Chapter 2
Molecular Pharmacology of CXCR4 Inhibition

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Abstract In recent years, the chemokine receptor CXCR4 has been shown to be implemented in the mobilization of progenitor cells from the bone marrow. This finding has prompted a search for CXCR4 antagonists acting as stem cell mobilizing agents. In accordance, it is important to look into the molecular pharmacology of well-known CXCR4 antagonists in order to augment the potency and affinity and to increase the specificity of future CXCR4-targeting compounds. In this chapter, binding modes of CXCR4 antagonists that have been shown to mobilize stem cells are discussed. In addition, comparisons between results obtained from structure–function studies and findings from newly released crystal structures are drawn.

The Activation of 7TM Receptors (G Protein-Coupled Receptors)

Chemokine receptors belong to the largest family of proteins in the human genome, namely the seven transmembrane spanning receptors (7TM receptors). Drugs targeting these receptors represent the majority of prescribed pharmaceuticals [1]. Therefore, it is expected that many more of these receptors are potential drug targets and numerous experiments have been performed in order to determine the exact activation mechanism.

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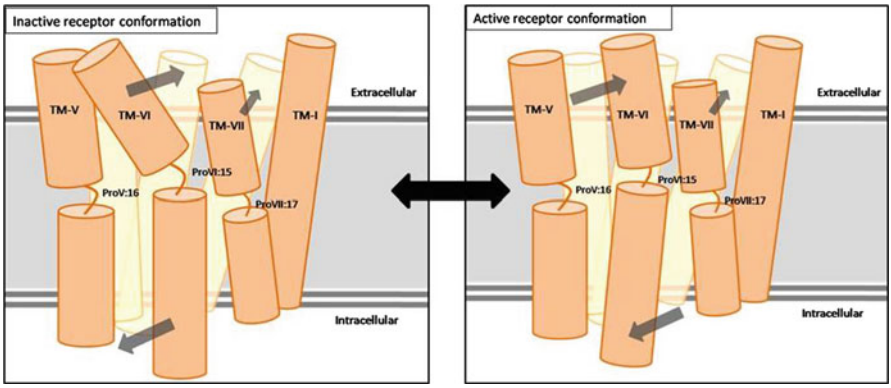

Fig. 2.1 Schematic drawing of the 7TM receptor activation mechanism as proposed by the Global Toggle Switch Model. TM-VI and TM-VII move around a weak point generated by the two highly conserved prolines in each helix. *Left panel*: The inactive receptor conformation. *Right panel*: The active conformation, in which the extracellular ends of TM-VI and TM-VII move toward each other, meanwhile the intracellular parts move away from one another, creating space for the binding of the signaling molecules. Redrawn from Schwartz et al. [2]

One theory of what occurs during the activation of 7TM receptors is the Global Toggle Switch Model described by Schwartz and coworkers [2–4]. Generally speaking, it is believed that all family A 7TM receptors are activated in much the same way, despite the fact that these receptors are activated by highly diverse ligands [5, 6]. Many of the studies that have been performed indicate that relatively large overall changes in the arrangement of the intracellular parts of the helices occur during receptor activation [7, 8]. This has led to the Global Toggle Switch Model, in which transmembrane domain VI (TM-VI) in particular performs a vertical "see-saw" movement, or a so-called toggle motion. This implies that the extracellular end of the helix tilts into the main ligand-binding pocket, meanwhile the intracellular end moves outward. In the assumed active state, an agonist binds and stabilizes this conformation. The extra space that is created on the intracellular side of the receptor allows for an associated molecule (e.g., the activated G protein or arrestin) to bind and elicit the signaling cascade (Fig. 2.1). However, due to the difficulty of crystallizing a stabilized active 7TM receptor, it has been difficult to provide proof of this theory. In fact, only recently rhodopsin (more specifically opsin, the ligand-free form of rhodopsin) was crystallized in what is presumed to be an active representation (despite the fact that the agonist, all-*trans* retinal, was lacking) [9, 10]. This crystal structure showed that compared to the dark inactive state of rhodopsin, the cytoplasmic half of TM-VI is tilted outward away from the helical bundle by 6–7 Å.

