

Computational analysis of the structural mechanism of inhibition of chemokine receptor CXCR4 by small molecule antagonists

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Abstract

Understanding the structural mechanism of receptor–ligand interactions for the chemokine receptor CXCR4 is essential for determining its physiological and pathological functions and for developing new therapies targeted to CXCR4. We have recently reported a structural mechanism for CXCR4 antagonism by a novel synthetic CXCR4 antagonist RCP168 and compared its effectiveness against the natural agonist SDF-1 α . In the present study, using molecular docking, we further investigate the binding modes of another seven small molecules known to act as CXCR4 antagonists. The predicted binding modes were compared with previously published mutagenesis data for two of these (AMD3100 and AMD11070). Four antagonists, including AMD3100, AMD11070, FC131 and KRH-1636, bound in a similar fashion to CXCR4. Two important acidic amino acid residues (Asp262 and Glu288) on CXCR4, previously found essential for AMD3100 binding, were also involved in binding of the other ligands. These four antagonists use a binding site in common with that used by RCP168, which is a novel synthetic derivative of vMIP-II in which the first 10 residues are replaced by *D*-amino acids. Comparison of binding modes suggested that this binding site is different from the binding region occupied by the N-terminus of SDF-1 α , the only known natural ligand of CXCR4. These observations suggest the presence of a ligand-binding site (site A) that co-exists with the agonist (SDF-1 α) binding site (site B). The other three antagonists, including MSX123, MSX202 and WZ811, are smaller in size and had very similar binding poses, but binding was quite different from that of AMD3100. These three antagonists bound at both sites A and B, thereby blocking both binding and signaling by SDF-1 α .

Keywords: chemokine receptors, CXCR4 structure, CXCR4 antagonists, HIV, molecular docking

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Introduction

Chemokines (chemoattractant cytokines) and their receptors play important roles in the normal physiology and pathogenesis of a wide range of human diseases, including multiple neurological disorders, cancer, and most notably, acquired immunodeficiency syndrome (AIDS).^{1–5} The human immunodeficiency virus (HIV-1) enters human cells through a fusion process in which the HIV-1 envelope glycoprotein gp120 binds to CD4, the main receptor for HIV-1 on the target cell surface. Two chemokine receptors, CXCR4 and CCR5, act as the principal co-receptors for HIV-1 entry.^{6–9} In 40–50% of HIV-infected individuals, the M-tropic strains of HIV-1 use CCR5 as the primary

entry co-receptor during the asymptomatic stage of disease.^{10–12} However, T-tropic strains that use CXCR4 eventually replace M-tropic strains and are associated with rapid disease progression.^{13–15} Natural chemokine ligands that bind to CXCR4 or CCR5 can inhibit HIV-1 infection^{16,17} by blocking virus-binding sites on the receptor and/or inducing receptor internalization.^{6,18} However, blocking the normal CXCR4 function raises concerns about undesired side-effects, since knockout mice lacking either CXCR4^{19,20} or its only natural ligand, SDF-1 α ,²¹ die during embryogenesis, with evidence of hematopoietic, cardiac, vascular and cerebellar defects. Consequently, the development of new inhibitors that target only the HIV-1

co-receptor function, but not the normal functions of SDF-1 α , is clearly desirable.

As a G-protein coupled receptor (GPCR), CXCR4 is classified as a member of the GPCR family-1 or rhodopsin-like GPCR family.^{22–24} It possesses seven transmembrane (7TM) helices with the N-terminus and three extracellular loops exposed outside the cell. The C-terminus and three intracellular loops face the cytoplasm. Since the identification of CXCR4 as a co-receptor for HIV entry, a number of peptide and low molecular weight pseudopeptide CXCR4 antagonists have been reported.^{25–28} Although disclosure of non-peptidic small molecule CXCR4 antagonists has been limited, a growing number of small molecule antagonists have been reported in recent years.^{29–32} The bicyclam AMD3100 was the first small molecule antagonist of CXCR4 to enter clinical trials for the treatment of HIV infection. AMD3100 is a specific CXCR4 antagonist that inhibits the membrane fusion step of the HIV-1 entry process.^{33,34} Unfortunately, this compound exhibited cardiac toxicity, precluding its further clinical development.^{30,31}

