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Fragment-Based Optimization of Small Molecule CXCL12 Inhibitors for Antagonizing the CXCL12/CXCR4 Interaction

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Abstract

The chemokine CXCL12 and its G protein-coupled receptor (GPCR) CXCR4 are high-priority clinical targets because of their involvement in metastatic cancers (also implicated in autoimmune disease and cardiovascular disease). Because chemokines interact with two distinct sites to bind and activate their receptors, both the GPCRs and chemokines are potential targets for small molecule inhibition. A number of chemokines have been validated as targets for drug development, but virtually all drug discovery efforts focus on the GPCRs. However, all CXCR4 receptor antagonists with the exception of MSX-122 have failed in clinical trials due to unmanageable toxicities, emphasizing the need for alternative strategies to interfere with CXCL12/CXCR4-guided metastatic homing. Although targeting the relatively featureless surface of CXCL12 was presumed to be challenging, focusing efforts at the sulfotyrosine (sY) binding pockets proved successful for procuring initial hits. Using a hybrid structure-based in silico/NMR screening strategy, we recently identified a ligand that occludes the receptor recognition site. From this initial hit, we designed a small fragment library containing only nine tetrazole derivatives using a fragment-based and bioisostere approach to target the sY binding sites of CXCL12. Compound binding modes and affinities were studied by 2D NMR spectroscopy, X-ray crystallography, molecular docking and cell-based functional assays. Our results demonstrate that the sY binding sites are conducive to the development of high affinity inhibitors with better ligand efficiency (LE) than typical protein-protein interaction inhibitors (LE = 0.24). Our novel tetrazole-based fragment **18** was identified to bind the sY21 site with a K_d of 24 μ M (LE = 0.30). Optimization of **18** yielded compound **25** which specifically inhibits CXCL12-induced migration with an improvement in potency over the initial hit **9**. The fragment from this library that exhibited

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the highest affinity and ligand efficiency (**11**: $K_d = 13 \mu\text{M}$, $LE = 0.33$) may serve as a starting point for development of inhibitors targeting the sY12 site.

Keywords

Chemokines; CXCL12/CXCR4 inhibitors; protein-protein interaction; metastasis; fragment-based and structure-guided drug design

Introduction

Cancer is believed to develop from the accumulation of genetic alterations. In turn, aberrant signal transduction results in several hallmarks of cancer including uncontrolled cell proliferation, evasion of apoptosis, angiogenesis and metastasis. Metastasis, the spread and growth of tumor cells to distant organ sites, is the most devastating attribute and plays a major role in patient morbidity and mortality. Chemokines are small soluble proteins (70-130 residues) that activate G-protein coupled receptors (GPCRs) and are involved in many physiological processes including cell trafficking, angiogenesis and embryogenesis [1-4]. Under normal conditions, they play pivotal roles in immune surveillance and response, inflammation, stem cell homing and other important physiological processes [5]. Their functions as chemoattractants and their effects on immune cells also underlie their involvement in many diseases such as cancer, inflammation, autoimmune and cardiovascular diseases [6, 7]. Chemokines as primary mediators of metastasis were first identified by Muller and colleagues who implicated the chemokine receptor C-X-C motif chemokine receptor 4 (CXCR4) in tumor cell trafficking [8, 9]. Different types of cancers express different chemokines and chemokine receptors [9, 10]. However, the chemokine receptor CXCR4 is the only one that is expressed by the majority of cancer types. At least 23 different cancers have been shown to express elevated levels of CXCR4 [10], sensitizing these cancers to C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor-1, SDF-1) gradients in distant tissues [11]. CXCL12 is constitutively expressed in the bone marrow, lungs and liver, which are common tissues of metastatic growth.

CXCL12 Inhibitors and CXCR4 Receptor Antagonists

Reagents that Modulate CXCL12/CXCR4 Interactions

In general, the three major classes of agents that can modulate the CXCL12/CXCR4 interaction are antibodies to CXCL12 or CXCR4, CXCR4 receptor antagonists, and CXCL12 inhibitors. Development of anti-CXCL12 antibodies is challenging because the chemokine sequence is nearly invariant among mammals. Therapeutic development of antibodies against CXCR4 is also very limited due to conformational heterogeneity of CXCR4 and posttranslational modifications that reduce antibody specificity and function [12].

