Molecular Mechanism of AMD3100 Antagonism in the CXCR4 Receptor

TRANSFER OF BINDING SITE TO THE CXCR3 RECEPTOR*

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Received for publication, August 27, 2003
Published, JBC Papers in Press, October 28, 2003, DOI 10.1074/jbc.M309546200

AMD3100 is a symmetric bicyclam, prototype non-peptide antagonist of the CXCR4 chemokine receptor. Mutational substitutions at 16 positions located in TM-III, -IV, -V, -VI, and -VII lining the main ligand-binding pocket of the CXCR4 receptor identified three acid residues: Asp171 (AspIV:20), Asp262 (AspVI:23), and Glu288 (GluVII:06) as the main interaction points for AMD3100. Molecular modeling suggests that one cyclam ring of AMD3100 interacts with Asp171 in TM-IV, whereas the other ring is sandwiched between the carboxylic acid groups of Asp262 and Glu288 from TM-VI and -VII, respectively. Metal ion binding in the cyclam rings of AMD3100 increased its dependence on Asp262 and provided a tighter molecular map of the binding site, where borderline mutational hits became clear hits for the Zn(II)-loaded analog. The proposed binding site for AMD3100 was confirmed by a gradual build-up in the rather distinct CXCR3 receptor, for which the compound normally was confirmed by a gradual build-up in the rather distinct CXCR3 receptor, for which the compound normally had no effect. Introduction of only a Glu at position VII:06 and the removal of a neutralizing Lys residue at position VII:02 resulted in a 1000-fold increase in affinity of AMD3100 to within 10-fold of its affinity in CXCR4. We conclude that AMD3100 binds through interactions with essentially only three acidic anchor-point residues, two of which are located at one end and the third at the opposite end of the main ligand-binding pocket of the CXCR4 receptor. We suggest that non-peptide antagonists with, for example, improved oral bioavailability can be designed to mimic this interaction and thereby efficiently and selectively block the CXCR4 receptor.

The CXCR4 receptor is expressed much more broadly than chemokine receptors in general, i.e. not only on a wide variety of leukocytes but also on cells outside the immune system; for example, in the central nervous system (1, 2). In contrast to many chemokine receptors, the CXCR4 receptor is only activated by a single chemokine ligand, stromal cell-derived factor (SDF-1α, also called CXCL12). Like many chemokine receptors, the CXCR4 receptor is involved in the control of migration and tissue targeting, i.e. homing of leukocytes. Importantly, SDF-1α and the CXCR4 receptor play a central role for the anchorage of CD34+ stem cells in the bone marrow. The importance of the receptor is emphasized by the fact that targeted deletion of either the gene for CXCR4 or for its ligand in both cases leads to embryologic lethality (3–5). This is only rarely observed in knockouts of genes for chemokine receptors and, in fact, also rarely for 7TM G protein-coupled receptors in general. The CXCR4 receptor is also expressed on many different types of cancer cells, and it seems to function both as a survival factor and to be responsible for the “chemotactic” spread of cancer cells as metastasis, for example, to the bone marrow where the SDF-1α ligand for the receptor is produced in large quantities.

AMD3100, which is composed of two 1,4,8,11-tetraazacyclotetradecane (cyclam) moieties connected by a conformationally constraining linker, is a prototype non-peptide antagonist of the CXCR4 receptor. The compound was discovered as an anti-HIV agent long before it was realized that it functioned through specific blockade of the CXCR4 receptor, which is used as a co-receptor for cell entry by so-called X4 strains of the AIDS virus. AMD3100 is a specific CXCR4 antagonist that inhibits the binding and function of SDF-1α with high affinity and potency (6, 7). AMD3100 has been shown to block the outgrowth of all X4 as well as dual-tropic (T cell- and macrophage-tropic) HIV variants in vitro. During the development of AMD3100, it was discovered that the compound increases white blood cell counts in the blood and, importantly, that the compound is able to mobilize stem cells from the bone marrow. Thus, currently the compound, in combination with G-CSF, is in clinical trials for stem cell mobilization for auto-transplantations in, for example, multiple myeloma patients. In fact, strong evidence has been presented which indicates that many other stem cell mobilizing regimes, such as cyclophosphamide and G-CSF, act through disruption of the SDF-CXCR4 system, thus emphasizing the crucial role of this system in the control of stem cell homing and mobilization (8).