Furthermore, the crystallization indicated that TM-V is longer, very straight, and more inclined with a resulting shift of the cytoplasmic end by 2–3 Å toward TM-VI. Unlike the activation of rhodopsin by light, agonists are very inefficient in stabilizing an active state of the β_2 adrenergic receptor (β_2AR), making it difficult to secure the receptor in this conformation [11]. Moreover, even in complex with an agonist,

the β_2AR crystallizes in an inactive conformation [12]. Experiments have shown that stabilization of the active state requires both binding of an agonist as well as the G protein [13]. In 2011, Kobilka and coworkers published the structure of a stabilized active conformation of the β_2 adrenergic receptor [11]. They employed a so-called nanobody that exhibits G protein-like behavior toward the β_2AR, preferentially binds to and stabilizes the active conformation, and furthermore, are more stable in detergent solutions the inherent G protein. Creating a complex of the β_2AR (coupled to a T4 lysosome which stabilizes the receptor [14]), the nanobody as well as a high-affinity agonist with favorable efficacy and a very slow off-rate made it possible to create a stabilized structure of an active β_2AR. When the β_2AR structure was superimposed with the opsin structure they were remarkably similar both in respect to the outward movement of the intracellular end of TM-VI as well as TM-V. Thus, these two active crystal structures provide further proof that overall conformational changes occur, including the toggle of TM-VI.

Molecular Pharmacology of CXCR4 Antagonists

Several CXCR4-targeting antagonists have been published since this chemokine receptor was discovered in 1996 (for recent review, see [15]). However, as the preponderance of these compounds are treated as anti-HIV compounds, only compounds which have been shown to act as stem cell mobilizing agents are included in the following sections.

The Cyclam Family

AMD3100 (Plerixafor/Mozobil™)

The cyclam-related compounds include the only CXCR4-targeting stem cell mobilizer that have reached the clinic (AMD3100 (Plerixafor, Mozobil™)). In general, these compounds are small-molecule antagonists of which AMD3100 is the prototype. It is composed of two 1,4,8,11-tetraazacyclotetradecane (cyclam) moieties linked together by a conformationally constraining aromatic linker (Fig. 2.2a). As the original indication of AMD3100 was anti-HIV therapy, the majority of published structure–function assays describe its potency in the inhibition of HIV-infection and not in the mobilization of stem cells.

Bicyclams are strongly basic at physiological pH due to the presence of four primary amines in each cyclam ring [16] and x-ray together with neutron diffraction structures have shown that the protonated cyclam ring has a tendency to form complexes with carboxylic acid groups by hydrogen bonds [17]. Accordingly, several studies have focused on the negatively charged domains facing the binding pocket of CXCR4.

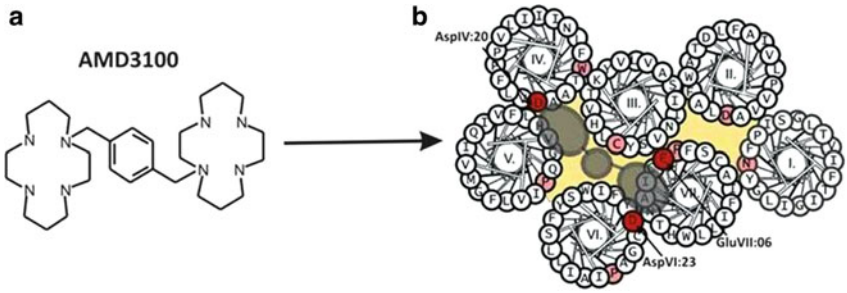

Fig. 2.2 (**a**) Chemical structure of the bicyclam AMD3100. (**b**) Schematic presentation of the proposed binding mode of AMD3100. Inserted is a helical wheel diagram of the chemokine receptor CXCR4. The *gray marking* shows the assumed position of the antagonist within the receptor and residues shown to be involved in the interaction are indicated in *red*. Conserved residues are colored *light red*