Although GPCRs have been one of the most important drug targets for the pharmaceutical industry, representing more than 30% of all US marketed therapeutics [13], no CXCR4 antagonist has been approved for therapy of metastatic cancer. Peptide-based CXCR4 antagonists lack efficacy with poor bioavailability. In spite of the apparent success of AMD3100 (**1**, Plerixafor, Chart 1) for stem cell mobilization [14], all of the CXCR4 antagonists [with the exception of a bicyclam mimetic MSX-122 (**2**)] in clinical trials have been withdrawn because of unmanageable toxicities as of August 2008 [15]. The cardiovascular toxicities from AMD3100 are presumably related to the metal-chelating properties of bicyclam [16]. Aside from the cardiotoxicity associated with long-term

administration of AMD3100, little is known of the drug-like properties of chemokine receptor antagonists or the origins of their toxicity [17].

Chemokines are generally viewed as “undruggable” proteins based on their size and lack of a well-defined pocket for small molecules to bind. However, recent studies have suggested otherwise, with the discovery of high-affinity inhibitors against several small proteins such as interleukin-2 and FKBP-12 [18-20]. The report of a chalcone molecule **5** binding to CXCL12 and inhibiting CXCR4 activation has further demonstrated that chemokines are viable targets for small molecule drug discovery although no structural information about chalcone binding has been revealed [21, 22]. The phosphate prodrug **6**, sulfate prodrug **7** and L-seryl prodrug **8** were recently reported to overcome the poor solubility of chalcone **5** [23]. More recently, our group used a structure-guided approach to identify a series of inhibitors that bound CXCL12 and inhibit the CXCR4-mediated calcium response [24].

Known CXCR4 Antagonists are not Approved for Treatment of Metastatic Cancers

CXCR4 receptors are expressed by various solid and hematologic tumors, such as breast cancer, lung cancer, prostate cancer, and leukemia [25, 26]. In spite of advances in surgery, chemotherapy and radiotherapy over the last decades, the death rate from lung cancer has remained unchanged mainly due to cancer metastasis. Lung cancer cells express CXCR4 while stromal cells within the tumor micro-environment constitutively secrete CXCL12. Activation of CXCR4 induces lung cancer cell migration and adhesion to stromal cells, which in turn provides growth- and drug- resistance signals to the tumor cells. CXCR4 antagonists were initially developed for treatment of HIV-1 infection. At the time of their discovery in the early 1990s, the mechanism of anti-HIV activity of the CXCR4 antagonists T140 and its analogs [27, 28], AMD3100 (**1**) [29, 30] and ALX-4C [31], was unknown. However, discovery of the co-receptor function soon demonstrated that the activity is due to the specific binding of the antagonists to the CXCR4 receptor [31-33].

Besides peptides [28, 34, 35] and antibodies [36-38], the most advanced small-molecule CXCR4 antagonists are represented by a bicyclam AMD3100 which was originally dropped from Phase II HIV clinical trials due to cardiotoxicity, but was later approved by FDA for treatment of non-Hodgkin's lymphoma and multiple myeloma [39]. AMD3100 was reported to inhibit CXCL12-induced chemotaxis by inhibiting the site 2 interaction with CXCR4 [40]. A series of bicyclam mimetics based on AMD3100 scaffold represented by MSX-122 (**2**) were reported towards development of an anti-metastatic agent [15, 41]. These series of bicyclam mimetics with non-chelating pyrimidyl heterocycles were designed to overcome safety issues of AMD3100 and improve pharmacokinetic properties for oral administration [42,43]. Benzimidazole-tetrahydroquinolineamine derivatives based on AMD070 (**3**) were a series of CXCR4 antagonists with potent and selective *in vitro* inhibition of X4 viral replication by blocking fusion and viral entry into the cell [44, 45]. Another series of CXCR4 antagonists are isothioureas derivatives, which block CXCR4/CXCL12 interactions *in vitro* and *in vivo* as well as the infection of target cells by X4-tropic HIV [46]. One of the isothioureas derivatives, IT1t (**4**), was co-crystallized with the CXCR4 receptor at 2.5 Å resolution [47]. Other miscellaneous small molecule CXCR4 antagonists can be found in a recent review [15]. However, this avenue has proven very challenging to move into the clinic [48, 49], emphasizing the need to explore alternative strategies that target the CXCL12 chemokine directly.

