Molecular Interactions of Cyclam and Bicyclam Non-peptide Antagonists with the CXCR4 Chemokine Receptor*

Received for publication, November 17, 2000

Published, JBC Papers in Press, January 11, 2001, DOI 10.1074/jbc.M010429200

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The non-peptide CXCR4 receptor antagonist AMD3100, which is a potent blocker of human immunodeficiency virus cell entry, is a symmetrical bicyclam composed of two identical 1,4,8,11-tetraazacyclotetradecane (cyclam) moieties connected by a relatively rigid phenylenebismethylene linker. Based on the known strong propensity of the cyclam moiety to bind carboxylic acid groups, receptor mutagenesis identified Asp171 and Asp262, located in transmembrane domain (TM) IV and TM-VI, respectively, at each end of the main ligand-binding crevice of the CXCR4 receptor, as being essential for the ability of AMD3100 to block the binding of the chemokine ligand stromal cell-derived factor (SDF-1α) as well as the binding of the receptor antibody 12G5. The free cyclam moiety had no effect on 12G5 binding, but blocked SDF-1α binding with an affinity of 3 μM through interaction with Asp171. The effect on SDF-1α binding of a series of bicyclam analogs with variable chemical linkers was found to rely either only on Asp171, i.e. the bicyclams acted as the isolated cyclam, or on both Asp171 and Asp262, i.e. they acted as AMD3100, depending on the length and the chemical nature of the linker between the two cyclam moieties. A positive correlation was found between the dependence of these compounds on Asp262 for binding and their potency as anti-human immunodeficiency virus agents. It is concluded that AMD3100 acts on the CXCR4 receptor through binding to Asp171 in TM-IV and Asp262 in TM-VI with each of its cyclam moieties, and it is suggested that part of its function is associated with a conformational constraint imposed upon the receptor by the connecting phenylenebismethylene linker.

Bicyclams derivatives composed of two cyclam (1,4,8,11-tetraazacyclotetradecane) units linked by an aliphatic or aromatic linker (see Fig. 1) have been identified as potent inhibitors of human immunodeficiency virus (HIV) type 1 and type 2 replication (1). The lead compound of the bicyclam series was discovered as an impurity being responsible for the antiviral effect in a preparation of cyclam (2). The prototype bicyclam, AMD3100, in which the two macrocyclic rings are connected by a phenylenebismethylene linker (Fig. 1), has a potency against HIV-1 and HIV-2 replication of 1–10 nM (3) and is currently used as the first coreceptor antagonist in Phase II clinical trials against AIDS (4).

The molecular mechanism of action of AMD3100 was initially believed to be related directly to the viral gpl20 protein (5). The problem was that the bicyclams were discovered in 1989, i.e. long before details of the molecular machinery on the target cell responsible for HIV cell entry had been characterized. Shortly after the discovery of the HIV coreceptors, it was shown that the bicyclams in fact inhibit HIV replication by binding to the CXCR4 chemokine receptor, which is the main coreceptor for gp120 used by X4, T-tropic strains of HIV for membrane fusion and cell entry (6–8). Thus, AMD3100 was the first non-peptide CXCR4 receptor antagonist discovered and optimized long before the receptor was shown to bind the SDF-1α chemokine.

The structure-function relationship with respect to the antiviral effect of the bicyclams has been worked out in great detail by De Clercq et al. (1) and Bridger et al. (2, 9–11). The optimum structural features required for potent antiviral activity of "bicyclam" analogs include the presence of two azamacroyclic rings containing 12–14 atoms/ring and a meta- or para-substituted phenylenebismethylene linker (para more active than meta) connecting the macrocyclic rings at nitrogen positions. The introduction of electron-withdrawing or -donating groups to the aromatic ring did not adversely affect antiviral activity; however, sterically hindering groups that restrict rotation of the macrocyclic rings at the benzyl position had a detrimental effect on antiviral potency.