While lacking an X-ray structure for binding of CXCR4 with any of its ligands (SDF-1 α or small molecule antagonists) hampers development of antagonists using structure-based design approaches, homologous molecular modeling could be useful in predicting binding mode and antagonistic activity of CXCR4. These types of approaches have been used previously for other GPCR family-1 members.³⁵ Recently, we used a similar approach to predict the binding mode of the N-termini of SDF-1 α and RCP168.^{36,37} While the results from this modeling study were in agreement with experimental results, the study used a homology model of CXCR4 that had been generated using the structure of bacterial rhodopsin as a template. In recent years, a few three-dimensional (3-D) structures of GPCR have been resolved, including bovine rhodopsin³⁸ and human β_2 adrenoceptor.^{39–41} In this paper, a new homology model of CXCR4 was built based on the 3-D structure of bovine rhodopsin (PDB code: 1f88).³⁸ This model was then used for docking studies on seven known small molecule antagonists of CXCR4 (Figure 1). The selected antagonists included AMD3100 and AMD11070, for which

binding data for CXCR4 have already been reported.^{23,24,42} We compared the predicted docking modes with the available experimental data in order to gain knowledge about the binding modes of CXCR4 antagonists.

Methods

Homology modeling of CXCR4

The amino acid sequence of human CXCR4 was obtained from the Swiss-Prot TrEMBL database (accession code P61073). The crystal structure of bovine rhodopsin (PDB code: 1f88) was selected as the template. 7TM segments were defined, essentially as described by Gerlach *et al.*²⁴ Sequence alignment of the 7TM α -helix (Figure 2) was made by aligning the 1f88 sequence to the multiple alignments of human GPCRs in GPCRDB (<http://www.gpcr.org/7tm/>). The modeler included in Discovery Studio 2.0 (Accelrys, San Diego, CA, USA) was used for the construction of the homology model. In total, 100 models were generated and the best model was then subjected to energy-minimization in Discovery Studio under the CHARMM force field. The system was first energy-minimized, using adopt basis NR algorithm with the protein backbone fixed, until it converged at an RMS gradient less than 0.10. The system was then minimized, with adopt basis NR algorithm with the C α atoms fixed, until it converged at an RMS gradient less than 0.05.

Preparation of ligand structures

The 3-D structure of seven ligands were constructed manually, assigned an Gasteiger-Hückel charge, and then energy-minimized in Sybyl 8.0 (Tripos Inc, St. Louis, MO, USA) under a Tripos force field until convergence at a gradient of 0.05 Kcal/(mol·Å). In the preparation of the PDBQT files required for AutoDock4,⁴³ Gasteiger-Hückel charges were retained and non-polar hydrogen atoms were merged with carbon atoms. The roots and rotatable bonds of ligands were detected automatically by