Fragment-Based and Structure-Guided Study of Small-Molecule CXCL12 Inhibitors

Sulfotyrosine Recognition is Critical for the CXCL12/CXCR4 Interaction

The chemokine CXCL12 uses a “two-step, two-site” process for binding and receptor activation [50]. First, the extracellular amino-terminal domain of the receptor, a flexible ~30-residue domain binds the conserved, disulfide stabilized core structure of the chemokine. Next, the amino terminus of the chemokine docks into a pocket within the transmembrane region of the GPCR to form a fully activated receptor complex. While amino acids at the chemokine N-terminus function as a low-affinity receptor agonist, the initial interaction provides a majority of the binding energy and much of the receptor-chemokine selectivity [51-53]. Crucial to these initial encounters are one or more sulfotyrosines (sY) on the receptor peptide [12, 54, 55]. The sulfate groups on these altered tyrosine residues, a result of post-translational modification of the receptor [56], have been shown to significantly enhance the binding affinity in chemokine recognition [57, 58]. In the case of CXCL12 the receptor affinity is improved 20-fold upon tyrosine sulfation [21]. To date, tyrosine sulfation has been biochemically characterized in a subset of the chemokine receptors, including CXCR3 [59], CXCR4 [12], CCR2B [60], CCR3 [61], CCR5 [62] and CX3CR1 [55].

Targeting the Sulfotyrosine Binding Sites for Drug Discovery

In contrast to the high-throughput type discovery method employed for the chalcone compounds, we hypothesized in silico screening could produce a higher hit rate as demonstrated for other targets [63]. The sulfotyrosine binding pockets on CXCL12 represent potential sites for small-molecule inhibitors because of their importance in receptor recognition and their unique structural features [64]. They consist of both hydrophobic and polar/charged groups that can be utilized for ligand affinity and specificity, as revealed by the NMR structure of a CXCL12 dimer bound by two CXCR4 N-terminal peptides (Fig. 1a). In the chemokine CXCL12, there are three sY binding sites: sY21, sY12 and sY7. Although members of the chemokine family share varying degrees of sequence homology (some as little as 20%), all members retain the canonical chemokine fold. Using NMR to monitor the titration of sulfotyrosine into four representative chemokines, we observed a sulfotyrosine recognition site analogous to the cleft on CXCL12 that binds sY21 of the receptor CXCR4 [65]. With an estimated 1% of all tyrosines in cell-surface and secreted human proteins modified by *O*-sulfation [66], inhibitor development against sY binding sites can have far reaching implications in targeting disease-related protein-protein interfaces. The discovery and optimization of a “privileged” small-molecule scaffold would be invaluable for the development of ligands against other sulfotyrosine recognition sites.

Small-Molecule CXCL12 Inhibitors Targeting the sY21 Binding Pocket

Although chalcone **5** prevents CXCL12 from binding to its CXCR4 or CXCR7 receptors [21], no structural information has ever been reported. Our recent report demonstrated that small molecule **9**, identified from in silico docking studies, bound CXCL12 with a $K_d = 64$ μ M and produced chemical shift perturbations localized to the sY21 binding site. A NMR structure of the CXCL12-inhibitor **9** complex has shown that this small molecule binds within the sY21 binding pocket and participates in both polar and nonpolar interactions that mimic CXCR4 D20 and sY21 contacts (manuscript in preparation; Fig. 2).

Design and Synthesis of a Fragment Library to Search for Better Starting Points of CXCL12 Inhibitors

A retrospective analysis of highly optimized protein inhibitors suggests maintaining a ligand efficiency (LE) 0.27-0.30 kcal/mol/non-hydrogen atom (LE: defined as the free energy of binding divided by the number of non-hydrogen atoms) [67]. However, the LE for protein-protein interaction (PPI) disruptors rarely exceeds 0.24 due to the limited number of chemical (such as buried salt bridges) and physical features (cavities) necessary for high affinity interactions [68]. The LE of compound **9** is 0.23 which is typical for PPI inhibitors. Therefore, our goal was to optimize hit **9** by advancing potency while simultaneously improving ligand efficiency. Unlike traditional hit-to-lead optimization by altering (usually adding) functionalities to improve the affinity, we decided to take a fragment-based approach to trim compound **9** in order to find a better starting molecule. The SAR analysis [24] and complex structure of compound **9**-CXCL12 suggests the carboxylic acid group is essential for high-affinity binding. We envisaged its bioisostere [69, 70], a 5-substituted-1*H*-tetrazole group, could not only exhibit efficient binding to the sY21 pocket but would also possess improved drug-like properties as the tetrazole moiety is comparable in both size and acidity to the carboxylic acid group while being more metabolically stable [71]. In addition, the carboxylic acid was replaced with its bioisostere, 5-substituted-1*H*-tetrazole to maintain IP privileges. To this end, a fragment library containing nine compounds was designed (Chart 2). The design aimed to verify if the acylthiourea linker/spacer is essential. The linker/spacer was shortened from four atoms (acylthiourea) to two (amide) or three (urea) atoms. This operation keeps some molecular features such as hydrogen bond donor/acceptor unchanged while smaller fragments with higher binding potential (larger LEs) are to be discovered.