In an initial study with just a few targeted mutations, we identified two acidic residues, Asp171 (AspIV:20) and Asp262 (AspVI:23), that are located in the main ligand-binding pocket of the CXCR4 receptor as being important for the binding of...
AMD3100 (9). That study was based on the knowledge of the strong preference of the cyclam moiety for interactions with carboxylic acid groups (21). Asp<sup>171</sup> and Asp<sup>262</sup> were also found to be essential for the function of the CXCR4 receptor as a co-receptor for HIV (10). The present study was aimed at performing a rather exhaustive mutational analysis (Fig. 1) of the molecular mechanism of action of AMD3100 using the monoclonal antibody 12G5 and not SDF-1α as the radioligand. The reason for this is that the binding of the endogenous chemokine ligand was affected by certain mutations down in the main ligand-binding pocket, which limited the number of residues that could be addressed with that ligand as a probe. The 12G5 antibody is a relevant probe to use because the interactions of bicycals with CXCR4 monitored by the inhibition of 12G5 binding follows a similar structure-activity relationship for the inhibition of HIV-replication (9, 11–13). Importantly, the rather simple binding mode of the prototype CXCR4 non-peptide antagonist AMD3100, which was found through mutational disruption of the binding of the non-peptide compound in the CXCR4 receptor, was proven through the transfer of the essential components of this binding site to the binding pocket of the otherwise rather distinct CXCR3 receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human chemokines CXCL12/SDF-1, CXCL10/IP10, and CXCL11/ITAC were purchased from Peprotech. The CXCR4-specific monoclonal antibody 12G5 was kindly provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA). Human CXCR4 was kindly provided by Timothy N. C. Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland), and human CXCR3 was kindly provided by Kuldeep Neote (Pfizer Inc., Groton, CT). The promiscuous chimeric G-protein Go<sub>a5</sub>emyr was kindly provided by Evi Kostenis (7TM-Pharma, Denmark). [<sup>3</sup>H]Nynoxy-inositol (PT6–271) and Bolton-Hunter reagent for iodination of 12G5 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). AG 1-X8 anion-exchange resin was obtained from Bio-Rad.

**Site-directed Mutagenesis**—Point mutations were introduced in the receptors by the polymerase chain reaction overlap extension technique (14) using the wild-type CXCR4 or the wild-type CXCR3 receptor 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<sup>+</sup>. The mutations were verified by restriction endonuclease digestion and DNA sequencing (ARI 310, PerkinElmer).

**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 Bolton-Hunter reagent in a total volume of 50 μl of 0.1 M borate buffer, pH 8.5, for 1 h. The reaction was terminated by the addition of 0.25 ml of the borate buffer supplemented with 0.2 M glycine and the Bolton-Hunter-labeled 12G5 separated from free Bolton-Hunter reagent by column chromatography (Econo-Pac DC10, Bio-Rad; Ref. 15).

**Transfections and Tissue Culture**—COS-7 cells were grown at 10% CO<sub>2</sub> and 37 °C in Dulbecco’s modified Eagle’s medium with glutamax (21885–025, Invitrogen) adjusted with 10% fetal bovine serum, 180 units/ml penicillin, and 45 μg/ml streptomycin (PenStrep). Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (16).

**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 × 10<sup>4</sup> to 1 × 10<sup>5</sup> 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 pmol [125I]12G5 plus unlabeled ligand in 0.5 ml of a 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed quickly twice 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.

**Phosphatidyl-inositol Assay (PI-turnover)**—COS-7 cells were transfected according to the procedure mentioned above. Briefly, 6 × 10<sup>5</sup> cells were transfected with 20 μg of receptor cDNA in addition to 30 μg of the promiscuous chimeric G-protein, Go<sub>a5</sub>emyr, which turns the Gai-
Dissection of the Binding Site for AMD3100 in the CXCR4 Receptor—Based on knowledge of cyclam-carboxylic acid interactions, two acid residues located at each end of the main ligand-binding pocket of the CXCR4 receptor, Asp<sup>171</sup> (AspIV-20) and Asp<sup>262</sup> (AspVI-23), have previously been identified as key interaction points for AMD3100 (9, 19). As shown in Table I and Fig. 2, these 2 positions plus 15 other positions in TM-III, -IV, -V, -VI, and -VII of the CXCR4 receptor were probed by mutational substitutions using the radio-labeled monoclonal antibody, 12G5, as a radioligand. In most cases, the side chain was substituted with the small methyl group of Ala. However, in some cases, a structurally similar but uncharged Asn residue was introduced instead of a charged Asp; in some cases, steric-hindrance mutations were performed through introduction of larger side chains such as Phe or Trp for Ala, Gly, or Ile (20). None of the substitutions impaired 12G5 binding to the CXCR4 receptor as reflected in the <i>K<sub>d</sub></i> and <i>B<sub>max</sub></i> values presented in Table I, indicating that the overall structure as well as the cell surface expression of the receptor was unaffected by the mutations. However, five of the substitutions impaired the binding of AMD3100 more than 10-fold. Substitution of Asp.

