and specific CXCR4 antagonist, it does not exhibit oral bioavailability presumably due to the positive charge (+2) of each ring at physiological pH (13–15). Long term treatment in HIV-infected persons may therefore be difficult. However, subcutaneous administration in combination with granulocyte-colony stimulating factor improves stem cell mobilization yields in patients undergoing transplantation (32).

In the present study we characterize the molecular mechanism of action of a novel series of monocyclam CXCR4 antagonists designed from the prototype symmetrical bicyclam AMD3100 (Fig. 1). In these compounds one of the cyclam rings was substituted with aromatic phenyl or pyridine rings (the “non-cyclam part”) linked by an N-substituted aliphatic chain and the original phenylenebismethylene linker to the remaining cyclam ring (i.e. the “cyclam part”). In the prototype monocyclam AMD3465, a high affinity CXCR4 antagonist that efficiently inhibits HIV cell entry via CXCR4 (16), the cyclam ring was substituted with a 2-pyridylmethylamine moiety (Fig. 1). We describe the binding of AMD3465 as well as its antagonistic properties in respect of blocking CXCL12-induced activation and HIV cell entry. The binding properties were determined by the use of radiolabeled monoclonal antibody 12G5 that interacts with a broader spectrum of CXCR4 mutants compared with the previously used radiolabeled CXCL12 (10). Importantly, the relative binding mode of the cyclam versus the non-cyclam part of AMD3465 in the CXCR4 receptor was characterized by use of a large library of receptor mutants and a series of selected analogs of this prototype monocyclam antagonist with modifications in each end of the molecule.

EXPERIMENTAL PROCEDURES

Materials—The human chemokine CXCL12 was purchased from Peprotech. The CXCR4-specific monoclonal antibody 12G5 was kindly provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA). Human CXCR4 cDNA was kindly provided by Timothy N. C. Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland). The promiscuous chimeric G-protein GΩ6q4myr was kindly provided by Evi Kostenis (7TM-Pharma, Denmark). myo-[3H]inositol (PT6–271) and Bolton-Hunter reagent for iodination of 12G5 were purchased from Amersham Biosciences (Uppsala, Sweden). AG 1-X8 anion-exchange resin was from Bio-Rad.

Site-directed Mutagenesis—Point mutations were introduced in the receptor by the polymerase chain reaction overlap extension technique (17) using the wild type CXCR4 as template. All reactions were carried out using the Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. The generated mutations were cloned into the eukaryotic expression vector pcDNA3+. The mutations were verified by restriction endonuclease digestion and DNA sequencing (ABI 310, PerkinElmer Life Sciences).

Iodination of 12G5—The Bolton–Hunter reagent was dried by a gentle stream of nitrogen for 30–60 min. 250 pmol of 12G5 was incubated on ice with 1 mCi of Bolton-Hunter reagent in a total volume of 50 μl of 0.1 mM borate buffer, pH 8.5, for 1 h. The reaction was terminated by addition of 0.25 ml of the borate buffer supplemented with 0.2 mM glycine and Bolton-Hunter-labeled 12G5 separated from free Bolton-Hunter reagent by column chromatography (Econo-Pac DC10, Bio-Rad) (18).

Transfections and Tissue Culture—COS-7 cells were grown at 10% CO2 and 37 °C in Dulbecco’s modified Eagle’s medium with glutamax (Invitrogen, catalog number 21885-025) adjusted with 10% fetal bovine serum, 180 units/ml penicillin, and 45 μg/ml streptomycin. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (19). Human astrogliaoma U87 cells expressing human CD4 (U87.CD4) (20) were kindly provided by Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York) and cultured at 10% CO2 and 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.01 μM Hepes, and 0.2 mg/ml gentamicin (G-418 sulfate). The CXCR4 encoding plasmids were cotransfected with the pPUR selection vector encoding puromycin resistance (Clontech) into U87.CD4 cells by the use of FuGENETM 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. Puromycin (1 μg/ml) selection was started after 18 h. After ~3 weeks, the puromycin-resistant cell cultures containing 10–30% CXCR4-positive cells were established for each of the CXCR4 variants. The CXCR4-expressing cells were isolated from these cell cultures as follows. Approximately 4 × 10⁶ cells in 500 μl of phosphate-buffered saline containing 2% fetal bovine serum were incubated with 20 μl of non-conjugated mouse anti-human CXCR4 antibody clone 12G5 for 30 min at room temperature. After two washing steps, the cells were incubated with 2 × 10⁶ sheep anti-mouse IgG-conjugated Dynabeads® M450 (Dynal, Oslo, Norway) in 8 ml of phosphate-buffered saline with 2% fetal bovine serum for 1 h at 4 °C under continuous gentle rotation. Thereafter, the CXCR4-positive cells (which had bound magnetic beads at their surface) were isolated from the cell suspension by magnetic separation and were thoroughly washed with phosphate-buffered saline and transferred to puromycin-containing growth medium. The CXCR4 expression was checked with phosphatidylethanolamine-conjugated anti-CXCR4 monoclonal antibody (clone 12G5) (BD Biosciences) and evaluated by flow cytometrically (FACSCalibur, BD Biosciences).