The tetrazole group was used as an anchor point to bind the sY21 pocket of CXCL12 from which fragments were then synthesized with phenyl substituted groups in *ortho*-, *meta*- and *para*-positions to make final compounds consisting of 15 to 21 non-hydrogen atoms (MW from 203 to 280). Fragments with *meta*- or *para*-substituents are relatively linear molecules while their *ortho*- counterparts were expected to adopt conformations that may be too voluminous for the sY21 pocket. This design was intentional as the steric tolerance near the narrow sY21 pocket may be revealed with the set of fragments.

Fragment libraries containing only nine members were designed and synthesized as shown in Scheme 1. *Ortho*-(1*H*-tetrazol-5-yl)aniline (**22**), *meta*-(1*H*-tetrazol-5-yl)aniline (**23**) and *para*-(1*H*-tetrazol-5-yl)aniline (**24**) were synthesized from the corresponding commercially available aminoben-zonitriles (**19-21**) via a cycloaddition of the corresponding nitriles with azide in the presence of triethylamine at 110 °C using nitrobenzene as solvent [72]. The isolated yields after column chromatography (silica/DCM-MeOH, up to 15% MeOH) on a gram scale are 77% for **22**, 87% for **23** and 88% for **24**, respectively. *N*-(2-(1*H*-Tetrazol-5-yl)phenyl)acetamide (**11**), *N*-(3-(1*H*-tetrazol-5-yl)phenyl)acetamide (**12**) and *N*-(4-(1*H*-tetrazol-5-yl)phenyl)acetamide (**14**) were prepared by acetylation of the corresponding tetrazole-anilines (**22-24**) with 2 equivalent (0.38 mmol) of acetic anhydride in the presence of triethylamine in DMSO at room temperature for 3 hr. Products were obtained in high yields (80% for **10**, 85% for **12** and 80% for **14**, respectively) after column chromatography (silica/DCM-MeOH, up to 10% MeOH). *N*-(2-(1*H*-Tetrazol-5-yl)phenyl)benzamide (**11**), *N*-(3-(1*H*-tetrazol-5-yl)phenyl)benzamide (**13**) and *N*-(4-(1*H*-tetrazol-5-yl)phenyl)benzamide (**15**) were synthesized by treating the corresponding tetrazole-anilines (**22-24**) with 2 equivalent of benzoyl chloride (0.38 mmol) in the presence of triethylamine in DMF at room temperature for 3 hr. Products were obtained as solid after column chromatography (silica/DCM-MeOH, up to 10% MeOH). The isolated yields are 75% for **11**, 82% for **13** and 70% for **15**, respectively. The tetrazole-phenylureas (**16-18**) were synthesized after a mixture of 2

equivalent of phenylisocyanate (0.38 mmol) and the corresponding tetrazole-aniline (**22-24**) was stirred at 50 °C overnight in DMSO and after the crude product was washed with DMC three times. The yields for 1-(2-(1*H*-Tetrazol-5-yl)phenyl)-3-phenylurea (**16**), 1-(3-(1*H*-tetrazol-5-yl)phenyl)-3-phenylurea (**17**) and 1-(4-(1*H*-Tetrazol-5-yl)phenyl)-3-phenylurea (**18**) are 75%, 85% and 75%, respectively. Similarly, compound **25** was synthesized by stirring a mixture of **24** (0.2 mmol) and 1-isocyanatonaphthalene (0.4 mmol) at 50 °C overnight in DMSO followed by column chromatography (silica/DCM-MeOH, up to 10% MeOH). The purity of the final compounds ranged from 96.3% to 99.9% (Table 1). The specifications of HPLC analysis are as follows: flow rate, 1 mL/min; column, Intertsil, 2.5 μ m, 4.6 $\times$ 150 mm; wavelength, 215, 254 and 280 nm; mobile phase, A: H₂O with 0.1% HCO₂H, B: MeOH, gradient of 30-95%B in 25 min. All compounds were fully characterized and confirmed by ¹H NMR and HRMS (ESI positive).

