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Targeting SDF-1/CXCL12 with a Ligand That Prevents Activation of CXCR4 through Structure-Based Drug Design

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Chemokines are a family of small secreted proteins that orchestrate cell migration by activating a set of G-protein-coupled receptors (GPCRs). The immune system relies on chemokine signaling to direct lymphocyte homing, orchestrate inflammatory responses, and stimulate wound healing.[1] Outside of these normal functions, chemokines and their receptors also participate in numerous disease states, including HIV/AIDS, asthma, autoimmune diseases, and cancer.[2,3] Most drug discovery research is directed at GPCRs,[4] and therapeutic modulation of chemokine signaling is correspondingly directed at the receptors rather than the ligands. Small-molecule antagonists targeting chemokine receptors are in various stages of development; the HIV entry inhibitor Maraviroc, which blocks the CCR5 coreceptor, was recently approved by the FDA for clinical use.[5] Chemokine variants and peptidomimetics are also viewed as potential inhibitors.[6]

Because it directs stem-cell homing[7] and participates in nearly every aspect of cancer progression—growth, metastasis, and neovascularization[8,9]—the CXCL12/CXCR4 signaling axis is of increasing interest for drug discovery. In principle, inhibitors targeting the chemokine ligand would also be useful, but small (<10 kDa) proteins traditionally have been considered too small to be "druggable". However, Fesik and co-workers[10] successfully used NMR-based fragment screening to identify micromolar ligands for FKBP12, a 12 kDa protein, and defined structure−activity relationships (SARs) that enabled the subsequent design of a potent nanomolar inhibitor. The serendipitous discovery by Wells and colleagues[11] of an IL-2 inhibitor that binds the cytokine ligand rather than the receptor demonstrated that shallow, solvent-exposed clefts on small, secreted proteins can serve as legitimate sites for drug discovery. The recent report of a chalcone that binds CXCL12 and prevents CXCR4 activation[12] suggests that chemokines are legitimate targets for inhibition.[13]

Tyrosine O-sulfation is an important post-translational modification in the N-terminal extracellular domain of chemokine receptors that contributes to specific chemokine recognition. CXCR4 sulfation at residues 7, 12, and 21 enhances its interaction with CXCL12,[14−16] and the NMR structure of a soluble dimeric CXCL12−CXCR4 complex revealed a specific binding pocket for each sulfotyrosine.[15] In a recent NMR study using full-length CXCR4, methyl-containing side chains in all three sulfotyrosine recognition sites exhibited saturation transfer effects,[17] reinforcing the functional relevance of the CXCL12−CXCR4 contacts we observed in the soluble complex and validated by mutagenesis.[15] Of the three CXCR4 sulfotyrosines, sY21 was reported to make the largest contribution to CXCL12 binding.[15,16] Consequently, we hypothesized that small molecules targeting the sY21 site could act as chemokine inhibitors and designed a structure-based screen for compounds that bind CXCL12 and prevent CXCR4 signaling.

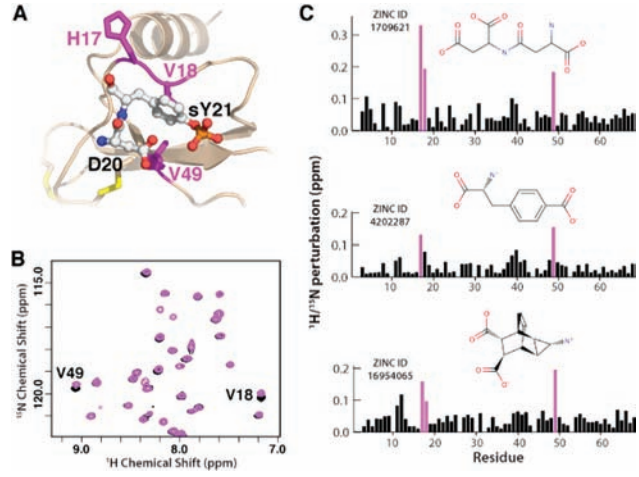

Figure 1. Correlation between chemical shift perturbations and docking of small molecules. (A) CXCR4 residues D20 and sY21 occupy a cleft on the CXCL12 surface bordered by residues in the N-loop (H17 and V18) and β3-strand (V49). (B) HSQC spectra of 250 μM [U-^{15}N]CXCL12 acquired in the presence of 0, 0.5, and 1.25 mM 1709621. (C) HSQC shift perturbations induced by binding of three ZINC compounds identify H17, V18, and V49 (purple bars).

We performed an in silico screen of compounds from the ZINC virtual compound library[18] using DOCK 3.5.54[19,20] at the site on CXCL12 occupied by CXCR4 residues D20 and sY21 (Figure 1A) in our NMR structure of the complex (PDB entry 2K05). After an examination of 1000 compounds with the best docking scores, the five that appeared most complementary to the sY21 site were selected for NMR titrations with [U-^{15}N]CXCL12 to assess the binding affinity and specificity.

Mapping of ligand-induced ^{1}H−^{15}N heteronuclear single-quantum correlation (HSQC) shift perturbations (Figure 1B) indicated that three of the five compounds (ZINC IDs 1709621, 4202287, and 16954065) bound weakly but specifically to CXCL12 in the CXCR4 sY21 site (Figure 1C). Perturbations induced by ZINC compound 4900356, while significant, were consistent with nonspecific interactions at multiple sites.