The relatively simple, symmetric structure of AMD3100 combined with the detailed knowledge of its structure-function relationship offer particular opportunities for understanding the molecular interaction of this non-peptide antagonist with its receptor target. It could, for example, be envisioned that the cyclam moieties by themselves could make strong enough interactions with the receptor for their binding sites to be identified by mutagenesis. The isolated 1,4,8,11-tetraazacyclotetradecane ring has an overall charge of +2 at physiological pH (12), and x-ray and neutron diffraction structures have shown that the protonated cyclam ring has the propensity to form a direct, hydrogen-bonded stabilized complex with carboxylic acid groups (13). In accordance with this, initial mutagenesis studies have shown that acidic residues in the second extracellular loop and in transmembrane domain (TM) IV of the CXCR4 receptor apparently are involved in the antiviral effect of AMD3100 (14). The cyclam rings are also known to be able to chelate metal ions (15); and recently, it has been shown that transition metal ions chelated by the two macrocyclic rings of AMD3100 increase the CXCR4-binding affinity and potency of AMD3100 by 10-fold (16). This could indicate that, for example,
metal ion-binding His residues, of which there are several in the main ligand-binding crevice of the CXCR4 receptor, could be involved in the binding of the bicyclam compounds (Fig. 2).

In this study, we have, based on the knowledge of the possible binding mode of the cyclam as such, initially targeted aspartate and histidine residues in the CXCR4 receptor for mutagenesis and combined this with studies of a series of bicyclam analogs with variable chemical linkers. The two major hits for AMD3100 binding (one of which was shown to be involved in cyclam binding) were located at each end of the main ligand-binding pocket, which we previously have analyzed in great detail in several other 7TM receptors, for example, by metal ion site engineering (17–19). In a molecular model of the CXCR4 receptor built over the recently published x-ray structure of rhodopsin (20), AMD3100 could be manually docked directly in the main ligand-binding crevice of the CXCR4 receptor, could be involved in the binding of the bicyclam compounds (Fig. 2).

The human chemokine Met-SDF-1 was kindly provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA). Met-SDF-1 was 125I-labeled using Bolton-Hunter reagent (Amersham Pharmacia Biotech) as described (26). AMD3100, AMD3106, AMD3108, AMD2763, AMD2849, AMD3389, and AMD2936 were synthesized as described (9, 11). Cyclam (1,4,8,11-tetraazaclotetradecane) was purchased from Aldrich.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—**Point mutations were introduced in the receptor by the polymerase chain reaction overlap extension technique (21) using the human wild-type CXCR4 receptor cDNA as template. All reactions were carried out using the Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. The generated fragments were subcloned into the pTEJ-8 eukaryotic expression vector (22) containing the wild-type CXCR4 receptor cDNA by substituting the wild-type cDNA fragment with the mutant cDNA fragment. The mutations were verified by restriction endonuclease digestion and DNA sequencing (ABI 310, PerkinElmer Life Sciences).

**Expression of Mutant Receptors—**COS-7 cells were grown at 10% CO2 and 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 µg/ml gentamycin. The wild-type and mutant CXCR4 receptors were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as described previously (23).

**Ligands—**The human chemokine Met-SDF-1α was kindly provided by Michael A. Luther (Glaxo Wellcome). This SDF-1α contains an additional NH2-terminal methionine; however, the protein shows the same binding properties as natural ligand SDF-1α (24, 25). 125I-Labeled Met-SDF-1α was prepared by oxidative iodination using IODO-GEN (Pierce), followed by high pressure liquid chromatography purification to separate unlabeled from labeled compound. The monoclonal antibody 12G5 was kindly provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA). 12G5 was 125I-labeled using Bolton-Hunter reagent (Amersham Pharmacia Biotech) as described (26). AMD3100, AMD3106, AMD3108, AMD2763, AMD2849, AMD3389, and AMD2936 were synthesized as described (9, 11). Cyclam (1,4,8,11-tetraazaclotetradecane) was purchased from Aldrich.