Study CXCL12-Tetrazole Interactions Using 2D NMR Spectroscopy and Molecular Docking

2D NMR was employed to study the fragment-induced CXCL12 chemical shift perturbations and determine the binding affinity of the synthesized fragments. [¹⁵N]-CXCL12 expression and purification was carried out as previously described [73]. Compounds **10-18** were titrated at 0, 25, 50, 250, 800, and 1600 μ M into samples containing 50 μ M [¹⁵N]-CXCL12 in 25 mM d-MES buffer (pH 6.8), 0.02% (v/v) NaN₃ and 10% (v/v) D₂O; compound **18** was titrated at 0, 25, 50, 200, 800, and 1600 μ M. 2D ¹H-¹⁵N heteronuclear single-quantum coherence (HSQC) spectroscopy experiments were performed on a Bruker 600 MHz spectrometer equipped with a TXI triple-resonance cryoprobe at 25 °C. Data was converted and processed using NMRPipe [74]. Previously published assignments for CXCL12 [73] were transferred by visual inspection and chemical shift values were tracked using CARA [75]. Combined ¹H/¹⁵N_H chemical shift perturbations were calculated as $((\delta_{\text{H}})^2 + (\delta_{\text{NH}})^2)^{0.5}$, where δ_{H} and δ_{NH} are the amide proton and nitrogen chemical shifts, respectively. Equilibrium dissociation constants (K_d) were determined by non-linear fitting to a quadratic equation that accounts for non-specific binding [24].

All molecules produced a subset of chemical shift changes distinct from the DMSO control titration – indicative of a specific binding interaction. Representative ¹H-¹⁵N HSQC spectra of A (**11**), B (**18**), C (**12**) and D (**17**) are shown in (Fig. 3).

CXCL12 chemical shift perturbations induced by nine fragments (**10-18**) are shown in (Fig. 4).

Chemical shift mapping suggests the *ortho*-substituents (**10**, **11** and **16**) cluster to the 1 and 2 strands near the CXCL12 dimer interface, consistent with the hypothesis that these molecules are too voluminous to occupy the narrow sY21 pocket. We hypothesize these reflect specific interactions as the perturbations are near the binding pocket for a different CXCR4 sulfotyrosine, sY12. Although further studies will be needed to authenticate this interaction, we used molecular docking to predict potential binding poses.

Docking was performed using DOCK3.4.54. Compounds **10**, **11** and **16** were docked against 20 NMR conformations and one X-ray structure in order to account for protein flexibility and capture the best conformation. Sulfotyrosine peptide ligands were used in the NMR structures (PDB ID 2K05) to create the docking environment for both the sY12 and sY21 sites, while heparin sulfate was used to generate the docking environment for the sY12 site in the X-ray structure (PDB ID 2NWG). The predicted binding poses for each compound were visually inspected and the pose that agreed the best with the NMR perturbations was chosen. Compounds **10**, **11** and **16** appear to bind to the sY12 site exploiting slightly different conformational space. As shown in (Fig. 5), docking predicts that the tetrazole in

compound **11** may form hydrogen bonds with Lys27, while the amide may hydrogen bond to Lys27. Compound **10** may hydrogen bond with Arg41 and His25, while the amide may hydrogen bond with Lys27 (docking poses not shown). For compound **16**, the tetrazole may form a hydrogen bond with His25, the urea nitrogen with Lys27 and the urea oxygen with Arg41 (docking poses not shown).

In contrast, the *para*- and *meta*-tetrazole fragments produce chemical shift changes near the predicted site suggesting relatively linear molecules are most compatible with the sY21 pocket. As shown in (Fig. 6), compound **18** appears to bind to the sY21 site. Docking predicts that the tetrazole in compound **18** may form hydrogen bonds with Ala19 and Asn22, while the urea nitrogen may form a hydrogen bond with Glu15 and the urea oxygen with Arg47.