Binding Experiments—COS-7 cells were transfected to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors and was aimed at obtaining 5–10% specific binding of the added radioactive ligand (2 × 10⁴ to 1 × 10⁵ cells/well for the different CXCR4 constructs). Two days after transfection, cells were assayed by competition binding for 3 h at 4 °C using 32 pm 125I-12G5 plus unlabeled ligand in 0.5 ml of a 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed quickly two times in 4 °C binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined as the binding in the presence of 0.1 μM unlabeled 12G5. Determinations were made in duplicates.

Phosphatidylinositol Assay (PI Turnover)—COS-7 cells were transfected according to the procedure mentioned above. Briefly, 6 × 10⁶ cells were transfected with 20 μg of receptor cDNA in addition to 30 μg of the promiscuous chimeric G-protein, GΩ6q4myr (abbreviated as Gq4myr), which turns the
i-coupled signal, most common pathway for endogenous chemokine receptors, into the $\alpha_i$ pathway (phospholipase C activation measured as PI turnover) (21). One day after transfection, COS-7 cells ($2.5 \times 10^4$ cells/well) were incubated for 24 h with $2 \mu$Ci of myo-[3H]inositol in 0.4 ml of growth medium per well. Cells were washed twice in 20 mM Hepes, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 10 mM glucose, and 0.05% (w/v) bovine serum albumin; and were incubated in 0.4 ml of buffer supplemented with 10 mM LiCl at 37 °C for 90 min in the presence of various concentrations of chemokines or AMD analogs together with a constant concentration of chemokine corresponding to 80% of maximal stimulation. Cells were extracted by addition of 1 ml of 10 mM formic acid to each well followed by incubation on ice for 30–60 min. The generated [3H]inositol phosphates were purified on AG 1-X8 anion-exchange resin (22). Determinations were made in duplicates.

**Measurement of Intracellular Calcium Mobilization**—On the day prior to the experiment, the U87.CD4.CXCR4 transfectants were seeded in 0.1% gelatin-coated 96-well black wall microplates (Costar, Cambridge, MA) at $2 \times 10^4$ cells per well. On the day of the experiment, the cells were loaded with the fluorescent calcium indicator Fluo-3 acetoxymethyl (Molecular Probes, Leiden, The Netherlands) at 4 $\mu$M for 45 min at 37 °C. After thorough washing with calcium flux assay buffer (Hanks’ balanced salt solution with 20 mM Hepes buffer and 0.2% bovine serum albumin, pH 7.4), the cells were preincubated for 15 min at 37 °C with AMD3100 or AMD3465 (1 $\mu$g/ml) in the same buffer. Then, the intracellular calcium mobilization in response to 2–50 ng/ml 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 (Molecular Devices, Sunnyvale, CA) (23).

**HIV-1 Infection Assays**—The CXCR4-using (X4) HIV-1 molecular clone NL4.3 was obtained from the National Institutes of Health NIAID AIDS Reagent program (Bethesda, MD), and the HIV-1 strain NDK was obtained from the Medical Research Council (MRC) (London, UK). Virus stocks of the clinical isolate (CI) number 10 was generated by co-culture of peripheral blood mononuclear cells from a healthy donor with lymphocytes from an HIV-1-infected person. U87.CD4 cells transfected with CXCR4 WT or mutants were digested using trypsin. These cells were washed and resuspended at $5 \times 10^4$ cells/ml in medium and seeded out at $2 \times 10^4$ cells per well in 24-well plates. The compounds (AMD3100 and AMD3465) were added at different concentrations together with the HIV-1 laboratory strain or CI number 10 and the plates were maintained at 37 °C in 10% CO$_2$. The cytopathic effect in the virus-infected cell cultures was evaluated microscopically at 5 days after infection. Then the supernatant was collected and analyzed for virus content based on the p24 core silver enzyme-linked immunosorbent assay (DuPont-Merck Pharmaceutical Co.).
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RESULTS