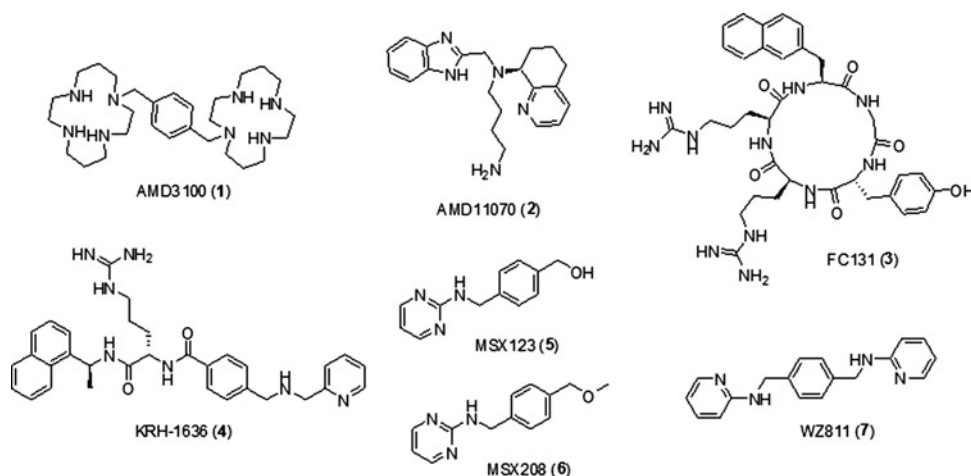


Figure 1 CXCR4 antagonists studied in this paper

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1F88 : SMLAAYMFLIMLGFPINFLTLYTV
CXCR4 : IFLPTIYSIIFLTGIVGNGLVLMG

1F88 : YILLNLAVADLFMVFGGFTTLYT
CXCR4 : KYRLHLSVADLLFVITLPFAVDA

1F88 : CNLEGGFATLGGEIALWSLVLAIERY
CXCR4 : CKAVHVIYTNLYSSVLILAFISLDYR

1F88 : HAIMGVAFTWMALACAAPPLVG
CXCR4 : AEKVYVGVWIPALLLTIPDFIF

1F88 : NESFVIYMFVWHFIPLIVIFFCYGQLV
CXCR4 : VVVFQFQHIMVGLILPGIVILSCYCI

1F88 : EVTRMVIIMVIAFLICWLPYAGVAFYIFTH
CXCR4 : KALKTTVILILAFFACWLPPYIGISIDSF

1F88 : IFMTIPAFFAKTSAVYNPVIYIMMNKQ
CXCR4 : KWISITEALAFFHCCLNPILYAFLGAK

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Figure 2 Alignment of the seven transmembrane helix regions of human CXCR4 to bovine rhodopsin. Sequence identity: 23.2%; sequence similarity: 52.4%

AutoDockTools-1.5.2 (Molecular Graphics Laboratory, The Scripps Research Institute, La Jolla, CA, USA).

Preparation of receptor files for molecular docking

The homology model of CXCR4 thus obtained was converted to a PDBQT file using AutoDockTools-1.5.2. The Gasteiger charges were added and non-polar hydrogen atoms were merged. In preparation of receptor grid files, a $62 \times 60 \times 60$ grid with a grid spacing of $0.375 \text{ \AA}$ was defined to cover the extracellular opening of the 7TM bundle. Grid maps were then generated by AutoGrid or docking simulations.⁴³

Docking simulations

Lamarckian genetic algorithm (GA) was adopted in AutoDock4 calculations, and 100 GA runs were performed for each ligand. The entire receptor structure was set to be rigid during the simulations. All other parameters for AutoGrid and AutoDock calculations were set to the default values of AutoDockTools-1.5.2.

Results

For each ligand, the 100 conformations resulting from AutoDock simulations were clustered. The representative model for binding to CXCR4 with a high predicted binding free energy is illustrated in Figures 3 and 4. The docking results indicated that all of the studied antagonists bind in the same pocket that seems to be important for the binding of RCP168.

The predicted mode for binding of AMD3100 (**1**, Figure 3a) indicated that one of the two cyclam rings is positioned between two important acidic amino acid residues, Asp262 and Glu288. Positively charged nitrogen atoms form electrostatic interactions with these two residues, while the second cyclam ring extends to the opposite side of the transmembrane bundle to form electrostatic interactions with Asp171. The predicted binding mode for AMD3100 agreed with the experimental results,^{23,24} which suggested that these three acidic residues, Asp171, Asp262 and Glu288, are the main interaction points between AMD3100 and CXCR4. Previous study suggested that one of the cyclam rings of AMD3100 should be sandwiched between Asp262 and Glu288, while the other cyclam interacts with Asp171 via positively charged nitrogen atoms,²³ which again is consistent with our predicted results based on docking calculations. In addition to the electrostatic interactions, AMD3100 also forms hydrophobic interactions with Ile259 and Ile284. However, the binding affinity is contributed primarily by electrostatic interactions.