One of the first studies described a series of mutations in the extracellular loop 2 (ECL2) and TM-IV and the association with the degree of drug resistance [18]. In this study, it was found that mutating aspartates to neutral amino acids (asparagine, alanine, or glycine) in the examined domains of CXCR4 markedly increased the resistance to AMD3100 and thus are important for the interaction between the compound and the receptor. Specifically, they suggested that Asp^{181}, Asp^{182}, Asp^{187}, and Asp^{193} in ECL2 and Asp^{171} (IV:20) in TM-IV were involved in AMD3100 interaction (the numbers in parentheses indicate the Schwartz generic numbering system modified from Baldwin's system [19, 20]). Furthermore, they noticed that mutating phenylalanines in the same two regions of CXCR4 (Phe^{172} (IV:21) and Phe^{174} (IV:23) in TM-IV and Phe^{199} and Phe^{210} in ECL2) to nonaromatic amino acids also decreased the efficacy of AMD3100. It was speculated that the importance of the phenylalanines was due to interactions with the aromatic linker of AMD3100. The authors argued that the reasons for this could be both direct interaction of the residues with the compound or that altering an amino acid could change the overall configuration of the receptor and indirectly affect the interaction between AMD3100 and CXCR4.

In 2001, Gerlach et al. performed a comprehensive mutagenesis study of all aspartate residues facing the binding pocket of CXCR4 [21]. Furthermore, in a previous study it had been shown that the cyclam rings are able to chelate metal ions and in fact that transition metal ion chelated by the two macrocyclic rings of AMD3100 increase the affinity [22–24]. Based on this finding, Gerlach et al. also included metal ion-binding histidine residues facing the binding pocket. Testing all the mutants in competition binding, it was clear that especially two aspartate residues in the extracellular ends of TM-IV (Asp^{171}) and TM-VI (Asp^{262} (VI:23)) are involved in the binding of AMD3100. Testing a single cyclam unit or a monocyclam against the same mutations indicated that only Asp^{171}, and not Asp^{262}, was important for monocyclam-based compounds. Taken together, these findings led to a proposal of the interaction mode, in which AMD3100 spans the ligand-binding pocket and each cyclam ring binds to the two aspartate residues located at the extracellular ends of the transmembrane domains.

The authors speculated that because of the conformationally constraining aromatic linker, AMD3100 could simply prevent the receptor from changing into an—at that time unknown—active conformation. The decreased affinity of the compounds containing only one cyclam ring in the receptor where Asp^{171} had been mutated could represent the affinity to Asp^{262}. Alternatively, the single cyclam does bind to Asp^{262}, but this does not interfere with binding of the radioactive-labeled competing ligand (CXCL12/SDF-1α) and would not be detected.