**Results**

**Binding of Cyclam Versus Bicyclam to CXCR4—**In COS-7 cells transiently expressing the human CXCR4 chemokine receptor, cyclam (1,4,8,11-tetraazaclotetradecane) competed for 125I-Met-SDF-1α binding with an affinity (Kd) of 13 µM, whereas it was unable to displace the radiolabeled monoclonal receptor antibody (125I-12G5) from CXCR4 even at millimolar concentrations (Fig. 3). In contrast, the bicyclam compound AMD3100, in which two identical cyclam rings have been joined by a phenylenebis(methylene) linker, showed a 200-fold higher affinity compared with the chemokine ligand (Fig. 3).

**Effect of Asp and His Mutations on Cyclam Binding—**Asp and His residues in the CXCR4 receptor were targeted for mutagenesis based on the assumption that each cyclam ring of AMD3100 and analogs would be doubly protonated at physiological pH or conceivably would be able to complex transition...
SDF-1 receptor expressed in COS-7 cells using either 125I-Met-SDF-1. Whole cell competition binding was performed on the wild-type CXCR4 or the isolated 1,4,8,11-tetraazacyclotetradecane (cyclam) (Fig. 2). In addition, four Asp residues (Asp 171, Asp 182, Asp 193, and Asp 262 (located in extracellular loops or in the transmembrane domains (TM) IV and V) were mutated to Asn (Fig. 3). Based on these mutagenesis results and on the assumption that the cyclam moiety could directly interact with the carboxylic acid group of Asp residues (2), it was presumed that AMD3100 would bind in between the transmembrane segments, with one cyclam moiety toward Asp 171 in TM-IV and the other interacting with Asp 262 in TM-VI. This binding mode for AMD3100 was also compatible with molecular models of the CXCR4 receptor (see “Discussion”). To probe this binding mode further, a series of analogs of AMD3100 with different linkers between the two cyclam moieties were tested in the Asp 171 and Asp 262 mutants.

**Binding Mode of Bicyclam Analogs**—In AMD2763 and AMD2849, the aromatic phenylene(ethylene) linker of AMD3100 was substituted with an aliphatic linker consisting of either three or six methylene groups. Of these, AMD2849 with the long aliphatic linker behaved almost like two “free” cyclam molecules, whereas AMD2763 with the short linker behaved more like the bicyclam AMD3100. Thus, the affinity of AMD2763 was only 3-fold lower than that of AMD3100 as monitored in competition with 125I-Met-SDF-1α binding (Table II), and like AMD3100, the high affinity binding of AMD2763 was dependent on both Asp 171 and Asp 262 (Fig. 6A), although the substitution at position 171 affected its binding more than the substitution at position 262. In AMD2849, the extension of the linker between the two cyclam moieties with three more methylene groups compared with AMD2763 decreased the affinity almost to that observed with cyclam, i.e., $K_i = 2.3 \, \text{versus} \, 13 \, \mu\text{M}$ (these figures are even closer when it is taken in account that AMD2849 in fact holds two cyclam moieties). Importantly, AMD2849 binding was (again like cyclam) dependent only on Asp 171 and was not affected by the substitution of Asp 262 (Fig. 6B).