Previously we have shown that AMD3100 is highly and almost solely dependent upon three acidic, anchor-point residues in the main ligand binding pocket of CXCR4: AspIV:20 (Asp171) in TM-IV, believed to interact with one of the cyclam moieties, as well as AspVI:23 (Asp262) and GluVII:06 (Glu288), which are believed to “sandwich” the other cyclam ring of AMD3100 between TM-VI and TM-VII (10, 11). Importantly, the high potency and efficacy of AMD3100 requires the presence of a rigid linker between the two cyclam moieties (10). Previously described monocyclams as well as bicyclams with flexible linkers that were less potent were shown only to be dependent upon AspIV:20 (10). AMD3465 is a novel, high potency monocyclam in which one of the cyclam rings of AMD3100 has been exchanged with a N-pyridinylmethylene moiety (Fig. 1) (16).

Common Interaction Points for AMD3465 and AMD3100 in the CXCR4 Receptor—In competition against the radiolabeled monoclonal antibody raised against CXCR4, 12G5, the affinity of AMD3465 was 8-fold higher compared with AMD3100 (Fig. 2, Table 1). Using this competition binding assay we initially probed the binding of AMD3465 in a library of 23 mutants of CXCR4 having substitutions in the main ligand binding pocket, including the three acidic anchor-point residues of AMD3100. In most cases the side chain was substituted with the small methyl group of Ala. Yet, in some cases a structurally similar but uncharged Asn residue was introduced instead of a charged Asp. In other cases steric hindrance mutagenesis was performed through introduction of larger side chains such as Phe or Trp for Ala, Gly, or Ile (24). The substitutions had no or a very limited effect on 12G5 binding to the CXCR4 receptor as reflected in the $K_d$ and $B_{max}$ values with less than 10-fold difference between the lowest and highest values (Table 1). This indicated that the overall structure as well as the cell surface expression of the receptor was generally unaffected by the mutations.

The binding of AMD3465 was affected more than 10-fold by 11 of the substitutions of which four were shared by AMD3100, i.e. the Asn/Ala substitutions of the three acidic anchor-point AspIV:20, AspVI:23, and GluVII:06 and the steric hindrance mutation of AlaIV:24 (Ala175) to Phe (A175F)-CXCR4. The magnitude of effects of the AspIV:20 and GluVII:06 substitutions (D171N)- and (E288A)-CXCR4, respectively, were similar for AMD3465 and AMD3100, whereas the effects of the AspVI:23 substitution (D262N)-CXCR4 and the steric hindrance substitution of AlaIV:24 (Ala175F)-CXCR4 were much higher for AMD3465 than for AMD3100. Thus, the affinity of AMD3465 was decreased 5000-fold in (D262N)-CXCR4 and 1913-fold in (A175F)-CXCR4, whereas these mutations only affected AMD3100 binding 52- and 40-fold, respectively (Fig. 3).

Mutations Affecting AMD3465 but Not AMD3100 Binding—Among the 7 mutations that specifically affected AMD3465 but not AMD3100 binding, His281 to Ala (H281A)-CXCR4, which is located in extracellular loop 3 close to the start of TM-VII (in position VII:−02), affected the binding affinity of AMD3465 4500-fold compared with WT CXCR4 (Table 1, Fig. 2). The affinity of AMD3100 was in fact improved slightly by this mutation (Table 1, Fig. 2). Ala substitutions of two other residues in TM-VII also impaired the binding affinity of AMD3465, but not AMD3100, namely IleVII:02 (Ile284) and ThrVII:05 (Thr287) and the Asp to Asn in position VI:23 (Asn262) and His to Ala in position VII:−02 (His281) on the binding of AMD3100 (A) and AMD3465 (B). Competition binding with $^{125}$I-12G5 as radioligand was performed in transiently transfected COS-7 cells. Increasing amounts of the bi- and monocyclam displaced the $^{125}$I-12G5 antibody from WT CXCR4 (●), (D262N)-CXCR4 (□), and (H281A)-CXCR4 (▲). Data are shown as mean ± S.E. (n = 4–22).

Calculations—IC$_{50}$ and EC$_{50}$ values were determined by nonlinear regression and $B_{max}$ values were calculated using the GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA).
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The data were obtained from competition binding with $^{125}$I (Bolton-Hunter)-labeled antibody 12G5 as radioligand on transiently transfected COS-7 cells. Values in parentheses represent number of experiments (n), and $F_{\text{mut}}$ indicates the difference between the affinities on mutant receptor compared to WT receptor. Red background indicates an $F_{\text{mut}} > 500$. Orange indicates an $F_{\text{mut}}$ from 50 to 500, and yellow indicates an $F_{\text{mut}}$ from 10 to 50.