The second antagonist AMD11070 (**2**) is also predicted to form electrostatic interactions with Asp262 and Glu288, by a terminal primary amino group and a nitrogen on its benzimidazole moiety, respectively (Figure 3b). Compared with AMD3100, AMD11070 is smaller and cannot simultaneously bind to Asp171 on the opposite side of the 7TM bundle; however, it is more lipophilic and shows more hydrophobic interactions with CXCR4 (i.e. with His113, Val112, Ile259, Tyr255, Tyr116).

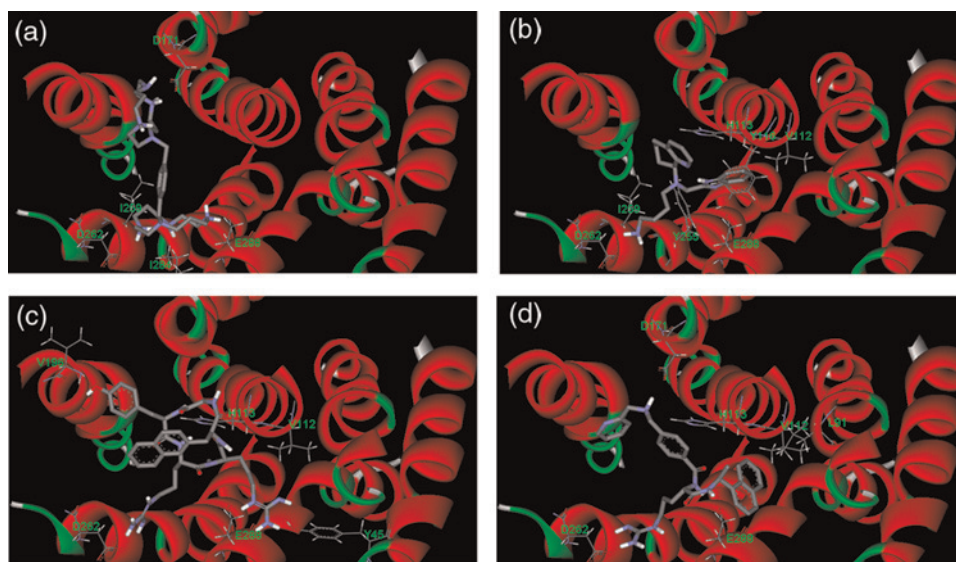


Figure 3 Predicted binding modes of CXCR4 antagonists. (a) AMD3100 (**1**); (b) AMD11070 (**2**); (c) FC131 (**3**) and (d) KRH-1636 (**4**). (A color version of this figure is available in the online journal)