**TABLE I**

| Residue | Position | B<sub>max</sub>± SEM | 12G5 | AMD3100 | AMD3479 |
|---------|----------|----------------------|------|---------|---------|
| Number  |          | fmax/10<sup>5</sup> cell | log <i>Kd</i>± SEM | <i>Kd</i> | (N) | log <i>Ki</i>± SEM | <i>Ki</i> | (N) | log <i>Ki</i>± SEM | <i>Ki</i> | (N) | fmut ± SEM |
| wt      |          | 137±37               | -8.90±0.09 | 1.57 (19) | 1.0 | 0.10±0.00 | 0.89 (17) | 1.0 | 0.02±0.00 | 0.02 (9) | 1.0 |
| TM-III  | H113A    | III-09               | 49±14   | 1.87±0.03 | 1.63 (4) | 1.0 | 0.57±0.01 | 0.10 (4) | 3.9 | 0.14±0.05 | 0.5 (18) | 1.0 |
| TM-IV   | T119A    | III-09               | 61±28   | 0.92±0.15 | 0.62 (5) | 0.4 | 0.34±0.09 | 0.46 (5) | 0.8 | 0.88±0.09 | 0.01 (4) | 0.6 |
| TM-V    | T168A    | V-17                 | 85±49   | -9.20±0.19 | 0.63 (6) | 0.4 | 0.13±0.12 | 0.74 (6) | 0.8 | 0.74±0.26 | 0.01 (4) | 0.6 |
| TM-V    | D171N    | IV-20                | 70±11   | -9.06±0.09 | 0.87 (14) | 0.6 | 0.87±0.13 | 0.13 (13) | 15 | 0.65±0.23 | 0.28 (11) | 12 |
| TM-V    | I177A    | IV-23                | 44±15   | -9.29±0.24 | 0.51 (5) | 0.3 | 0.56±0.16 | 0.31 (5) | 3.5 | 0.17±0.31 | 0.68 (4) | 28 |
| TM-V    | A175P    | IV-24                | 42±21   | -8.81±0.12 | 1.55 (4) | 1.0 | 0.45±0.24 | 0.36 (4) | 40 | 0.07±0.34 | 0.86 (3) | 250 |
| TM-V    | V196A    | V-01                 | 27±6    | -9.05±0.10 | 0.89 (4) | 0.6 | 0.53±0.13 | 0.46 (4) | 5.2 | 0.78±0.17 | 0.17 (3) | 68 |
| TM-V    | V200A    | V-05                 | 139±15  | -9.31±0.14 | 0.49 (3) | 0.3 | 0.62±0.10 | 0.56 (3) | 0.6 | 0.75±0.07 | 0.02 (3) | 0.7 |
| TM-V    | V207W    | V-12                 | 117±76  | -9.03±0.32 | 0.93 (4) | 0.6 | 0.52±0.33 | 0.32 (4) | 3.4 | 0.6±0.05 | 0.25 (2) | 10 |
| TM-V    | V255A    | V-16                 | 235±101 | -8.87±0.13 | 1.36 (4) | 0.9 | 0.5±0.30 | 0.34 (3) | 5 | 0.63±0.23 | 0.19 (4) | 7.6 |
| TM-VII  | Y265A    | V-17                 | 172±73  | -8.87±0.16 | 1.36 (6) | 0.9 | 0.5±0.30 | 0.34 (3) | 5 | 0.63±0.23 | 0.19 (4) | 7.6 |
| TM-VII  | D259A    | VI-20                | 40±8    | -9.52±0.12 | 0.30 (3) | 0.2 | 0.81±0.05 | 1.5 (3) | 1.7 | 0.76±0.06 | 0.03 (3) | 1.1 |
| TM-VII  | I259W    | VI-20                | 74±6    | -8.99±0.16 | 1.03 (3) | 0.7 | 0.34±0.33 | 0.46 (3) | 5.2 | 0.68±0.36 | 0.84 (2) | 34 |
| TM-VII  | D262N    | VI-23                | 162±9   | -9.94±0.13 | 1.15 (13) | 0.7 | 0.14±0.15 | 0.46 (13) | 52 | 0.49±0.58 | 0.13 (3) | 522 |
| TM-VII  | S263A    | V-24                 | 101±44  | -9.44±0.20 | 0.36 (3) | 0.2 | 0.27±0.14 | 0.54 (3) | 6.1 | 0.7±0.17 | 0.08 (3) | 3.2 |
| TM-VII  | H258A    | VII-01               | 116±35  | -9.08±0.16 | 0.83 (8) | 0.5 | 0.8±0.21 | 0.16 (11) | 0.2 | 0.74±0.14 | 0.03 (5) | 1.2 |
| TM-VII  | I234A    | VII-02               | 116±16  | -8.83±0.14 | 1.47 (5) | 0.9 | 0.96±0.36 | 1.1 (4) | 1.2 | 0.64±0.07 | 0.23 (3) | 9.3 |
| TM-VII  | E288A    | VII-06               | 140±46  | -9.02±0.06 | 0.96 (16) | 0.6 | 0.20±0.17 | 0.63 (15) | 71 | 0.56±0.32 | 0.23 (3) | 93 |

