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Benzenesulfonamides: A Unique Class of Chemokine Receptor Type 4 Inhibitors

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Abstract

The interaction of CXCR4 with CXCL12 (SDF-1) plays a critical role in cancer metastasis by facilitating the homing of tumor cells to metastatic sites. Based on our previously published work on CXCR4 antagonists, we have synthesized a series of aryl sulfonamides that inhibit the CXCR4/CXCL12 interaction. Analog bioactivities were assessed with binding affinity and Matrigel invasion assays. Computer modeling was employed to evaluate a selection of the new analogs docked into the CXCR4 X-ray structure and to rationalize discrepancies between the affinity and Matrigel in vitro assays. A lead compound **5a** displays subnanomolar potency in the binding affinity assay ($IC_{50} = 8.0$ nM) and the Matrigel invasion assay (100% blockade of invasion at 10 nM). These data demonstrate that benzenesulfonamides are a unique class of CXCR4 antagonists with high potency.

Keywords

CXCR4 inhibitors; metastasis; sulfonamides; inflammation

Introduction

G-protein coupled receptor (GPCR) CXCR4 and the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) play a crucial role in physiological processes such as leukocyte migration/trafficking and hematopoiesis.^[1] The interaction of CXCL12 with CXCR4 has implications in cancer metastasis^[2] and CXCR4 is a co-receptor for HIV-type1 infection.^[3] CXCR4 is frequently overexpressed in solid tumors as compared with normal tissue.^[4] Activation of the CXCR4/CXCL12 pathway can lead to recruitment of distal stroma by tumor cells to facilitate tumor growth and metastasis, as well as promote homing of tumor cells to metastatic sites,^[5] angiogenesis,^[6] cancer cell survival and invasion.^[4, 7] Therefore, disruption of the interaction of CXCL12 with CXCR4 could potentially block or delay metastasis.

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Thus far, the most explored non-peptidic anti-CXCR4 agents are bicyclams such as AMD3100 and its derivatives.^[8] However, their metal chelating properties may be the cause of cardiotoxicity and therefore may well limit the use of these compounds clinically.^[8] Recently, we identified a novel class of CXCR4 antagonists^[9] that led to a potent dipyridine – WZ811 (**1**) (Figure 1). In addition, potent dipyrimidine analogs were also synthesized.^[10] Exploration of tunable areas around the dipyridine pharmacophore led us to prepare a new class of benzenesulfonamide analogues (Figure 2). The central phenyl ring was retained because it has been shown to be important for inhibitory activity.^[9] A binding affinity assay against the potent CXCR4 antagonist TN41003 (**2**) (Figure 1) was employed as a primary screening method for the new analogs.^[9, 11] Some of these compounds were further analyzed in the Matrigel invasion assay using full length CXCL12.

Chemistry

A small series of mono- and di-sulfonamide analogs of **1** (Figure 2) were prepared with a selection of substituents to test the viability of this structural class as CXCR4 antagonists. Compounds **3a** and **3b** were synthesized in one step by the reaction of xylenediamine with the corresponding sulfonylchlorides (Scheme 1). Compounds **5a** – **5n** were prepared by the treatment of 4-(bromomethyl)benzenesulfonylchloride with the corresponding secondary amine to give compounds **4a** – **4d**, which were treated with the appropriate amine to give the final products (Scheme 2). Compound **7** was obtained similarly by treating 4-(bromoethyl)-benzenesulfonyl chloride with N-methyl-2-pyridinemethanamine to give **6**, which was subsequently coupled with pyrrolidine in the presence of K₂CO₃ to give the final target (Scheme 2). As shown in Scheme 3, N-Boc-4-hydroxyaniline was treated with 2-chloro-N, N-diethylethanamine to yield **8** and subsequently deprotected with HCl in dioxane to give **9** as the hydrochloride salt. Compound **9** was then combined with 4-(bromomethyl)-benzenesulfonyl chloride to give **10**, which yielded **11a** – **11d** upon treatment with the corresponding amines.