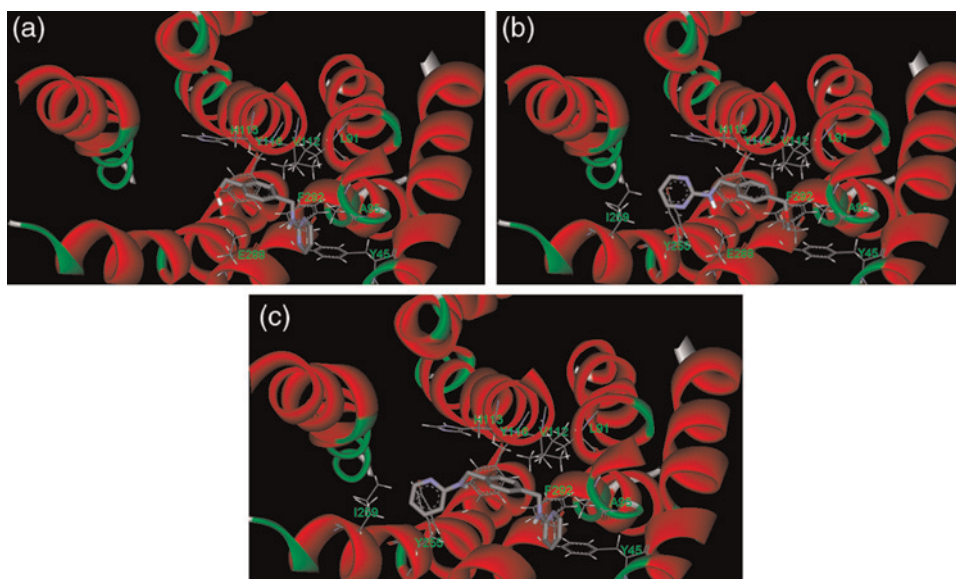


Figure 4 Predicted binding modes of CXCR4 antagonists. (a) MSX123 (5); (b) MSX208 (6) and (c) WZ811 (7). (A color version of this figure is available in the online journal)

The cyclopentapeptide FC131⁴⁴ (3) is large in size. In our simulation results (Figure 3c), two guanidinium groups in FC131 extend close to Asp262 and Glu288 and form electrostatic interactions with them. Hydrogen bonds are formed between one guanidinium group of FC131 and a phenol hydroxyl group of Tyr45, and between a phenol of FC131 and Val196; while the sidechain of one arginine of FC131 binds to Val112 and His113 of CXCR4 through hydrophobic interactions.

The KRH-1636⁴⁵ (4) compound is a peptidomimetic CXCR4 antagonist. It is a linear molecule bearing an arginine sidechain. We were surprised to find that the binding mode of KRH-1636 (Figure 3d) was similar to that of AMD3100. This compound forms a salt bridge with Asp262 via a guanidinium group on the arginine sidechain, a hydrogen bond with Glu288 by a benzoylamide NH, and electrostatic interactions with Asp171 by the secondary amine next to the terminal pyridine ring. In this case, the terminal pyridine and secondary amine act as the equivalent of one of the cyclam rings of AMD3100, while the arginine residue acts like the other cyclam ring. Although electrostatic interactions between KRH-1636 and CXCR4 may be weaker than those of AMD3100, the terminal lipophilic naphthyl moiety enables it to bind CXCR4 through stronger hydrophobic interactions with Leu91, Val112, His113 and Glu288.

The antagonists MSX123 (5) and MSX208 (6) differ by a methyl group on the benzyl alcohol. These compounds belong to the MSX series, which contain derivatives of 4-aminomethyl benzyl alcohol with different substituents on the amine and hydroxyl functionalities.⁴⁶ The antagonist WZ811³² (7) shows nanomolar EC₅₀ values in SDF-1 α antagonism assays and possesses a central 4-methyl amine benzylamine moiety similar to that found in AMD3100. However, probably due to its smaller size, its predicted mode for CXCR4 binding is quite different from that found for AMD3100, but is very similar to that found for compounds 5 and 6 (Figure 4). As illustrated in Figure 4a,

MSX123 forms a hydrogen bond with Glu288 through its free benzyl alcohol, and a hydrogen bond with Tyr45 through its 2-aminopyrimidine group. However, the benzyl alcohol group is blocked by methylation in MSX208 and thus cannot bind Glu288 via hydrogen bonding. Therefore, MSX208 adopts an orientation opposite to that predicted for the MSX123 binding mode (Figure 4b) so that it can form two hydrogen bonds with CXCR4: between an aminopyrimidine group and Glu288 and between a methoxyl group and Tyr45. For WZ811 (Figure 4c), one benzylamino group forms a hydrogen bond with Tyr45. Although their overall orientation differs, the binding modes of these three small ligands are very similar, and hydrophobic interactions contribute the majority of the binding affinity. The residues Ala95, Leu91, Val112, His113, Tyr116 and Phe292 play important roles in hydrophobic binding of all three small antagonists. In addition, Tyr255 and Ile259 are also important in hydrophobic binding of MSX208 and WZ811.