Fig. 2. Residues identified through mutagenesis to be important for the binding of AMD3100 and AMD3479 (Zn<sup>II</sup>) 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 the Zn<sup>II</sup>-loaded version of AMD3100 (AMD3100[Zn<sup>II</sup>]) (right panel) as shown in fold decrease in affinity compared with wt CXCR4, as determined in competition binding experiments on transiently transfected COS-7 cells with <sup>125</sup>I<sub>2</sub>G5 as the radioligand. Gray background indicates <10-fold decrease in affinity; yellow, 10- to 50-fold decrease; orange, 50- to 100-fold decrease; and red, >100-fold decrease. The actual binding affinities are shown in Table I.
Dissection and Transfer of AMD3100 Site from CXCR4 to CXCR3

Fig. 3. Effect of Glu to Ala substitution at position VII:06 (Glu288) in CXCR4 on the binding of AMD3100 (A) and AMD3100(Zn2) (B). Competition binding experiments with [3H]12G5 as radioligand were performed in transiently transfected COS-7 cells. The competition curves for the unlabeled 12G5 are indicated with squares (A and B): □, wild-type receptor, and ■, E288A-CXCR4. A, displacement with AMD3100 in wild-type receptor (○) and E288A-CXCR4 (●). B, displacement with AMD3100(Zn2) in wild-type receptor (△) and E288A-CXCR4 (▲). Data are shown as means ± S.E. (n = 11–19).

Fig. 4. Effect of Glu to Ala substitution at position VII:06 (Glu288) in CXCR4 on SDF-1-induced PI-turnover and inhibition by AMD3100. 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 in detail under “Experimental Procedures”). Dose-response curves for SDF-1 are shown in A: wild-type receptor (○) and E288A-CXCR4 (▲). Inhibition of SDF-1-induced PLC activity by AMD3100 are shown in B: wild-type receptor (○), E288A-CXCR4 (●), AMD3100(Zn2), wild-type receptor (□), and E288A-CXCR4 (▲). Data are shown as means ± S.E. (n = 3–5).

Ala175, located four residues after AspIV:20 (Asp171) to Asn has previously been shown to affect AMD3100 38-fold in competition against the natural chemokine ligand SDF-1α (9). However, when using 12G5 as a radioligand, this substitution only resulted in a 15-fold impairment of AMD3100 binding. In contrast, substitution of AlaIV:24 (Ala175, located four residues after AspIV:20) with a Phe residue affected AMD3100 binding 40-fold. In the x-ray crystal structure of rhodopsin, the residue which corresponds to Ala175 is located at the start of extracellular loop 2 facing down into the main ligand-binding pocket. The relatively large effect of the steric-hindrance substitution of Ala175 with Phe is in good agreement with the assumption that one of the cyclam rings of AMD3100 should be located in the pocket between TM-III, -IV, and -V, i.e. in front of Asp171, which is where the large aromatic side chain of the introduced Phe residue would be expected to be located in the (A175F) mutant.