In AMD3106 and AMD3108, the meta-disubstituted phenyl group of the linker of AMD3100 was exchanged with either a 2,6-disubstituted pyridine (AMD3106) or with a 2,4-disubstituted pyridine (AMD3108) (Fig. 1). Of these, the pyridine nitrogen of the 2,4-disubstituted analog (AMD3108) has a strong tendency to form pendant interactions with the adjacent macrocyclic (cyclam) ring, which apparently constrains the conformation of the compound in a way that impairs its function (2, 11). For sterical reasons, this pendant interaction is not possible in the 2,6-disubstituted analog (AMD3106), which therefore should be able to function in a manner very similar to AMD3100 (2). In accordance with this, AMD3106 in the transfected COS-7 cells bound with an affinity almost identical to that of AMD3100, and its binding was affected by the Asp 171 and Asp 262 substitutions in a manner almost identical to that which was observed for AMD3100 (Fig. 7A). In contrast, the affinity of the AMD3106 isomer (AMD3108) was decreased 10-fold compared with AMD3100 and AMD3106, and importantly, the binding of AMD3108 was dependent only on Asp 171 and not on Asp 262 (Fig. 7B). Thus, although AMD3108 binds with a higher affinity than cyclam (albeit still reduces compared with AMD3100), the binding mode of the 2,4-disubstituted pyridine derivative appears to be similar to that of a cyclam, *i.e.*, being independent of Asp 262.

**A High Affinity Monocyclam Analog**—Deletion of one of the macrocyclic rings of AMD3100 resulted in a monocyclam analog (AMD3389), which bound with surprisingly high affinity. AMD3389, which is a cyclam with the attached para-xylylene group (the “linker” in AMD3100), displaced 125I-Met-SDF-1α from the wild-type CXCR4 receptor with an affinity $K_i = 170 \, \text{versus} \, 74 \, \text{nM}$ (Fig. 8A and Table III). Importantly, the binding mode of AMD3389 was similar to that of the cyclam, as it was highly dependent on Asp 171 and not impaired by substitution of Asp 262 (Fig. 8B). As the binding...
Non-peptide Bicyclam Antagonists of the CXCR4 Receptors

The data were obtained from competition binding on COS-7 cells expressing the wild-type and mutant receptors using $^{125}$I-Met-SDF-1 as radioligand. Values in parentheses represent number of experiments (n). ND, not determined. The D171N/D262N construct showed too low expression to be investigated.

### Table I

| Human wild-type | Met-SDF-1a | AMD100 | Cyclam |
|-----------------|-----------|--------|--------|
|                 | $K_d$ (nM) | $K_i$ (nM) | $K_i$ (nM) |
| CXCR4           |          |        |        |
| H113A           | 9.2 ± 2.5 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| D171N           | 10.0 ± 3.4 | 9.4 ± 0.3 | 9.4 ± 0.3 |
| D182N           | 20.0 ± 7.8 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| D193N           | 24.0 ± 10.0 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| D262N           | 20.0 ± 7.8 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| H203A           | 7.8 ± 2.6 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| H262N           | 20.0 ± 7.8 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| H281A           | 7.2 ± 1.5 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| H294A           | 73 ± 14 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| H113A/H281A     | 9.2 ± 5.4 | 9.6 ± 0.3 | 9.6 ± 0.3 |
| D171N/D262N     | Low expression | 9.6 ± 0.3 | 9.6 ± 0.3 |

### Figure 4

**Effect of Asp-to-Asn substitution at positions 171 and 262 in the CXCR4 receptor on AMD3100 (A) and cyclam (B) competition for SDF-1a binding.** Whole cell competition binding was performed on wild-type CXCR4 (WT; - - -), D171N CXCR4 (□), and D262N CXCR4 (△) receptors expressed in COS-7 cells using $^{125}$I-Met-SDF-1a as radioligand. Data are shown as means ± S.E. (n = 3–6).

### Figure 5

**Effect of Asp-to-Asn substitution at positions 171 and 262 in the CXCR4 receptor on competition for 12G5 antibody binding.** Whole cell competition binding was performed on wild-type CXCR4 (WT; □), D171N CXCR4 (△), and D262N CXCR4 (△) receptors expressed in COS-7 cells using $^{125}$I-12G5 as radioligand. Data are shown as means ± S.E. (n = 3–6).