### TABLE 1
Affinity of 12G5, AMD3100, and AMD3465 for the wild-type CXCR4 and various CXCR4 mutations

| Residue | Bmax ± SEM | 12G5 | AMD3100 | AMD3465 |
|---------|------------|------|---------|---------|
|         | fmol/100,000 cell | log Kd ± SEM | (nM) | log Kd ± SEM | (nM) | Fmut | log Kd ± SEM | (nM) | Fmut |
| wt      |          |         |        |         |       |      |        |       |      |
| wt      |         |         |        |         |      | 1.0  | 0.11  |       | 1.0  |
| TM-III  |         |         |        |         |      | 1.1  | 2.2   |       | 3.0  | 230  |
| TM-IV   |         |         |        |         |      | 1.2  | 3.5   |       | 5.0  |
| ECL-II  |         |         |        |         |      | 1.3  | 3.5   |       | 8.7  |
| TM-V    |         |         |        |         |      | 1.4  | 3.5   |       | 16   |
| TM-VI   |         |         |        |         |      | 1.5  | 3.5   |       | 4.7  |
| TM-VII  |         |         |        |         |      | 1.6  | 3.5   |       | 1.4  |

| Residue | V12G5A | V228A | V228T | V228A |
|---------|--------|-------|-------|-------|
|         |        |       |       |       |
|         |        |       |       |       |
|         |        |       |       |       |
|         |        |       |       |       |

### FIGURE 3
Residues identified through mutagenesis to be important for the binding of AMD3100 and AMD3465 shown in a helical wheel diagram of the CXCR4 receptor. The background color indicates the magnitude of the effect of the mutation on the binding of either AMD3100 (left panel) or AMD3465 (right panel). Gray background indicates <10-fold decrease in affinity; yellow, 10–50-fold decrease; orange, 50–500-fold decrease; and red, >500-fold decrease in affinity. The actual binding affinities are shown in Table 1.

Thus, the major result of the mutational analysis using the 12G5 monoclonal antibody as a radioligand was the identification of several additional residues of importance for the binding of AMD3465 compared with AMD3100. The three acidic, anchor-point residues for AMD3100 were shared by AMD3465. However, several additional residues facing right into the main binding pocket were uncovered as being selectively important for AMD3465. Of these, the His$^{113}$ identified one residue in TM-III, His$^{113}$ (His$^{113}$), as being important for AMD3465 binding. This residue is facing right into the middle of the main ligand binding pocket. Despite this, Ala substitution of His$^{113}$ (H113A)-CXCR4 did not affect on the binding of AMD3100, whereas the affinity of AMD3465 was decreased 23-fold (Table 1).
Correlation between Inhibition of Receptor Activity and Affinity Measured against Radiolabeled Monoclonal Antibody—The affinity of bicyclam analogs measured against 125I-12G5 as radioligand has previously been shown to correlate to the affinity obtained using 125I-CXCL12 (stromal cell-derived factor-1α) as a radioligand (10). However, we have observed mutants with impaired binding of 125I-CXCL12, e.g., the Ala substitution of GluVII:06 in CXCR4 (11). Therefore, to get a complete picture of the ability of AMD3465 to antagonize CXCL12-induced signaling activity we decided to probe the library of CXCR4 mutants in a functional assay previously used to map the interaction mode of AMD3100 (11). Thus, COS-7 cells were transiently transfected with the receptor construct of interest together with the promiscuous G-protein Goi4myr (21), and the ability of AMD3465 and AMD3100 to inhibit PI turnover induced by submaximal doses of CXCL12 were assessed. Using this approach, the overall picture of residues of importance for AMD3465 binding, compared with AMD3100 binding, was basically the same as identified in competition against 125I-12G5 (Table 2 compared with Table 1). Thus, again the three acidic key residues for AMD3100 binding were identified as being important also for AMD3465. Among these AspVI:23 again had the biggest impact for AMD3465 function as compared with AMD3100 because the inhibitory potency of AMD3465 was decreased 804-fold for (D262N)-CXCR4 compared with WT CXCR4 and only 21-fold for AMD3100.