Through mutational substitutions of 16 residues located in TM-III, -IV, -V, -VI, and -VII of CXCR4, it was shown that AMD3100 depends strongly on not only the two aspartate residues previously mentioned (Asp^{171} and Asp^{262}), but also a glutamate (Glu^{288} (VII:06)) in TM-VII [25]. When chelating zinc to AMD3100 it increased the dependency on Asp^{262} tenfold and a number of other residues on the inner face of TM-IV, -V, and -VI also appeared to be involved. The combination of these three acidic residues is unique to CXCR4, which is in agreement with the fact that AMD3100 is known to be highly selective for CXCR4 [26]. However, when aligning the chemokine receptors, it was discovered that two of the three residues (Asp^{171} and Asp^{262}) are present in the CXCR3 receptor, which otherwise is structurally rather distinct in the remaining binding pocket. On the other hand, a lysine is present in position 300 (LysVII:02), which possibly forms a neutralizing salt bridge with Asp^{278} (corresponding to Asp^{272} (VI:23) in CXCR4). To verify the assumed binding site of AMD3100, the construction of two mutations was necessary in CXCR3 (Ser^{304} (VII:06) to Glu and Lys^{300} to Ala (to interrupt the salt bridge)). Testing the mutant receptors in an inositol phosphate-signaling assay showed that AMD3100 was not able to inhibit activation induced by the endogenous chemokines on the CXCR3 wild-type receptor or on a receptor where only one of the mutations had been introduced. However, when combining the two mutations in CXCR3, and thereby reconstituting the tri-dentate acidic binding site in CXCR4, partial inhibition was seen when testing AMD3100 and the zinc-chelated form acted as a full antagonist. This finding supports the notion that these three acidic residues are essential and sufficient for AMD3100 binding. Previous studies have shown that the function of the linker is not based on its aromatic properties, but rather that it constrains the mobility and distance between the cyclam moieties [27, 28]. Based on this knowledge and the fact that the mutational analysis did not suggest that the side chains of any of the surrounding amino acids in proximity of the aromatic linker are involved in AMD3100-interaction, a binding mode of the compound was suggested: one cyclam ring interacts with the aspartate in TM-IV, while the other is sandwiched in between the aspartate in TM-VI and the glutamate in TM-VII. The linker then acts, as previously suggested, as a constraint, preventing TM-VI from moving inward and into the active conformation (Fig. 2.2b).

AMD3465 (A Monocyclam)

In the pursuit of orally bioavailable compounds, eradicating the cyclam moieties of AMD3100 is essential, because as mentioned before each cyclam ring has a

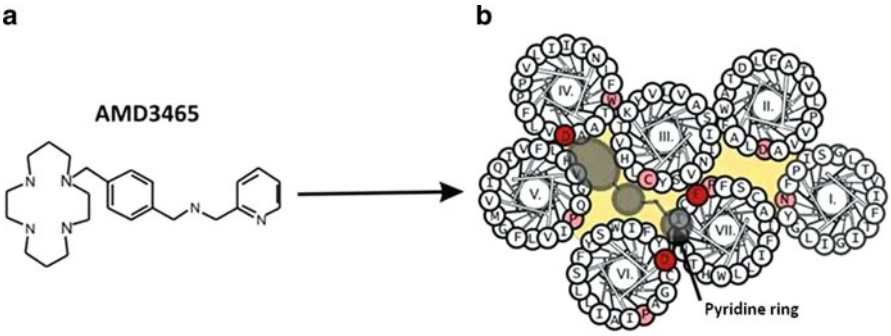

Fig. 2.3 (**a**) Chemical structure of the monocyclam AMD3465. (**b**) Schematic presentation of the proposed binding mode of the antagonist shown in a helical wheel diagram of CXCR4. The position of the compound is shown in *gray*, the pyridine ring of the molecule is indicated. Residues shown to be involved in the interaction are shown in *red*. In addition, His^{281}, placed at the extracellular end of TM-VII, is believed to be involved in the binding of the pyridine ring. Conserved residues are indicated in *light red*

positive charge of +2. As the original indication was as an antiviral therapeutic, a related compound with a higher oral bioavailability was sought after. However, as a stem cell mobilizing compound the lack of oral bioavailability does not pose any hindrances.

One such compound is AMD3465, the prototype monocyclam—constructed as a derivative of AMD3100 where one of the cyclam rings has been substituted by a pyridinemethylene moiety (Fig. 2.3a). In a publication from 2007, the complete binding mode of AMD3465 was described [29]. By mutational analysis it was shown that the monocyclam was dependent on the same three acidic residues as AMD3100 (Asp^{171}, Asp^{262}, and Glu^{288}). Additionally, a number of mutational hits were discovered in the extracellular ends of TM-III, -V, -VI, and -VII, especially mutation of a histidine residue in the top of TM-VII (His^{281} (VII:-02)) decreased the binding affinity >4,500-fold of AMD3465. By employing monocyclam compounds that varied in the "non-cyclam" and cyclam part, respectively, the authors were able to establish a very likely interaction mode between AMD3465 and CXCR4. As mentioned previously, Gerlach et al. [21] suggested that the cyclam ring in a monocyclam compound interacts with the aspartate in TM-IV. On the basis of this, it was expected that the pyridine moiety would bind to residues in the extracellular ends of TM-VI and -VII. Indeed, testing the compounds that varied in the "non-cyclam" part showed that they were not influenced by mutations made in the pocket surrounded by TM-III, -VI, and -VII but the mutational hits found for AMD3100 and AMD3465 in TM-IV and -V were still present. Moreover, these alternative monocyclam compounds displayed a much lower affinity than AMD3465 and this indicates that the pyridine interaction of AMD3465 with the residues in TM-VI and -VII—especially His^{281}—is necessary for high-affinity