Correlation with Antiviral Activity—Antiviral potency for the analogs employed in this study has previously been reported (27), and a good correlation was found between their antiviral potency and the affinity measured in competition with the 12G5 receptor antibody (Fig. 9A). Interestingly, however, the monocyclam analog AMD33389 clearly fell outside this correlation, as its high affinity in potentiating 12G5 binding (Fig. 8B) did not correlate with its relatively poor ability to inhibit HIV-1 cell entry (Fig. 9A). A reasonable correlation was found also between the antiviral potency of the cyclam and bicyclam analogs and their affinity as antagonists of SDF-1a binding, $r = 0.86$ (Fig. 9B). However, in absolute numbers, the correlation was not really good because the span of antiviral potencies covered 5 orders of magnitude, i.e. from $10^{-3}$ to $10^{-4}$ μ, whereas the affinities for these compounds as competitors of SDF-1a binding only covered 2 orders of magnitude, from $10^{-7}$ to $10^{-5}$ μ (Fig. 9B). An interesting highly significant correlation was found between the potency of the compounds in HIV cell entry assays and their dependence on Asp$^{262}$ for binding, again with ADM3389 being a clear exception (Fig. 9C). However, it should be noted that concerning Asp$^{262}$, the affinity of AMD3389 was in fact increased by substitution of this residue, whereas its ability to potentiate 12G5 binding was eliminated, indicating a complex interaction mode. Nevertheless, for the rest of the compounds, the very clear correlation shown in Fig. 9C indicates that interaction with Asp$^{262}$ could be particularly important for the ability of these compounds to function as anti-HIV agents.

### Discussion

In this study, the binding of the non-peptide CXCR4 receptor antagonist AMD3100 has been characterized by combining...
TABLE II
Affinity of 12G5 and AMD3100 for the wild-type CXCR4 receptor and His- and Asp-substituted CXCR4 receptor mutants

The data were obtained from competition binding on COS-7 cells expressing the wild-type and mutant receptors using $^{125}$I-12G5 as radioligand. Values in parentheses represent number of experiments (n).

| Receptor Mutant | $B_{max}$ ± S.E. (fmol/100,000 cells) | $\log K_d$ ± S.E. (nM) | $K_i$ (nM) |
|-----------------|-------------------------------------|------------------------|------------|
| Human wild-type | 12G5                                | AMD3100                |
| CXCR4           | 39 ± 10                             | 6.29 ± 0.12            | 0.29 ± 0.01|
| H113A           | 25 ± 7.4                            | 6.03 ± 0.17            | 0.16 ± 0.01|
| D171N           | 28 ± 7.9                            | 7.58 ± 0.23            | 0.01 ± 26  |
| D182N           | 79 ± 46                             | 7.11 ± 0.19            | 0.22 ± 2100|
| D193N           | 91 ± 24                             | 4.5 ± 2               | 0.19 ± 19  |
| H203A           | 85 ± 71                             | 1.3 (2)                | 0.23 ± 17  |
| D262N           | 77 ± 33                             | 1.0 (4)                | 0.11 ± 150 |
| H281A           | 5.5 ± 1.6                           | 5.68 ± 0.22            | 0.17 ± 550 |
| H294A           | 133 ± 59                            | 6.03 ± 0.16            | 0.14 ± 550 |
| H113A/H281A     | 6.0 ± 1.4                           | 6.29 ± 0.29            | 0.14 ± 550 |

![Fig. 6. Competition binding experiments using AMD2763 (A) or AMD2849 (B) in the wild-type CXCR4 receptor compared with the D171N and D262N mutants.](image62x323to285x546)

![Fig. 7. Competition binding experiments using AMD3106 (A) or AMD3108 (B) in the wild-type CXCR4 receptor compared with the D171N and D262N mutants.](image320x315to543x546)