None of the substitutions in TM-V affected AMD3100 more than 5-fold, whereas in TM-VI, the mutational analysis (as expected) pointed to AspVI:23 (Asp262) but also to TyrVI:16 (Tyr261) as being interaction points for AMD3100. Surprisingly, substitution of IleVI:20 (Ile259, located in between these two residues and facing right into the middle of the main ligand-binding pocket) had only minimal effect on AMD3100 binding (Table 1 and Fig. 2). Even the introduction of a large Trp residue in position VI:20 only gave a 5-fold effect on AMD3100 binding.

In TM-VII, substitution of IleVII:02 (Ile284) had no effect on AMD3100 binding, despite the fact that this residue (like IleVI:20) is facing directly into the main pocket and at a location close to AspVI:23. In contrast, substitution of AspVII:06 (Asp288) to Ala affected AMD3100 around 70-fold. Moreover, a number of substitutions which had given small, single-digit effects for AMD3100 now gave clear, 5.2-fold (ValV:01 or Val196), 10-fold, and 5.2-fold (ValV:01 or Val196), and 34-fold (IleVI:20 or Ile259) (Table 1 and Fig. 2).

Important Acidic Residue in TM-VII for the Binding of AMD3100—GlufVII:06 (Glu288) in the cyclam rings of AMD3100 is known to increase its affinity for the CXCR4 receptor around 35-fold (Table 1; Refs. 21 and 22). Previously, we have found that this increased affinity in fact is determined by only one of the GlufVII:06 ions in one of the cyclam rings and that the effect is achieved through interaction with the carboxylic group of AspVII:23 (Asp282) (22). When the GlufVII:06-loaded AMD3100 (also called AMD3479) was probed in the library of mutated CXCR4 receptors, an interesting picture emerged. The four to five residues that were identified as interaction points for AMD3100 itself were all positive, but the effects of the mutations were in all cases (except for Asp171) larger for the GlufVII:06-loaded version than for AMD3100 alone (Table 1 and Fig. 2).

Moreover, a number of substitutions which had given small, single-digit effects for AMD3100 now gave clear, i.e. > 10-fold, effects for the GlufVII:06-loaded version: 28-versus 3.5-fold (PheIV:23 or Phe174), 68-versus 5.2-fold (ValIV:01 or Val196), and 34-versus 5.2-fold (IleVI:20 or Ile259) (Table 1 and Fig. 2).
sidered to be a reflection of steric hindrance. As shown in Fig. 3, by using $^{125}$I12G5 as a radioligand, the Glu$^{288}$ to Ala substitution shifted the competition binding curve for AMD3100 with or without Zn(II) 70- to 90-fold to the right. We were unable to use $^{125}$I-SDF-1α as radioligand in binding experiments in this construct because of a lack of specific binding (data not shown). This is very likely a reflection of a decreased affinity of SDF-1α on the Glu$^{288}$ to Ala mutant receptor, as the potency of SDF-1α in signaling was impaired 87-fold in this construct (Fig. 4A). Thus, Glu$^{288}$ seems to be a residue that is critical for the function of SDF-1α as an agonist on the CXCR4 receptor. By using appropriate sub-maximal doses of SDF-1α on the wild-type and on the Glu$^{288}$ to Ala mutant form of CXCR4, respectively, it was possible to demonstrate that, not only the affinity (Table I and Fig. 3), but also the potency of AMD3100 (with or without Zn(II)) were highly dependent on the presence of a Glu in position VII:06. Thus, in the wild-type CXCR4, receptor potencies of 79 nM and 17 nM were observed for AMD3100 and the Zn(II)-loaded version, respectively, whereas right shifts of 23- to 116-fold of the bicyclic inhibition curves were observed in the Glu$^{288}$ to Ala mutant form of CXCR4 (Fig. 4B).

Thus, the overall picture for the AMD3100 binding mode is that it is critically dependent on two acid residues at the extracellular ends of transmembrane segments VI and VII, AspV:23 and GluVII:06, respectively, plus one Asp residue located at the opposite end of the main ligand-binding pocket, i.e. in position IV:20. Surprisingly, few other residues that are known to be lining each end of the pocket as well as the space in between seem to be directly involved in the recognition of the relatively large bicyclic compound. For the Zn(II)-loaded version of AMD3100, a similar picture is observed; however, the dependence on Asp$^{288}$ is increased 10-fold, and a number of other residues on the inner face of TM-IV, -V, and -VI are picked up in the mutational analysis.