It is interesting to note that several compounds induced shift perturbations in CXCL12 N-terminal residues 4-9 (Fig. 4). Unfortunately, further studies are required to gain structural insight as the N-terminus is highly flexible and its length permits contact with a ligand bound at either the dimer interface or the sY21 cleft. Non-linear fitting of chemical shift perturbations yielded affinities ranging from 13 to 327 μ M (Table 1). Compounds **11** and **18** possess the highest affinities of 13 and 24 μ M and exhibit corresponding LE improvements of 0.33 and 0.30, respectively. Fragments **10**, **12** and **14** induced small chemical shift perturbations, resulting in large fitting errors and an inability to generate meaningful affinity values. We hypothesize the small perturbations result from the size of the acetyl group on the phenyltetrazole moiety rather than the large hydrophobic groups present in the other fragments.

As shown in (Table 1), most compounds possess improved LEs and demonstrate an enhanced binding potential compared to compound **9**. The compounds with an amide linker (**11**, **13** and **15**) uniformly demonstrated higher binding potentials than their urea counterparts (**16** – **18**), implying that they may be other appropriate starting points for the next round of optimization. The *para*-substituted molecules (**14**, **15** and **18**) are the only set that exhibited a positive correlation between molecular weight and affinity suggesting this to be the optimal tetrazole orientation. In contrast, the affinities of *ortho*- and *meta*-substituted tetrazoles decreased 25- and 2.5-fold, respectively, from 265 to 280 Da. Overall, these results support the tetrazole moiety as an effective anchor point for targeting sulfotyrosine binding pockets and developing more efficient lead molecules.

Optimization of Tetrazole-Based CXCL12 Inhibitor **18**

With compound **18** as a novel hit binding to the sY21 site of CXCL12 in hand, a subsequent library containing a dozen derivatives was designed and synthesized. In this library, both the *para*-substitution and urea linker were retained while different substituted aromatic functionalities replaced the benzene ring in **18**. Molecular docking predicts that compound **25** binds the sY21 site similarly to that of **18** (Fig. 7). Similar to **18**, compound **25** is found to not only bind in the sY21 site determined by 2D NMR (Fig. 8A – 8C) but also inhibit CXCL12-induced chemotaxis (Fig. 8D).

The activity of the tetrazole fragments was tested using an *in vitro* chemotaxis assay. THP-1 monocytes, which endogenously express the CXCR4 receptor, were incubated with CXCL12 in the absence or presence of each fragment. At 250 μ M, compound **9** was unable to completely inhibit 30 nM of CXCL12-induced chemotaxis and yielded an IC₅₀ of ~800 μ M. Although not directly comparable, compound **25** significantly diminished cell migration and inhibited chemotaxis to 10 nM CXCL12 with an IC₅₀ = 111 $\pm$ 24 μ M. Compounds **13** - **16** also significantly inhibited migration toward 10 nM CXCL12 at 250 μ M. No compounds affected cell viability. To determine whether compound **25** specifically

inhibits CXCL12, the chemotaxis assay was repeated with CCL2 (also known as monocyte chemoattractant protein-1, MCP-1), a distinct chemokine that activates the CCR2 receptor (data not shown). Our results indicate that compound **25** specifically inhibits CXCL12-induced migration with an improved potency compared to compound **9**.

Concluding Remarks and Future Perspectives

The CXCL12/CXCR4 interaction is a high-priority clinical target because of its involvement in a broad spectrum of pathologies, including: autoimmune disease, cardiovascular disease and cancer. Current drug discovery efforts focus on identifying molecules that bind the transmembrane region of the chemokine receptor. However, antagonizing the large protein-protein interface has proven difficult for traditional high-throughput approaches and emphasizes the need for alternative strategies. We recently demonstrated the effectiveness of rationally designing small molecules that target the chemokine ligand [24]. From our published SAR analysis of initial hit **9** [24] and the unpublished complex structure with CXCL12 (manuscript in preparation), we designed a fragment library with improved affinities, efficiencies, and potencies. The best overall compound **25** retains a specific interaction with the sY21 site and exhibits a substantial improvement in potency (Fig. 8). In addition, the identification and development of compounds that bind the sY12 site provided a second target for future CXCL12-directed inhibitors and, therefore, fragment **11** serves as another starting point for ligand expansion or fragment-linkage in the development of high-affinity molecules.