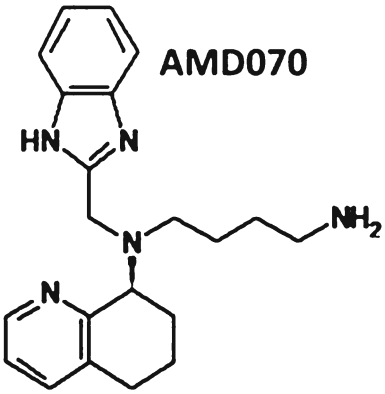

Fig. 2.4 Chemical structure of the noncyclam AMD070

binding to CXCR4. Accordingly, neither of the compounds varying in the cyclam part (by incorporation of a Cu^{2+} or a Ni^{2+}) of the molecule differed from AMD3465 in respect to affinity and was influenced by the same mutations as AMD3465. In conclusion, AMD3465 is believed to mimic the binding mode of AMD3100, with the cyclam ring interacting with Asp^{171} in TM-IV and neighboring residues in TM-III and -V, whereas the pyridine domain interacts with residues in the extracellular end of TM-VI and -VII, in particular His^{281}, which are not involved in AMD3100 binding (Fig. 2.3b).

AMD070 (A Noncyclam)

Because of the "remaining" cyclam moiety in AMD3465, the compound still needs to be administered by a parental route. The monocyclam could therefore be considered to constitute a medical intermediate compound—a step on the way to accomplish orally bioavailable compounds. Indeed, such a molecule has been developed, namely AMD070 (or AMD11070), in which the second cyclam ring has been replaced by a more drug-like chemical moiety (Fig. 2.4).

Not much work has been published on the molecular pharmacology of AMD070, but one publication has suggested that the compound overlaps with the binding sites that are shared between AMD3100 and AMD3465, i.e., Asp^{171}, Asp^{262}, and Glu^{288} [30]. In addition to the shared tri-acidic motif, the authors found that AMD070 also exhibited dependence on residues in the corner between TM-I, -II, -III, and -VII, namely Asp^{97} (II:23), Trp^{94} (II:20) (both in TM-II), and Tyr^{45} (I:07) (in TM-I). AMD070 has been tested in Phase II clinical trials as an anti-HIV agent, and has in general been well tolerated, but is currently on clinical hold due to histologic changes to the liver observed in long-term animal studies [31]. Additional preclinical safety assessments are pending.