Among the acidic residues located in the main ligand-binding crevice of the CXCR4 receptor, Asp$^{171}$ was identified as being important for the ability of the isolated cyclam moiety to compete for SDF-1a binding. Substitution of the other Asp residue in this main pocket, Asp$^{262}$ in TM-VI, did not affect binding of the free cyclam. Importantly, however, the binding of the bicyclam AMD3100 was dependent not only on Asp$^{171}$, but also on Asp$^{262}$. It is possible that the free cyclam moiety also binds to Asp$^{262}$, but that its affinity for Asp$^{262}$ is relatively low compared with the binding to Asp$^{171}$. If so, the binding to Asp$^{262}$ will not be appreciated in the competition binding experiments. When the supposedly main binding site for the free cyclam moiety is eliminated in the D171N mutation, the affinity of the monocyrclam was determined to be $\sim 400$ μM. This could possibly represent the affinity of the cyclam at the Asp$^{262}$ site, which could have been confirmed by the D171N/D262N double mutation. However, the low expression level of this construct unfortunately prohibited this. Another possibility is that although cyclam binds to Asp$^{262}$, this binding could be silent with respect to the effect on SDF-1a binding. Nevertheless, the fact that the bicyclam AMD3100 clearly is dependent on both Asp$^{171}$ and

Proposed Binding Mode for the Bicyclam AMD3100—The isolated 1,4,8,11-tetrazacyclotetradecane ring (cyclam) has an overall charge of +2 at physiological pH (12), and x-ray and neutron diffraction structures have shown that the protonated cyclam ring has the propensity to form a direct, hydrogen-bonded stabilized complex with carboxylic acid groups (13).
Asp^{262} and the fact that cyclam has a strong propensity to interact with carboxylic acid groups indicate that each of the cyclam moieties of the bicyclam binds to each of these two Asp residues located in TM-IV and TM-VI, respectively.

The suggested binding mode for AMD3100 versus cyclam is supported by the results obtained with analogs having different linkers. When the aromatic, conformationally constraining phenylenebisethylene linker of AMD3100 was exchanged with an aliphatic propylene linker (AMD2849), the bicyclam behaved in the binding assay almost exactly like two extended by three more methylene groups (AMD2936), the compound was decreased, and it became more dependent on Asp^{171} and less dependent on Asp^{262} for competition against SDF-1, i.e. more “cyclam-like.”

When the aliphatic linker was extended by three more methylene groups (AMD2849), the bicyclam behaved in the binding assay almost exactly like two molecules of free cyclam (Fig. 6). Furthermore, the disubstituted pyridine-linked analog, which has a strong propensity to interact with carboxylic acid groups indicate that each of the pyridine moieties of cyclam is essential for its antiviral activity (10) could be manually docked into the main ligand-binding crevice of the CXCR4 receptor in a manner that positioned each of the cyclam moieties in close proximity to Asp^{171} in TM-IV and Asp^{262} in TM-VI, respectively (Fig. 10A). If AMD3100 binds like this, how does it then act as an antagonist? The x-ray structure is based on the dark-state, inactive conformation of rhodopsin. Thus, conceivably AMD3100 can interact directly with the corresponding inactive state of the CXCR4 receptor, as indicated in Fig. 10A.

Because of the conformationally constraining spacer, AMD3100 could simply prevent the receptor from changing into a yet unknown active conformation. The free cyclam moiety conceivably can interact with both Asp^{171} and Asp^{262} (Fig. 10B); however, the presumed interaction with Asp^{262} is not detected in the binding assay.