Although our work focused on inhibition of CXCR4 activation, CXCL12 also interacts with C-X-C motif chemokine receptor 7 (CXCR7). CXCR7 is a non-canonical receptor that promotes arrestin recruitment and downstream signaling without activation of heterotrimeric G proteins. Following its re-classification as a chemokine receptor aberrant CXCR7 expression has been implicated in breast, lung, and B cell lymphoma [76-78]. Nonetheless characterization both *in vitro* and *in vivo* remains substantially hindered by the inability to discriminate between CXCR4- and CXCR7- mediated signaling. Indeed, two commonly used CXCR4 inhibitors (compound **1** and peptidomimetic TC14012) also activate CXCR7 [79, 80]. Several specific small molecule CXCR7 inhibitors have been identified through high throughput screening [76], but application of structure-based approaches could enhance the identification of novel antagonists [63, 81]. An obvious step in developing CXCL12 antagonists is assessing their potential targeting the CXCL12/CXCR7 interaction. Chalcone molecule **5** competes with CXCR7 binding to CXCL12 and suggests a similar inhibition may be observed using compounds targeting the sulfotyrosine binding sites [21]. Perhaps more interesting, a structural understanding of the CXCL12/CXCR7 interface may enable the development of specific inhibitors as potential therapeutics or molecular probes to dissect CXCL12 effects on respective receptors.

The success of our previous structure-based discovery of a CXCL12 antagonist [24] and its rapid optimization, reported herein, suggest the sulfotyrosine binding moiety may serve as a 'natural small molecule' site to guide drug discovery and development. The conservation of both sulfotyrosine binding sites and receptor tyrosine sulfation throughout the chemokine family highlights the potential for expansion of chemokine-directed discovery [65, 82]. Rational drug discovery campaigns targeting the chemokine ligand may prove more effective than traditional strategies focused at the receptor. Whereas further studies are required to gain structural information of interactions at the sY12 site of CXCL12, both NMR and mutagenesis have characterized the interface analogous to the CXCL12 sY21 recognition site for numerous chemokines [83-85]. Targeting the ligand may also enable modulation of homeostatic and pathologic processes. Chemokine signaling is generally considered to be polygamous with receptors recognizing multiple ligands. For example,

although CXCL12/CXCR4 was identified as a monogamous pair, CXCR4 is now known to additionally interact with extracellular ubiquitin and MIF [86, 87]. Recent evidence suggests these systems are not redundant, but rather display signs of ligand selectivity and biased agonism in which chemokine ligands signal disparate physiologic outcomes dependent upon specific structural interactions with the associate receptor [88-90]. Targeting a specific ligand may empower regulation, rather than simply elimination, of receptor activity. Futures perspective to the discovery of antagonists for CXCL12/CXCR4 interactions, may include the use of computational methodologies such as Quantitative-Structure Activity Relationships (QSAR) [91-119], especially those focused on the simultaneous classification and prediction of compounds with different biological activities [120-137], including the discovery of inhibitors for proteins associated with one or more diseases [138-143]. Alternatively, complex network theory could be used to gain a better understanding about ligand-protein and protein-protein interactions [143-152].

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Fig. (1).

Fig. (2).

Fig. (3).

Fig. (4).

Fig. (5).

Fig. (6).

Fig. (7).
The docking pose of compound 25.

Fig. (8).
Tetrazole fragments bind CXCL12.

Chart 1.
Structures of Representative Small Molecule CXCR4 Antagonists and CXCL12 Inhibitors.

Chart 2.

Scheme 1.
Synthesis of fragment library.

Table 1

Structure and Activity of Fragments

ID	Structure	MW	HPLC (%)	K _d (μM)	LE
9		350	NA ^a	64 ± 15	0.23
10		203	98.9	ND ^b	ND
11		265	97.7	13 ± 7	0.33
12		203	99.9	ND	ND
13		265	99.7	50 ± 16	0.29

ID	Structure	MW	HPLC (%)	K _d (μM)	LE
14		203	98.7	ND	ND
15		265	98.7	64 ± 23	0.29
16		280	97.5	327 ± 92	0.23
17		280	96.3	126 ± 23	0.25
18		280	99.3	24 ± 28	0.30

^aNot available^bNot defined.