Results and Discussion

Binding Affinity Assay

The compounds were initially screened with a binding affinity assay involving competition of peptide **2** with the putative antagonists as described in our previous publications.^{[11], [9]} MDA-MB-231 cells were pre-incubated with compounds at concentrations of 1, 10, 100, and 1000 nM, then incubated with biotinylated **2** and streptavidin-conjugated rhodamine to determine the binding efficiency of the new analogs to the CXCL12 binding domain of CXCR4. The effective concentration (EC) is defined as the lowest concentration at which a significant reduction in the rhodamine fluorescent color is observed as compared to the control reflecting the competitive displacement by peptide **2**. Thus, this screening is a semi-quantitative, first pass measure of the level of activity and should not be confused with IC₅₀.

Initially, five compounds (**3a**, **3b**, **5a**, **5b** and **11a**) were synthesized and analyzed for binding. As shown in Table 1, the disulfonamides **3a** and **3b** were found to be relatively inactive. However, the mono-substituted sulfonamides **5a**, **5b** and **11a** were more effective with EC values of 10, 1 and 10 nM, respectively. Hence, we pursued analogues with the mono-substituted sulfonamide motif as potential CXCR4 blockers using the preliminary and less time-consuming EC binding assay.

Thus, the molecular sectors to the left (Region A) and to the right (Region B) of the mono-substituted sulfonamide structure (Figure 3) were explored by substitution and binding affinity screening against antagonist **2**. The central phenyl ring was left untouched since previous work in our group showed that this ring is critical for activity.^[9–10] Several cyclic

and acyclic amines were introduced to region A of the benzenesulfonamide motif (Scheme 2). The morpholine derivative (**5c**) and the pyrrole derivative (**5f**) were not well tolerated and showed an EC of over 1000 nM. However, the potencies of diethyl (**5d**) and piperazine (**5e**) derivatives were comparable to the initially synthesized **5a** and **5b**. Small non-aromatic rings appeared to be favored in region A.

Next, region B was explored by adding substituted aromatic rings (fluorine, tert-butyl or methoxy groups) or a pyridine ring, while retaining the piperidine, pyrrolidine or the diethylamine groups in region A. Surprisingly, addition of fluorine or methoxy at the para-position, as well as fluorine ortho and meta (**5m** and **5n**, respectively), resulted in a significant reduction in binding as compared to **5b**. However, addition of a tert-butyl moiety to the para-position produced the potent compounds **5j** and **5k**. Replacement of the phenyl ring of region B with a pyridine ring (**7**) likewise enhanced potency. Due to the success of **11a**, additional analogues were prepared that retained the diethyl amine fragment in region B, but varied region A with cyclic, acyclic or aromatic substituents. In this case, the 5- and 6-membered rings were well tolerated in region A (**11c** and **11d**). However, the morpholine substituted **11b** showed poor activity, as was the case for the other morpholine analog **5c**.

Since we used only four concentrations (1, 10, 100 and 1000 nM) for the TN binding assay to determine the effective concentration (EC), we performed seven point evaluations for several compounds with EC values ranging from 1 to 1000 nM (Figure 4). The comparative results (Table 2) illustrate that the rhodamine assay is semi-quantitative and able to demonstrate binding at a minimum within a factor of 5–10.