In comparison with the other four compounds, ZINC 310454 produced larger shifts for more CXCL12 residues (Figure 2A). While the pattern of shift perturbations is distinct from those for the other molecules, their distribution on the CXCL12 surface is

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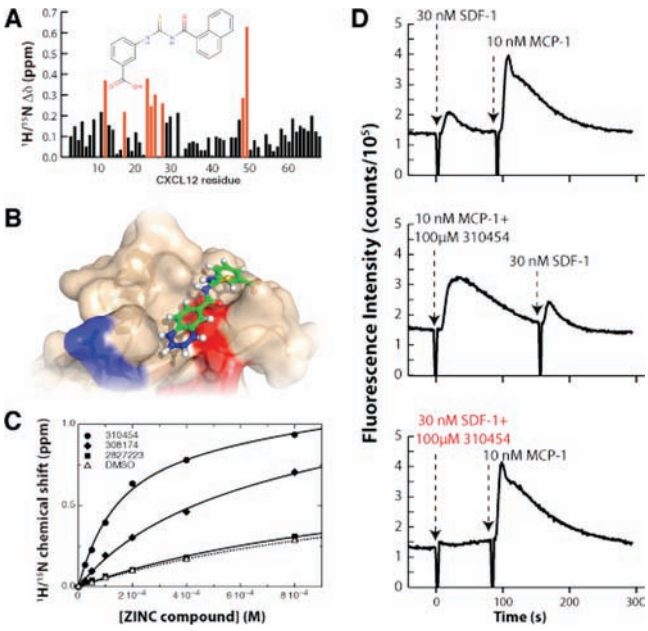

Figure 2. 3-(Naphthalene-2-carbonylthiocarbamoylamino)benzoic acid (ZINC ID 310454) binds CXCL12 and inhibits CXCR4-mediated Ca^{2+} flux. (A) CXCL12 chemical shift perturbations induced by ligand binding. (B) A consistent set of perturbed residues (red) surround the docked 310454 (naphthyl) and 308174 (phenyl) compounds, but R12 (blue) is shifted only when the naphthylene is present. (C) Modifications to the naphthyl (308174) or benzoic acid (2827223) groups reduced the CXCL12 binding affinity relative to that of 310454. (D) Ca^{2+} flux of THP-1 cells in response to (top) 30 nM CXCL12 followed by 10 nM chemokine CCL2/MCP-1, (middle) 10 nM CCL2/MCP-1 preincubated with ZINC ID 310454 followed by 30 nM CXCL12, and (bottom) 30 nM CXCL12 preincubated with 100 μM ZINC ID 310454 followed by 10 nM CCL2/MCP-1.

consistent with the docking pose for this compound (Figure 2B), which is considerably larger than the others.

Nonlinear fitting of the chemical shift perturbations showed that 310454 bound CXCL12 significantly more tightly ($K_d = 64 \pm 15$ μM) than the other compounds, with affinities similar to those exhibited by successful "hits" from other NMR-based drug discovery efforts.[21] To further probe the SARs of 310454-mediated CXCL12 inhibition, we measured the binding of five related compounds by NMR spectroscopy (Figure 2C and Supporting Figure 2). Removal or replacement of the carboxylic acid with a methyl ketone or bromine established the critical importance of this functional group, as evidenced by complete loss of binding. Indeed, the presence of one or more carboxylic acid groups was a common feature of the original five compounds identified through in silico screening. Substitution of the naphthyl group with a phenyl ring lowered the affinity by ~10-fold and altered the pattern of shift perturbations in a manner consistent with the predicted binding mode (Figure 2B). Thus, both charged and hydrophobic interactions contribute to the affinity and specificity of 310454 for the CXCL12 sulfotyrosine binding pocket.

To test 310454 as an inhibitor of CXCL12-mediated signaling, we measured CXCR4 activation by monitoring intracellular Ca^{2+} levels in THP-1 cells, which express high levels of CXCR4 and CCR2.[22] Addition of 100 μM 310454 alone induced no Ca^{2+} flux in THP-1 cells. Preincubation of the chemokine with 100 μM 310454 had no effect on MCP-1/CCR2 signaling but abolished the CXCL12-mediated Ca^{2+} flux response (Figure 2D). We conclude that 310454 is a selective inhibitor of CXCL12 that acts by blocking a key interaction with sY21 in the CXCR4 N-terminus.

Our results reinforce the concept that small cytokines are viable drug targets.[11,12] The uncertainty in side-chain positions associated with NMR structure ensembles is often viewed as a barrier to successful in silico ligand screening. However, using a single NMR-derived conformer, we exploited details of CXCR4 sY21 recognition by CXCL12 to search for compounds that would occupy the same site and satisfy a similar set of polar and hydrophobic contacts. Four unrelated compounds bind the selected site, and one, 3-(naphthalene-2-carbonylthiocarbamoylamino)benzoic acid, inhibits CXCL12 activation of its CXCR4 receptor at micromolar concentrations. SAR analysis confirmed the orientation of the bound ligand and demonstrated a requirement for the benzoic acid functional group, suggesting that it may mimic the negatively charged sulfotyrosine. We speculate that sulfotyrosine binding sites on other chemokines and elsewhere on the CXCL12 surface could be similarly targeted with small molecules. Linking micromolar ligands that bind adjacent sites may enable the design of novel, high-affinity inhibitors of CXCL12 and other chemokines.

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Supporting Information Available: Complete ref 12, detailed methods, CXCL12-associated docking poses of all compounds, and HSQC spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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