The complete picture of residues with selective importance for the interaction of AMD3465 (but not AMD3100) with CXCR4 was somehow simpler in the functional test. Thus, only 3 residues, HisVII:-02, GlnV:05, and HisIII:05, of the 7 residues identified in the competition binding setup (described above) were identified in respect of inhibition of CXCL12-induced CXCR4 activation. Consistent with the identified impact of (H281A)-CXCR4 in the competition binding (Fig. 2), this substitution again had the biggest impact with a 164-fold decrease in the potency of AMD3465, whereas the corresponding inhibitory potency for AMD3100 was unaffected (Fig. 4). The steric hindrance substitution (Q200W) and the Ala introduction (Q200A) resulted in a 23- and 15-fold reduction, whereas (H113A) resulted in a 13-fold decrease in potency for AMD3465. In contrast, <10-fold decrease was obtained for the ThrVII:05, IleVII:20, and ValV:01 substitutions (Table 2), which all appeared as “hits” (>10-fold decrease) in the competition against 125I-12G5 (Table 1).
Thus, the overall picture for the AMD3465 binding mode, like AMD3100, is critically dependent on AspVI:23 and Glu-VII:06 at the extracellular ends of TM-VI and -VII, respectively, plus AspIV:20 located at the opposite end of the main ligand binding pocket. In addition AMD3465, in contrast to AMD3100, appears to interact with HisVII:−02 at the extracellular end of TM-VII (at the interface to extracellular loop 3) and HisIII:05. Both of these His residues are facing right into the main binding pocket of CXCR4.

Correlation with Antiviral Activity for the Monocyclam AMD3465—The affinity measured against 125I-12G5 has previously been shown to correlate well with the antiviral potency of bicyclam analogs (10). However, this was not the case for the first high affinity, but low potency monocyclam compound AMD3389, that in contrast, potentiates the binding of 12G5 with high potency and display a very low ability to inhibit HIV-1 cell entry (10). In contrast to the low potency (IC50 ~ 1 μM) in respect of HIV cell entry blockade previously observed for AMD3389 (10, 25), we observed a high potency in the nanomolar range for AMD3465 (Table 3) for three different X4 viral strains (IC50 of 2.7, 4.7, and 36 nM for the NDK, C1 number 10, and NL3.3, respectively). Similar inhibitory potencies were obtained for AMD3100 (Table 3). The importance of the three acidic key residues AspIV:20, AspVI:23, and GluVII:06, respectively, and of HisVII:−02 were probed for AMD3465 in parallel with AMD3100, and again HisVII:−02 displayed a selective importance for AMD3465, but not for AMD3100, whereas the mutations of the acidic residues affected both compounds similarly (Table 3).

Binding Mode for Different Monocyclam Analogs—To identify the relative binding mode in the CXCR4 receptor of the cyclam versus the non-cyclam part of the prototype monocyclam, AMD3465, we included four analogs of this in the mutational mapping. Two of these differed from AMD3465 in respect of the non-cyclam moiety; i.e. in AMD3529, the pyridine ring of AMD3465 was substituted with a phenyl ring (Figs. 6 and 7), whereas in AMD3389, the pyridine ring was totally removed (Fig. 6). In contrast, AMD8721 and AMD8899 were modified in the cyclam ring by incorporation of either Cu2+ or Ni2+, respectively (Fig. 6). These two latter compounds were both affected by the various CXCR4 mutations in a very similar manner as AMD3465 (Table 4 and Fig. 6). The two compounds with modifications in the non-cyclam part, AMD3529 and AMD3389, displayed much lower potencies for the wild type CXCR4 receptor than AMD3465 (i.e. 178-fold decreased potency for AMD3529 and 2090-fold for AMD3389 as compared with AMD3465). Importantly, both of these analogs were totally unaffected by the HisVII:−02 and AspVI:23 substitutions, and only slightly affected by the GluVII:06 substitution all located in the TM-III-VI-VII pocket of the receptor. In contrast, both analogs with modifications in the non-cyclam part of the molecule displayed a relatively minor decrease (<15-fold) in potency in the AspIV:20 and HisIII:05 substitutions, i.e. they were affected in a relatively similar manner as AMD3465 (18-fold). Thus, these AMD3465 analogs with modified or lacking non-cyclam moieties appear to have a similar dependence of residues in the pocket around AspIV:20 but appear to have lost

Inhibition of calcium release is a classic functional assay for Gt-coupled 7TM receptors, and very often used for structure-function analyses in chemokine receptors. We therefore decided to probe the impact of the acidic key residues (AspIV:20 and AspVI:23) and the identified HisVII:−02 on the inhibition of CXCL12-induced PI turnover by AMD3100 (A) and AMD3465 (B). Phospholipase C activity (PI turnover) was measured in COS-7 cells after co-transfection of receptor cDNA with a promiscuous, chimeric G-protein Gqi4myr (as described under “Experimental Procedures”). Increasing amounts of the bi- and monocyclam inhibited the CXCL12-induced activity in WT CXCR4 ( ), (D262N)-CXCR4 ( ), and (H281A)-CXCR4 ( ). Data are shown as mean ± S.E. (n = 3–10).