Transfer of the Binding Site for AMD3100 to the CXCR3 Receptor—A survey of the main ligand-binding pocket of all human 7TM receptors revealed that the combination of AspIV:20, AspV:23, and GluVII:06 is unique to the CXCR4 receptor, which is in agreement with the fact that AMD3100 is known to be a highly selective antagonist for the CXCR4 receptor (23). However, among the chemokine receptors, we verified that the CXCR3 receptor had two of the three residues, i.e. AspIV:20 and AspV:23, but that it had a Ser residue in position VII:06 instead of a Glu as in CXCR4 (Fig. 5). Interestingly, at position VII:02, a Lys residue is located in the CXCR3 receptor (Lys$^{300}$), which very likely will form a neutralizing salt bridge with AspV:23 (Asp$^{278}$) because of the close proximity of the extracellular ends of TM-VI and VII (Fig. 5). Thus, in the CXCR3 receptor, it is very likely that of the three proposed key residues, only one, AspIV:20 (Asp$^{186}$), is available for interaction with AMD3100. To try to verify the proposed binding site for AMD3100, which in the CXCR4 receptor is based on mutational “destruction” of the binding and function of the non-peptide antagonist, we attempted to use the binding pocket of the CXCR3 receptor as a scaffold to gradually build up the binding site for the antagonist.

As expected, AMD3100 had no effect on the inositol phosphate accumulation induced by the agonist chemokines ITAC or IP10 in the CXCR3 receptor. Moreover, neither introduction of Glu at position VII:06 nor substitution of LysVII:02 with a non-neutralizing Ala residue changed this (Fig. 6). However, in the CXCR3 construct, where these two substitutions were combined to ensure the presence and exposure of all three proposed key interacting residues for AMD3100, the bicyclic antagonist inhibited in a dose-dependent manner both the ITAC and the IP10-induced signaling, with IC$_{50}$ values of 1.1 and 9.3 μM, respectively (Fig. 6). However, only a partial inhibition down to 35–40% of the maximal stimulation was observed with AMD3100. As shown in Fig. 7, this was not the case for the Zn(II)- or Ni(II)-loaded analogs of AMD3100 which acted as full antagonists.

Metal Ion-loaded Versions of AMD3100 Are More Potent and Efficacious Antagonists—Besides the fact that the metal ion-loaded versions of AMD3100 completely inhibited the agonist activity in the “CXCR4-mutated” CXCR3 receptor (Fig. 7), a surprisingly high potency of these compounds was observed. Thus, for the Zn(II)-loaded AMD3100, left-shifts of 19- to 69-fold were observed in the inhibition of the ITAC- and IP10-induced activity, whereas for the Ni(II)-loaded AMD3100, left-shifts of 4- to 70-fold were observed (Fig. 7).

Gradual Construction of the AMD3100-binding Pocket in CXCR3—When the Zn(II)-loaded AMD3100 was probed, it became clear that its binding site could gradually be built up in
the CXCR3 receptor to as close as 4- to 8-fold from the affinity of the CXCR4 receptor (Fig. 8). Thus, just the Ala substitution of LysVII:02 (K300A), which is proposed to "neutralize" AspVI:23 (Fig. 5), shifted the dose-response curve for AMD3100 43-fold to the left in inhibition of IP10-induced signaling (Fig. 8B). Introduction of Glu in position VII:06 (S304E) had an even larger effect, 113-fold, and the combination of the two substitutions (K300A,S304E) gave a potency for AMD3100 of 1420-fold improvement for this construct.

**Probing Additional Presumed Interaction Points for AMD3100 in the CXCR3 Receptor**—It was assumed above that in the CXCR3 receptor, the two naturally occurring Asp residues in positions IV:20 (Asp186) and VI:23 (Asp278) formed the basis for the high affinity binding of the AMD3100 bicyclam when they were combined with the Glu, which was introduced at position VII:06 (and when the disturbing LysVII:02 had been removed). This assumption was supported by the observation that the dose-response curve for the Zn(II)-loaded AMD3100.