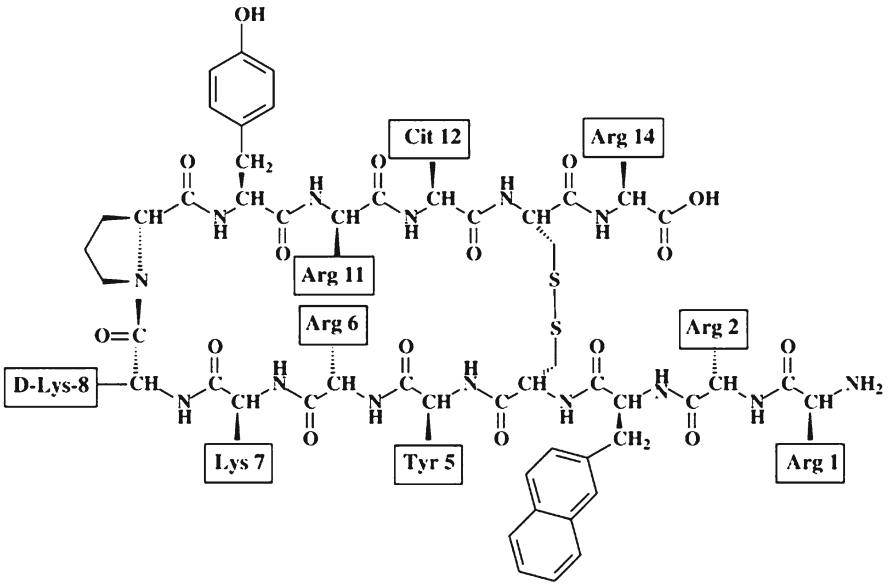

Fig. 2.5 Chemical structure of T140

Peptidic Compounds

T22, T140, and Derivatives

T22 is an 18-mer and a potent inhibitor of CXCR4 through an interaction with the N terminus and extracellular loop 1 and 2 [32–34]. However, the compound is highly basic and has poor oral bioavailability. Hence, smaller, more potent derivatives of T22, e.g., T140, have been constructed in which some of the basic amino acids have been replaced (Fig. 2.5). In a study from 2009, it was shown that administration of T140 to bone marrow-transplanted mice increases the production of progenitor and mature cells and exit to the periphery [35].

In 2003, a computational model of CXCR4 in a solvated lipid bilayer in complex with T140 was published [36]. The authors identified key residues by mutagenesis and used these as a basis for a plausible docking mode of T140. The computational model showed that the N terminal of T140 interacted with ECL2, while the C terminal was oriented inferiorly and interacted with residues in TM-IV as well as in ECL2 and ECL3. Four key residues of T140 had previously been identified as indispensable for CXCR4 antagonism, namely Arg^{2}, Nal^{3}, Tyr^{5} (in the N terminus of T140), and Arg^{14} (in the C terminus) [37] and were predicted to act directly with CXCR4. In fact, the four key residues all had multiple interactions with residues in CXCR4, including amino acids in the N terminus, TM-IV, TM-V, ECL2, and ECL3, mainly in the extracellular regions. The modeling showed that Arg^{14} of T140 forms strong hydrogen bonds with Asp^{171} of CXCR4 (also seen to be important for binding

of compounds belonging to the cyclam family). The authors also showed that mutation of Asp^{262} to alanine decreased the efficiency of T140. They speculated that this was due to a crucial role of ECL3 for the interactions with the C terminal of T140.

FC131 is a cyclic pentapeptide, derived from the critical residues of T140 for CXCR4 inhibition. In an extensive study from 2006, 11 derivatives of FC131, which were believed to share a common binding mode, were docked to a three-dimensional model of the transmembrane region of CXCR4 [38]. The authors had previously published a minimalistic 3D pharmacophore model for cyclopentapeptides suggesting the spatial arrangement of the domains required for CXCR4 binding [39], and the ligands were docked according to this model to further elucidate the atomic details of the CXCR4 interaction. By comparing the result of the ligand poses only two of the binding modes were common for all 11 compounds indicating likely binding modes.

The two proposed binding modes partly overlapped. However, the first binding mode indicates that the compounds mostly interact with residues in the "minor" ligand-binding pocket, i.e., the area that is defined by TM-I, -II, -III, and -VII, albeit part of the cyclopentapeptides might interact with hydrophobic residues in the "major" binding pocket (delimited by TM-III, -IV, -V, -VI, and -VII). On the other hand, the second proposal for a binding mode places the ligands in the CXCR4 "hotspot", in between TM-III, -V, -VI, and -VII. Interestingly, in both binding modes Arg^4 of the ligands forms a salt bridge with Glu^{288}, which also had been shown to be highly involved in the interaction between CXCR4 and the cyclam compounds [25]. However, in the computational modeling of the complex between CXCR4 and T140, the residue did not seem to interact with the compound [36].