FIGURE 4. Effect of Asp to Asn in position VI:23 (Asn262) and His to Ala in position VII:−02 (His281) on the inhibition of CXCL12-induced PI turnover by AMD3100 (A) and AMD3465 (B). Phospholipase C activity (PI turnover) was measured in COS-7 cells after co-transfection of receptor cDNA with a promiscuous, chimeric G-protein Gqi4myr (as described under “Experimental Procedures”). Increasing amounts of the bi- and monocyclam inhibited the CXCL12-induced activity in WT CXCR4 ( ), (D262N)-CXCR4 ( ), and (H281A)-CXCR4 ( ). Data are shown as mean ± S.E. (n = 3–10).
dependence on the residues at the extracellular ends of TM-VI and VII, AspVI:23, HisVII:02, and GluVII:06, i.e., interactions that are essential for AMD3465.

**DISCUSSION**

In this study we have characterized the binding and molecular mechanism of action of the prototype monocyclam CXCR4 receptor antagonist AMD3465 by combining receptor mutagenesis with studies of different chemical analogs of AMD3465. AMD3465, in which one of the four-nitrogen cyclam moieties has been replaced by a two-nitrogen N-pyrrolidinylmethylene moiety, binds with higher affinity and inhibits the CXCR4 signaling with higher potency than the classic, symmetrical bicyclam antagonist AMD3100. The identification of the relative binding mode of the cyclam versus the non-cyclam part of the AMD3465 monocyclam molecule together with the previous characterization of the general molecular mechanism of action of the bicyclam AMD3100, provides the structural knowledge base for subsequent replacement of also the “other” cyclam with a more drug-like chemical moiety providing, for example, oral bioavailability.

**Mutational Mapping of AMD3465 Versus AMD3100 Binding**—The key interaction points appear to be similar for AMD3465 and AMD3100 with mutational hits located in, respectively, the III-IV-V pocket (AspIV:20) and the III-VI-VII pocket (AspVI:23 and GluVII:06) of the main ligand-binding crevice of the CXCR4 receptor. However, as previously observed for the metal-loaded version of AMD3100 (26), the mutational map for AMD3465 is “tighter” with more clear hits and larger effects of the mutations observed both in the competition against iodinated monoclonal antibody ^125^I-12G5 (Table 1, Fig. 2) and in the inhibition of CXCL12-induced CXCR4 activation (Table 2, Fig. 3). For instance, the effect of AspVI:23 mutation on the binding of AMD3465 was 100-fold higher than the effect on AMD3100 binding. Importantly, in the III-VI-VII pocket a number of clear mutational hits located more toward the extracellular ends of TM-VI and -VII were observed for AMD3465. Of these, the HisVII:02 was most important with a >1000-fold decrease in affinity and a 161-fold decrease in the potency of AMD3465 for (H281A)-CXCR4 as compared with the WT CXCR4 receptor (Table 1, Figs. 2 and 3). However, also residues in TM-III (HisIII:05), TM-V (ValV:01 and GlnV:05), TM-VI (IleVI:02), and TM-VII (IleVII:02 and ThrVII:05) appeared to be
involved in binding of AMD3465, but not AMD3100, to CXCR4.

**Proposed Binding Mode of AMD3465**—Based on the previously published observation that the free cyclam moiety as well as the basic AMD3100-like, monocyclam analog AMD3389 both were affected by substitution of AspIV:20 but not AspVI:23 (10), it is expected that the cyclam ring (the “left side”) of AMD3465 binds in the III-IV-V pocket with interactions to AspIV:20 and surrounding residues. The “right side” of AMD3465 with the pyridine ring moiety would then be expected to interact with residues at the extracellular ends of TM-VI and especially TM-VII. In accordance with this, the mutational map for AMD3529 (in which the pyridine ring of AMD3465 was substituted with a phenyl ring), was rather similar to that of the AMD3389 compound, in which the “other cyclam ring” is totally lacking (as compared with AMD3100) or in which the 2-pyridylmethylamine moiety is lacking (as compared with AMD3465). Thus for both AMD3389 and AMD3529 basically all of the mutational hits, which for AMD3100 and AMD3465 are found in the III-VI-VII pocket, are lacking; whereas the hits in the III-IV-V pocket are still present. This supports the notion that the “remaining” cyclam moiety of these compounds is binding in the III-IV-V pocket (i.e., around AspIV:20). The low affinity of AMD3529 and AMD3389 (as compared with AMD3100 and AMD3465) indicates that the pyridine interaction of AMD3465 with the III-VI-VII pocket is necessary for high-affinity interaction with CXCR4. The difference in the mutational maps for AMD3465 and AMD3529 indicates that the pyridine ring of AMD3465 is interacting mainly with AspVI:23 and HisVII:-20 in a mode that cannot be mimicked by the corresponding phenyl group of AMD3529 (Fig. 7).