**Differentiation between Inhibition of Binding of Various CXCR4 Ligands**—The three different ligands for CXCR4, i.e. the endogenous chemokine SDF-1α, the monoclonal receptor antibody 12G5, and HIV virions or rather the gp120 envelope protein, are not affected by AMD3100 in an identical manner. Importantly, receptor mutations affect AMD3100 function differently for the different ligands. In this study, the non-peptide compounds have mainly been probed as non-peptide antagonists normally are, i.e. for their effect on binding of the endogenous receptor ligand, in this case, the chemokine SDF-1α. However, AMD3100 was originally developed as a blocker of HIV replication, which turned out to be blockade of HIV binding to the CXCR4 receptor. Blocking SDF-1α binding, antibody binding, and binding of the HIV gp120 envelope protein is not necessarily the same function. For example, in this study, we found that mutations of Asp^{262} and Asp^{165} in extracellular loop 2 (Fig. 2) have no effect on the ability of AMD3100 to antagonize SDF-1α binding, whereas these residues in an initial mutational analysis of the CXCR4 receptor have been reported to be partially important for the ability of AMD3100 to block HIV replication (14). An interesting observation along these lines is that AMD3389 inhibition of SDF-1α binding is highly depend-

![Figure 8](image)

**Fig. 8.** Competition binding experiments using AMD3389 in the wild-type CXCR4 receptor compared with the D171N and D262N mutants. Competition binding was performed on the wild-type CXCR4 (WT; □), D171N CXCR4 (■), and D262N CXCR4 (△) receptors expressed in COS-7 cells using either [125I]-Met-SDF-1α (A) or [125I]-12G5 (B) as radioligand. Data are shown as means ± S.E. (n = 3).

|     | Log $K_i$ ± S.E. | $K_i$ (n) | Log $K_i$ ± S.E. | $K_i$ (n) | Log $K_i$ ± S.E. | $K_i$ (n) |
|-----|----------------|----------|----------------|----------|----------------|----------|
| AMD3389 | -7.12 ± 0.10 | 0.020 (1) | -5.56 ± 0.31 | 2.8 (3) | -5.95 ± 0.10 | 1.1 (5) |
| AMD3100 | -7.08 ± 0.07 | 0.053 (4) | -4.76 ± 0.10 | 17 (3) | -5.76 ± 0.37 | 1.8 (3) |
| AMD3108 | -6.07 ± 0.10 | 0.055 (3) | -4.12 ± 0.10 | 21 (3) | -5.76 ± 0.12 | 2.2 (3) |
| AMD2938 | -6.19 ± 0.07 | 0.055 (3) | -4.24 ± 0.36 | 58 (3) | -5.47 ± 0.06 | 3.4 (3) |
| AMD2849 | -6.74 ± 0.10 | 0.053 (4) | -4.55 ± 0.19 | 45 (3) | -5.81 ± 0.19 | 1.6 (3) |
| AMD3389 | -6.78 ± 0.10 | 0.17 (3) | -4.08 ± 0.17 | 84 (3) | -7.21 ± 0.19 | 0.062 (3) |
| Monocyclam | -4.90 ± 0.11 | 13 (6) | -3.43 ± 0.11 | 370 (3) | -5.02 ± 0.22 | 9.6 (4) |

|野生型 | $K_i$ (n) | $K_i$ (n) | $K_i$ (n) | $K_i$ (n) |
|-------|----------|----------|----------|----------|
| AMD3100 | -7.12 ± 0.10 | 0.020 (1) | -5.56 ± 0.31 | 2.8 (3) | -5.95 ± 0.10 | 1.1 (5) |
| AMD3106 | -7.08 ± 0.07 | 0.053 (4) | -4.76 ± 0.10 | 17 (3) | -5.76 ± 0.37 | 1.8 (3) |
| AMD3108 | -6.07 ± 0.10 | 0.055 (3) | -4.12 ± 0.10 | 21 (3) | -5.76 ± 0.12 | 2.2 (3) |
| AMD2938 | -6.19 ± 0.07 | 0.055 (3) | -4.24 ± 0.36 | 58 (3) | -5.47 ± 0.06 | 3.4 (3) |
| AMD2849 | -6.74 ± 0.10 | 0.053 (4) | -4.55 ± 0.19 | 45 (3) | -5.81 ± 0.19 | 1.6 (3) |
| AMD3389 | -6.78 ± 0.10 | 0.17 (3) | -4.08 ± 0.17 | 84 (3) | -7.21 ± 0.19 | 0.062 (3) |
| Monocyclam | -4.90 ± 0.11 | 13 (6) | -3.43 ± 0.11 | 370 (3) | -5.02 ± 0.22 | 9.6 (4) |

**Table III**

Binding of various ligands to the wild-type, D171N, and D262N CXCR4 receptors expressed in COS-7 cells using [125I]-Met-SDF-1α as radioligand.