The First Crystal Structure of a Chemokine Receptor: CXCR4

In 2010, the first crystal structure of a chemokine receptor, namely CXCR4, was published [40]. Stevens and coworkers described five independent inactive structures (three different constructs) of CXCR4 where either a small-molecule compound derived from isothiourea, IT1t, or a cyclic peptide, CVX15 was bound. In order to stabilize the receptor structure, they employed a T4 lysozyme fusion inserted between TM-V and -VI on the cytoplasmic side as well as additional thermostabilizing mutations. The authors describe the most distinctive differences between the structure of CXCR4 and other published crystal structures of 7TM receptors and indicate that homology models of CXCR4 based on these previously published structures might give a wrong impression of which residues are in the proximity and interacting with the ligands.

The ligand-binding pocket differs from other published structures of 7TM receptors in that it is larger, more open, and is located more proximal to the extracellular surface. The small-molecule antagonist, IT1t, occupies the part of the pocket that is delimited by side chains of TM-I, -II, -III, and -VII (the so-called "minor" binding pocket) and makes no contact with TM-IV, -V, and -VI. This is in stark contrast to

ligands complexed to other 7TM receptor structures and is not consistent with the molecular pharmacology for other CXCR4 small-molecule antagonists predicted by mutational analysis, as discussed above. IT1t contains nitrogen molecules that could be protonated with a net positive charge. One of them forms a salt bridge with Asp^{97} in TM-II while another could make a polar interaction with a cysteine in ECL2 (residue 186). Both cyclohexane rings of IT1t fit into small pockets and form hydrophobic contacts with CXCR4. The imidazothiazole ring is connected to the rest of the molecule by a short flexible linker and appears to make a salt bridge to Glu^{288}.

The bulky 16-residue cyclic peptide compound, CVX15, fills most of the binding pocket volume. The peptide forms a β-hairpin loop and both the N terminal and C terminal of the compound are buried in the pocket. The first four amino acids in the N terminus of the peptide form hydrogen bonds to CXCR4 with backbone residues in ECL2. The first residue in the peptide, an arginine, makes polar interactions with Asp^{187} (in ECL2), while the second amino acid, also an arginine, interacts with Thr^{117} (III:09), Asp^{171}, and possibly His^{113} (III:05). An arginine in the C terminal of the peptide makes a salt bridge with Asp^{262} and a salt bridge is also observed between Asp^{193} in the top of TM-V and a lysine at position 7 in the peptide. Finally, a proline in the C terminal makes a water-mediated interaction with the Glu^{288}.

Concluding Remarks

Studying the molecular pharmacology of a compound is vital when optimizing the properties of the compound. Learning the exact binding mode and which residues the compound interacts with is crucial for the drug development process. The knowledge provides clues in the further pursuit of a more potent compound. Furthermore, increasing the specificity of the compound to its target will decrease the possibility of side effects. The experimental methods to obtain knowledge about the specific interactions between a compound and a corresponding receptor are plentiful, and the amount of publications of crystal structures has, since the first published crystal structure of rhodopsin in 2001, increased substantially and provides a unique insight into how ligands may bind and 7TM receptors are activated. However, the crystal structures are instant images of an inactive or an active receptor and not of the dynamic process that happens when a receptor is activated. This is why the functional receptor studies are also important and over the last two decades the functional studies have led to, in combination with crystal structures, a unifying mode of 7TM receptor activation, namely the Global Toggle Switch Model.

As can be deduced from the crystal structures of CXCR4 in complex with the small-molecule antagonist IT1t, the binding mode of the compound clearly stands out from what has been published previously. The interaction pattern between CXCR4 and IT1t differs from results obtained from mutational studies as well as homology models of CXCR4 based on crystal structures of other 7TM receptors. This shows that there is still a need for a more specific description of the molecular requirements for receptor activation and the detailed molecular interaction and

binding modes of different ligands. The recently presented crystal structure of CXCR4 constitutes an important step toward further understanding of the molecular requirements for CXCR4 action.

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