**Fig. 7.** Improvement of the inhibitory potency and efficacy of AMD3100 in inhibiting agonist-induced IP turnover in the K300A,S304E-CXCR3 receptor. Dose-dependent inhibition of 10 nM ITAC (A and B) or 10 nM IP10 (C and D) induced IP turnover by AMD3100 (■) and by the Zn(II)-loaded version (▲) (A and C) or the Ni(II)-loaded version (●) (B and D). Data are shown as means ± S.E. (n = 3–5).
inhibition of ITAC was shifted respectively 51-fold and 38-fold to the right when either AspIV:20 (D186N) or AspVI:23 (D278N) were mutated to Asn in the K300A,S304E-CXCR3 receptor. For comparison, the location of the dose-response curve for AMD3100(Zn2) on SDF-1-induced PI-turnover in the CXCR4 receptor is shown as a dotted line. Data are shown as means ± S.E. (n = 3–4).

**DISCUSSION**

In the present study, a rather exhaustive mutational analysis of essentially all residues facing the main ligand-binding pocket of the CXCR4 receptor identified only three residues, all acidic, located in TM-IV, -VI, and -VII as being critical for the binding of the bicyclam non-peptide antagonist AMD3100 (Fig. 10). The assumption that AMD3100 acts through binding to essentially only these three anchor-point residues was confirmed through the construction of the tri-acidic motif in the binding pocket of the CXCR3 receptor, which is structurally rather distinct in the remaining part of the main ligand-binding pocket. Nevertheless, it bound AMD3100 with an affinity within 10-fold of that of the CXCR4 receptor after the three acidic residues were all made accessible in the correct CXCR4-like positions in the CXCR3 pocket.

**The Binding Mode of AMD3100 with and without Zn(II) in the CXCR4 Receptor**—From a chemical point-of-view, AMD3100 is a rather atypical non-peptide antagonist, which probably relates to the way it was originally discovered (24). A characteristic feature of the vast majority of non-peptide ligands for 7TM receptors is that they are composed of two or usually more aromatic, hydrophobic moieties connected through some conformationally constraining bonds. However, the only aromatic part of AMD3100 is the phenyl moiety in the 1,4-dimethylene(phenylene) linker, which connects the two cyclam rings. Previous structure activity relationship analysis demonstrated that the function of this linker is not based on its aromaticity but rather on the fact that it simply constrains the mobility and distance between the two macrocycles (12, 13). In agreement with this, our mutational analysis of the CXCR4 receptor did not point to a dependence of AMD3100 upon side chains of any residue located in the part of the main ligand-binding pocket that would be expected to be where the linker was located (Table I), i.e. on the inner face of TM-III, -V, or -VI, for example. This point is emphasized by the observation that an affinity of almost the same magnitude could be obtained in the mutated CXCR3 receptor, despite the fact that this receptor has, for example, two Args on the inner face of TM-V where CXCR4 has a Val and a Gln, and that CXCR3 has a Gly and a Phe on the inner face of TM-III where CXCR4 has a His and a Leu, respectively (Figs. 1 and 5).

The two large, symmetrical cyclam macrocyclic rings are also very atypical chemical moieties in 7TM small molecule ligands. However, although these rings are complex structures, the fact that they have a strong preference for binding to carboxylic acid groups and that they can do so in a couple of different but strong manners we believe offers a unique key to understanding the overall binding mode and molecular mechanism of action of AMD3100 in the CXCR4 receptor. At physiological pH, the cyclam ring has an overall charge of +2 and can adopt the most stable so-called trans-III R,R,S,S type of conformation at the four nitrogen atoms (25, 26). X-ray and neutron diffraction analysis of a protonated cyclam complex with 4-tert-butylbenzoic acid demonstrated a direct complex of the ring system with a carboxylic acid group (27). Three non-equivalent hydrogen bonds (one strong, one intermediate and one weak) could be formed through the oxygens of the carboxylic acid and the amines of the cyclam ring (27). With Zn(II) or another divertal transition metal ion bound in the middle of the ring, cyclam adopts the thermodynamically favored trans-III configuration to also give a complex with an overall charge of +2 (6, 28, 29). In complexation with carboxylic acids, the metal ion coordinates the uni-dentate C-O , whereas the non-coordinated oxygen of the carboxylate group forms a hydrogen-bond with a secondary amine proton of the ring (7, 28). Recently, x-ray and NMR analysis of the bis-zinc complex of AMD3100 demon-
As indicated above, there are several possible conformations of the cyclam ring with and without metal ions. With a bound metal ion, one possible configuration of the ring that is bound to the two acidic residues (one on each side) would be the cis-V conformation suggested by Sadler and coworkers, where the Asp262 should be the one coordinating the metal ion and GluVII:06 the one binding through hydrogen-bonds to the opposite face of the ring (30). Conceivably, AspVII:06 would interact mainly through hydrogen-bond formation to the other cyclam ring or at least in a manner in which the metal ion does not provide any further advantage (22). Although the carboxylates of each of the acidic residues should, in principle, be able to interact optimally with a metal-loaded free cyclam ring, it is only Asp262 which, in fact, is able to do so in the bicyclam AMD3100. Conceivably, steric clashes of the ring or conformational constraints induced by the binding of both of the rings at the same time in the binding pocket apparently prevent an optimal interaction of both of the rings at the same time.