In good agreement with this, we find in the present study that neither of the two AMD analogs with modifications in the “cyclam” part (AMD8721 and AMD8899) differed from AMD3465 in respect of receptor recognition because they were high potency antagonists for the WT CXCR4 receptor and were influenced by the same mutations as AMD3465 (Table 4 and Fig. 6). AlaIV:24, i.e., four residues after AspIV:20, is located at the start of extracellular loop 2 facing down into the main ligand binding pocket in the x-ray crystal structure of rhodopsin. The relatively large and similar effect on AMD3465 and AMD3529 indicates that the pyridine ring of AMD3465 is interacting mainly with AspVI:23 and HisVII:-20 in a mode that cannot be mimicked by the corresponding phenyl group of AMD3529 (Fig. 7).

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![FIGURE 6. Dependence of HisIII:05 (His113) and HisVII:-02 (His281) for bi- and monocyclams. Whole cell phospholipase C activity (PI turnover) was measured in COS-7 cells after co-transfection of receptor cDNA with a promiscuous, chimeric G-protein Gqi4myr (as described under “Experimental Procedures”). Increasing amounts of the different monocyclams, AMD3465 (C and D), AMD8721 (E and F), AMD3529 (G and H), and AMD3389 (I and J) are shown together with the dose-response curves for the bicyclam AMD3100 (A and B). Inhibition of CXCL12 (10 nM)-induced activity in WT CXCR4 by increasing amounts of the bi- and monocyclam (○), (H281A)-CXCR4 (●), and (H113A)-CXCR4 (■). Data are shown as mean ± S.E. (n = 3–10).](image-url)
Non-peptide Monocyclam Antagonists for the CXCR4 Receptor

FIGURE 7. Residues identified through mutagenesis to be important for the action of AMD3100, AMD3465, and AMD3529 shown in a helical wheel diagram of the CXCR4 receptor. The background color indicates the magnitude of the effect of the mutation on the action of AMD3100 (left panel), AMD3465 (middle panel), or AMD3529 (right panel). Gray background indicates <5-fold decrease in inhibitory potency; yellow, 5–50-fold decrease; orange, 50–100-fold decrease; and red, >100-fold decrease in inhibitory potency. The actual inhibitory potencies are shown in Tables 2 and 4.

TABLE 4
Functional analysis of the interaction between CXCR4 WT and mutants with selected AMD monocyclams

| Residue | AMD3465 (IC50 ± SEM, nM) | AMD8721 (IC50 ± SEM, nM) | AMD8899 (IC50 ± SEM, nM) | AMD3529 (IC50 ± SEM, nM) | AMD3388 (IC50 ± SEM, nM) |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|
| wt      | 125 ± 15        | 165 ± 20        | 230 ± 30        | 75 ± 10         | 110 ± 20        |
| wt      | 125 ± 15        | 165 ± 20        | 230 ± 30        | 75 ± 10         | 110 ± 20        |

Measured by inhibition of CXCL12-induced activity

However, it cannot be excluded that certain analogs in fact
are binding in an “opposite mode.” Because the chemical mod-
ifications in the non-macrocyclic, right side of the monocyclam
compounds, such as AMD3465, were introduced to mimic the
other cyclam ring of AMD3100, these chemical moieties will
very likely have some propensity to bind in either of the two
pockets in which the two cyclam rings of AMD3100 are bind-
ing. However, based on the analysis of the mutational maps of the AMD3465 analogs we believe that the preferred binding mode of the monomacrocyclic compounds will be with the cyclam ring in the III-IV-V pocket.