Discussion

The CXCR4 antagonists, AMD3100 (1), a bicyclam-containing small molecule, was the first drug to enter clinical trials for treatment of HIV infection. Although 1 binds specifically to CXCR4, it was withdrawn from the clinical trials due to cardiotoxicity and lack of bioavailability.^{30,31} Mutagenesis experiments^{23,24} revealed that mutation D171N of CXCR4 leads to a 10–50-fold reduction of AMD3100 binding activity, while mutants D262N and E288A show 50–100-fold reductions in binding, which suggested that these three anchor-point acidic amino acid residues are essential for AMD3100 binding. A second antagonist, AMD11070 (2), which shows better oral bioavailability, is currently in phase I/II clinical trials. Experimental data for AMD11070 also indicate Asp262 and Glu288 as the most

important residues for AMD11070 binding to CXCR4.⁴² Again, the binding modes predicted in our docking studies are in agreement with experimental data.

While no experimental data are available for the remaining compounds, much information can be obtained from their predicted binding modes. For compounds **3–7**, the predicted binding modes suggest that these antagonists also bind to important residues in site A (Figure 5), specifically Glu288 and Tyr255, which have been proposed to be important in RCP168-binding. In addition, Asp262, which is crucial for the binding of AMD3100 and AMD11070, is also very important for the predicted binding of **1**, **2**, **3** and **4**. Interestingly, the large ligands such as FC131 and KRH-1636 show similar binding modes to that adopted by AMD3100; that is, each molecule binds to the two key residues in one end of the 7TM bundle (Asp262 and Glu288) and to a residue in the opposite end of the 7TM bundle (Asp171 or Val196).

On the other hand, for small ligands, such as **5**, **6** and **7**, the binding modes are quite different from that adopted by AMD3100: these molecules form hydrogen bonds with Glu288 and Tyr45, while hydrophobic interactions with the neighboring residues, such as Leu91, Val112, His113, Tyr116 and Phe292, significantly increase the binding affinity. Although WZ811 possesses a central 4-aminomethyl benzylamine moiety similar to that found in AMD3100, its binding conformation differs from AMD3100, but is very similar to that of the MSX series compounds.

Our previous mutagenesis and molecular modeling studies^{36,37,47} suggested that RCP168, which has the first 10 amino acids in the N-terminus replaced by *D*-amino acids, binds differently than SDF-1 α , the only known natural ligand for CXCR4. In fact, molecular docking simulations revealed different orientations of the N-termini of RCP168 and SDF-1 α . Comparison of modeling data with available experimental data revealed the interesting observation that the N-terminus of RCP168 occupies a binding pocket that contains residues shown to be important for RCP168 binding to CXCR4 (site A, indicated by the red oval in Figure 5).⁴⁷ Mutations in the residues of this site, such as Tyr255 and Glu288, can diminish binding affinity for

RCP168 by 13–40%. The N-terminus of SDF-1 α also occupies a binding site that is different from the one important for RCP168 binding.^{36,37} This binding site (site B, indicated by the yellow oval in Figure 5) contains residues Phe87 and Phe292, which are important for the binding of SDF-1 α to CXCR4.⁴⁷ Thus, the modeling data, in tandem with the experimental data, suggested the possible existence of two different binding pockets in the 7TM bundle: one is important for the binding of antagonistic ligands and the other is important for SDF-1 α -CXCR4 signaling (i.e. binding of N-terminus of SDF-1 α). With this hypothesis in mind, we further analyzed our modeling data for CXCR4 antagonists and compared our finding with available experimental data.

In general, AMD3100, FC131 and KRH-1636 appear to have similar binding poses, although AMD11070 is too small to bind to Asp262 and Glu288 on one side of the 7TM bundle as well as to Asp171 on the opposite end. However, these three all share the common feature that strong electrostatic attractions with Asp262 and Glu288 are predominant contributors to ligand–receptor binding. Both Asp262 and Glu288 are located in site A (Figure 5), which is important for the binding of RCP168. These ligands also undergo hydrophobic interactions with Tyr255, Ile259 and Ile284, also located in site A, and mutation of Tyr255 (Y255A) decreases the binding affinity of RCP168.⁴⁷ These observations suggest that these CXCR4 antagonists share a common binding site that differs from the SDF1 α signaling site (site B), and further support the notion that a ligand-binding pocket may co-exist with an SDF-1 α signaling site in the 7TM bundle of CXCR4.