It should be noted that, although the combination of AspIV:20, AspVI:23, and GluVII:06 is unique to the CXCR4 receptor, GluVII:06 (i.e. GluVII:06 in the CXCR4 receptor) is a residue which is conserved among the majority of chemokine receptors. Interestingly, it is not only in the CXCR4 receptor that GluVII:06 is an essential interaction point for non-peptide ligands. Thus, it has been shown that GluVII:06 is the most important interaction point for non-peptide antagonists in the CCR2 receptor (31, 32). Similarly, in the homologous CCR5 receptor, it has been found that several classes of non-peptide antagonists are highly dependent upon GluVII:06 (33–35). Thus, it seems that GluVII:06 is a very useful anchor-point for non-peptide antagonists in chemokine receptors and possibly can be used in basically all receptors that express this residue. In this context, it is very interesting that an acidic residue in this position (VII:06) is rather specific for chemokine receptors and, thus, only rarely found in 7TM receptors in general (36). In addition to the CCR2, CCR5, and the CXCR4 receptors, non-peptide antagonists have been discovered and developed for several other chemokine receptors, as for instance the CCR1, CCR3, and the CXCR2 receptor (37–39).

Development of Novel CXCR4 Antagonists—AMD3100 is an efficient CXCR4 antagonist, which currently is in clinical trials as a stem cell mobilizing agent as an adjunct to G-CSF. The compound has a total of four positive charges at neutral pH and is not bioavailable orally. However, stem cell mobilization is an indication where parenteral administration is well accepted. Nevertheless, the CXCR4 receptor is potentially a very interesting target for several other indications such as blockade of HIV cell entry and chronic inflammation (1, 3, 40); for these indications, an orally active compound would be preferred. AMD3100 has some excellent properties as a CXCR4 antagonist, which is currently is in clinical trials as a stem cell mobilizing agent as an adjunct to G-CSF. The compound has a total of four positive charges at neutral pH and is not bioavailable orally. However, stem cell mobilization is an indication where parenteral administration is well accepted. Nevertheless, the CXCR4 receptor is potentially a very interesting target for several other indications such as blockade of HIV cell entry and chronic inflammation (1, 3, 40); for these indications, an orally active compound would be preferred. Therefore, it is very interesting that an acidic residue in this position (VII:06) is rather specific for chemokine receptors and, thus, only rarely found in 7TM receptors in general (36). In addition to the CCR2, CCR5, and the CXCR4 receptors, non-peptide antagonists have been discovered and developed for several other chemokine receptors, as for instance the CCR1, CCR3, and the CXCR2 receptor (37–39).

Fig. 10. Molecular model of the presumed binding mode of AMD3100(Zn2+). The receptor model is built over the x-ray crystal structure of rhodopsin (43) and shows the interaction of one of the cyclam rings of AMD3100(Zn2+) with AspIV:20 (AspVII:06 in CXCR4 and AspVII:06 in CXCR3), whereas the other cyclam ring is “sandwiched” between AspVI:23 (AspVII:06 in CXCR4 and AspVII:27 in CXCR3) and GluVII:06 (GluVII:06 in CXCR4, which is a Ser residue in CXCR3 and part of the reason why AMD3100 does not bind to the wild-type CXCR3 receptor).

As presented in this paper could serve as the basis for the design of non-macrocyclic (i.e. chemically distinct) compounds which nevertheless could mimic the action of AMD3100 on the CXCR4 receptor.

Acknowledgment—We thank Lisbet Elbak for excellent technical assistance.
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