Values in parentheses represent number of experiments (n).
assessed by linear regression analysis. HIV-1 antiviral potency (data obtained from Bridger and Skerlj (2)) is plotted against the binding affinity (Ki) measured either by 125I-12G5 (A) or 125I-Met-SDF-1α (B) or against the difference in binding affinity (ΔKi) between the wild-type (WT) and D262N mutant CXCR4 receptors measured by 125I-Met-SDF-1α (C).

FIG. 9. Correlation of HIV-1 antiviral potency and interaction with the CXCR4 receptor of the different bicyclam analogs as assessed by linear regression analysis. HIV-1 antiviral potency (data obtained from Bridger and Skerlj (2)) is plotted against the binding affinity (Ki) measured either by 125I-12G5 (A) or 125I-Met-SDF-1α (B) or against the difference in binding affinity (ΔKi) between the wild-type (WT) and D262N mutant CXCR4 receptors measured by 125I-Met-SDF-1α (C).

FIG. 10. Molecular model of the main ligand-binding pocket of the CXCR4 receptor with either AMD3100 (A) or two cyclam molecules manually docked into favorable interactions with Asp171 in TM-IV and Asp262 (B) in TM-VI. The receptor model is built over the rhodopsin model of Palczewski et al. (20). The conformation of AMD3100 is based on structural requirements of high antiviral effect of AMD3100 (9, 10) and the crystallographic x-ray structure of 6,6-bis(1,4,8,11-tetraazacyclotetradecane)-dinickel(II)tetraperchlorate (30), obtained from the Cambridge Structural Data Base.

ent on Asp171, but independent of Asp262, whereas its potentiation of 12G5 antibody binding is totally dependent on Asp262 (Fig. 8). One explanation could be that the monocyclam analog can in fact bind in two fashions (perhaps even at the same time). In one binding mode, the compound binds mainly to Asp171 and blocks SDF-1α binding. In the other binding mode, AMD3389 interacts mainly with Asp262 and thereby presumably presents the overlying extracellular loop 2, which holds important epitopes for 12G5 (28) in a favorable way for antibody recognition.

Interaction with Asp262 in TM-VI appears to be an important feature for this series of compounds with respect to their function as anti-HIV agents. All of the bicyclam compounds were highly dependent on interaction with Asp171, but varied in their dependence of Asp262. Importantly, a strong correlation could be demonstrated between the antiviral potency of the compounds and the apparent loss of their binding energy observed when Asp262 was substituted with a non-charged Asn residue (Fig. 9C). It could be interesting to probe whether the good pharmacological properties of AMD3100 and close analogs and the apparent requirement for forming a connection across the main ligand-binding crevice can be transferred to other classes of CXCR4 antagonists or other chemokine receptor antagonists in general. It should be emphasized that one of the hallmarks of AMD3100 is that it has a very broad specificity with respect to blocking basically all types of X4 envelopes, which obviously is a requirement for a good anti-HIV drug (29). This compound has been optimized for its antiviral activity through the cumbersome testing of every analog in HIV cell entry assays (2). For the moment, this appears to be the safest approach. Although there is a correlation between the potency of the compounds as anti-HIV agents and their potency in inhibiting SDF-1α or 12G5 antibody binding, the correlation is not close enough, and there are outliers that deviate many orders of magnitude.

Acknowledgments—We thank Dr. Michael A. Luther and colleagues at Glaxo Wellcome for providing Met-SDF-1α.

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