In conclusion, the mutational analysis of AMD3465 as well as a series of analogs of this strongly supports the notion that this prototype monocyclam compound is mimicking the general binding mode of AMD3100 in the CXCR4 receptor in a way where the macrocyclic cyclam moiety of AMD3465 is binding in the III-IV-V pocket in a rather similar way as the corresponding cyclam moiety of AMD3100, whereas the 2-pyridylmethylamine moiety of AMD3465 interacts with residues in the III-VI-VII pocket. However, the 2-pyridylmethylamine moiety of AMD3465 apparently picks up interactions especially with residues located more toward the extracellular end of TM-VI and VII, for example, HisVII—02, which are not noted in the mutational map for AMD3100.

Displacement of Radiolabeled Monoclonal Antibody Is a More Sensitive Assay Than the Inhibition of Chemokine-induced CXCR4 Activation—We chose the competition binding assay with the radiolabeled 12G5 receptor antibody for the “screening” of the CXCR4 mutational library because it was observed that 125I-CXCL12 failed to bind to several of the mutations (11). In addition, 12G5 is a relevant probe to use because the interactions of bicyclams with CXCR4 monitored by the inhibition of 12G5 binding follow a similar structure-activity relationship as for the inhibition of HIV cell entry (10, 27–29). Interestingly, in general the displacement of 125I-12G5 binding was more sensitive and resulted in larger fold changes in the affinities (Table 1) compared with the changes observed in the inhibitory potencies measured in the PI turnover assay (Table 2). Examples are the >4500-fold decrease in affinity for the binding of AMD3465 to (D262N)-CXCR4 and the >5000-fold decrease in affinity for the binding to (E288A)-CXCR4 (Table 1), in contrast to the 800- and 160-fold decreases observed in inhibitory potencies for AMD3465 in these two mutations (Table 2). Conceivably, proper interaction with the key residues for mono- and bicyclams is more important for the displacement of monoclonal antibody than for the inhibition of CXCL12-induced activation. The displacement of 125I-CXCL12, which was employed for a selection of mutations in addition to 125I-12G5, resulted in small affinity changes (for example, decreases from 17- to 58-fold in the affinity of AMD3465 and AMD3100 for (D262N)- and (H281A)-CXCR4 compared with WT CXCR4, data not shown) that were closer to the changes in inhibitory potencies (Table 2), than to the larger affinity changes obtained in competition against 125I-12G5 (Table 1). This indicates that it is the antibody (structure, size, binding epitope?), and not the chosen method (competition binding versus signaling assay) that determines the observed sensitivity.

Not One, but Several, Different CXCR4 Blocking Agents for Clinical Use—The bicyclams were in fact discovered as antiviral HIV compounds before the identification of CXCR4 as a HIV cell entry co-receptor (7, 30). Structure-function studies have shown that optimal conditions for potent antiviral effects of the bicyclam analogs require that the two cyclam moieties are linked by a para-(like AMD3100) or meta-disubstituted phenylenebismethylene linkers at the nitrogen positions (Fig. 1). Various aliphatic linkers have also been probed, yet the conformational constraint imposed by heterearomatic linkers results in higher affinity and potency of the bicyclam compounds (7, 10, 28, 29, 31). Despite the potent antiviral effects of AMD3100, the requirements for parenteral administration limit its long-term use as an anti-HIV compound (13–15). However, for short-term treatment AMD3100 is highly efficient, for instance, when applied together with granulocyte-colony stimulating factor for stem cell mobilization in patients undergoing bone marrow transplantation (32). For long-term use, a novel series of monocyclam compounds have been developed to improve the pharmacokinetic and eliminate the cytotoxic properties (16). The prototype monocyclam AMD3465, described here, contains a 2-pyridylmethylamine moiety replacing one of the four nitrogen cyclam rings. Like AMD3100, AMD3465 suffers from lack of oral bioavailability, and the monocyclams could therefore be considered to constitute “medicinal chemical intermediates” toward the development of orally active non-cyclam CXCR4 antagonists, exemplified by AMD11070, in which the second cyclam ring has been substituted with a more drug-like chemical moiety (33). In the present study we find that the monocyclam AMD3465 is a highly efficient inhibitor of CXCR4 signaling and of HIV cell entry through CXCR4 as compared with AMD3100. In general, AMD3465 appears to mimic the general binding mode of AMD3100, but in a mode where the non-cyclam part picks up novel interactions especially with residues located more toward the extracellular end of TM-VI and VII. It is envisioned that AMD11070 and other non-macrocyclic CXCR4 antagonists, which recently have been developed on the basis of initially AMD3100 and subsequently AMD3465 as “intermediates” (33) may in fact mimic the interaction pattern of these two compounds with essential interactions at each end of the main ligand binding pocket of the CXCR4 receptor (10, 11, 26).

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