Ligands **5**, **6** and **7** are much smaller in structure than compounds **1–4** and conceivably their binding modes are substantially different from those of **1–4**. As shown in Figure 4, these small ligands bind both site A and site B, especially with Phe292, which is proposed to be important in SDF-1 α signaling.^{36,37,47} Nevertheless, **5–7** also share some common features with RCP168 in that they bind to Glu288 and Tyr45, both of which have been shown to be important for RCP168 binding.⁴⁷

During the completion of the modeling studies presented here, the crystal structures of CXCR4 in complex with a small molecule and cyclic peptide antagonists were published by others.⁴⁸ We compared our homology model with the experimental structure and found that helices 3 and 4 in our model deviated somewhat from the presented crystal structure, while other transmembrane helices fitted well with the experimental structure. Important residues in sites A and B, which were located in helices 2, 6 and 7, were found to have similar side chain conformations to the crystal structure, especially for site A.

Conclusions

Chemokines and their receptors play important roles in normal physiology and in the pathogenesis of a wide range of human diseases. Since the identification of CXCR4 as a co-receptor of HIV-1, several peptidic as well as non-peptidic antagonists have been reported. However, structural aspects of the mechanism of antagonism of

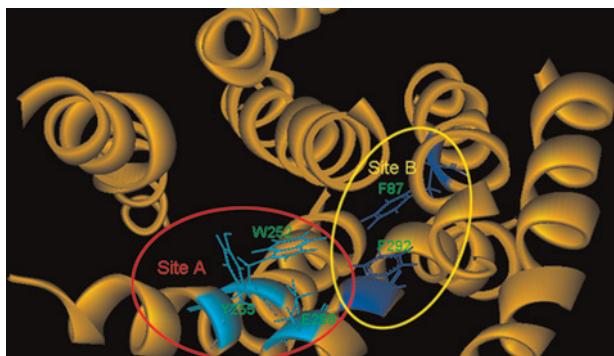


Figure 5 Cartoon view of CXCR4 from the top displaying two regions: site A (red oval) and site B (yellow oval), inside the transmembrane domain of CXCR4. The residues in site A (Trp252, Tyr255 and Glu288) that are important for binding of RCP168 are shown in cyan, whereas residues in site B (Phe87 and Phe292) that are important for binding of SDF-1 α are shown in blue. (A color version of this figure is available in the online journal)

CXCR4, particularly by small molecule antagonists, have not yet been clearly established. In this study, we have probed the binding modes of seven representative CXCR4 antagonists using homology modeling and molecular docking. Four antagonists, AMD3100, AMD11070, FC131 and KRH-1636, have similar binding modes that involve three important acidic amino acid anchor-point residues (Asp171, Asp262 and Glu288), as suggested by previous mutagenesis studies.^{23,24} These four ligands share a common binding site with RCP168 (site A) in the transmembrane helix bundle. Comparison of these binding modes with experimental data also suggested that this binding site is different than the region occupied by the N-terminus of SDF-1 α , the only known natural ligand of CXCR4. On the other hand, three other small CXCR4 antagonists, MSX123, MSX208 and WZ811, bind CXCR4 in another pose that occupies both a ligand-binding site and the SDF-1 α signaling site. This study provides insights into the way that CXCR4 antagonists interact with the receptor and would be useful in structure-based design and lead optimization for therapies involving CXCR4 antagonists. Furthermore, the co-existence of an antagonistic ligand-binding site with an SDF-1 α signaling site is indicated in CXCR4, which may be helpful in understanding the interaction between CXCR4 and its chemokine and synthetic ligands.

Author contributions: ZH and JA supervised the project. SPK, MY, HG, MYL, SE and XZ performed the experiments and wrote the paper. SPK and MY contributed equally to this work.

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