Results of Matrigel Invasion Assay

The Matrigel invasion assay, a functional probe using full length CXCL12, was employed as a secondary functional assay to test whether the compounds can block CXCR4/CXCL12-mediated chemotaxis and invasion at two concentrations (10 and 100 nM).^[11] The reason for selecting these two concentrations was to identify potent compounds that are reasonably active at least at 100 nM and to show concentration-dependence. The results of both binding affinity and Matrigel invasion for all analogues are depicted in Table 1 except for compounds showing a binding affinity > 1000 nM. Previously, we demonstrated that the dominant effects of our Matrigel invasion arise from a CXCR4/CXCL12-mediated process.^[10b, 11] The results show that **5a** and **5b** perform well in both the affinity binding assay and invasion assay at 95 to 100% inhibition of invasion at 100 nM as compared to peptide **2** as the positive control (set as 100% inhibition of CXCR4/CXCL12-mediated invasion). Compound **5d** also delivered a reasonable result of 71% and 84 % at 10 nM and 100 nM, respectively. Compound **5g** exhibited a binding affinity of 100 nM, although the results in the Matrigel invasion were moderate at 48% (10 nM) and 72% (100 nM). Compound **5k** showed an EC of 1 nM in the binding affinity assay and also gave moderate results in the Matrigel invasion assay. For the series **11a** – **11d**, **11a** delivered a satisfying result in both the binding affinity assay (EC 10 nM) and the Matrigel invasion assays (77% at 10 and 100 nM). Compound **11d** gave a binding affinity of EC of 10 nM and performed moderately in the Matrigel invasion assay with 82% inhibition of invasion at 100 nM.

One striking feature of the data as it pertains to results across both binding and Matrigel assays is the behavior of six compounds: **3b**, **5c**, **5h**, **5i**, **5m** and **5n**. Each substance furnishes a binding assay value of 1000 nM (EC, Table 1), but a contrary Matrigel invasion reduction of >75%. A consistent proposal for the behavior of this subset (29% of the compounds prepared) is provided below in the context of antagonist orientation in the binding pocket of CXCR4. One final unusual and unique compound is **7**, which evidences a

binding concentration of 1 nM, but a Matrigel invasion outcome of 44%. We regard this compound as an outlier that needs further examination.

Computational Modeling

Mapping CXCR4 Antagonists and CXCL12 N-terminus Binding Sites—

Computational docking of the synthesized ligands into the binding pocket of the recently disclosed CXCR4 X-ray structure^[12] was performed to furnish insights into the discrepancies between the binding affinities (EC) and the Matrigel invasion assay for the subset of six analogs mentioned above. Compared with previously solved GPCR X-ray structures, the binding cavity of CXCR4 is larger and more open with 3322 Å³ cavity volume.^[12] The small-molecule antagonist IT1t only occupies part of the pocket. Several functional studies of mutant CXCR4 revealed that Asp97, Asp187, Glu288, F87, D171 and F292 are required for CXCL12 binding, while the first three residue mutants impair CXCL12 signaling.^[13] The cyclic peptides **2** and CVX15 (**12**) are CXCR4 antagonists of known structure. CXCR4 alanine scanning for mutants identified residues required for the binding of **2** to be Asp171, Arg188, Tyr190, Gly207 and Asp262.^[14] By combining the mutational outcomes with crystal structure analysis, we can map the binding sites of the CXCR4 antagonists and the CXCL12 N-terminus. In this way, antagonist peptides **2** and **12** are shown to occupy similar sectors of the CXCR4 binding cavity, since most of the key residues sensitive to the binding of **2** are in close contact with **12**. However, mutational analysis also shows that the CXCL12 N-terminus binds in another sector of the binding pocket, leading to only partial overlap between peptide antagonist and CXCL12 N-terminal binding (Figure 5).

Prediction of Benzenesulfonamide Analog Binding Poses—In order to explore the possible structural basis behind the binding/Matrigel discrepancy for certain benzenesulfonamides, we arbitrarily classify the analogs of Table 1 as active (EC < 100 nM) or inactive (EC > 1000). In general, we find that the unscaled binding free energies from Prime MM-GBSA calculations correlate with the two categories of effective concentrations. For compounds regarded as active, the unscaled energies are > |30| kcal/mol (Table 3). For example, the best Glide docking pose of **5a** (Figure 6a, EC = 10) is protonated on the pyrrolidine nitrogen, forms a salt bridge (2.8 Å) to Asp97 and delivers a predicted binding free energy of −36.4 kcal/mol. One oxygen of the sulfonamide engages in a hydrogen bond with Arg188. Both the pyrrolidine and phenyl rings fit into small subpockets and make hydrophobic contacts with CXCR4 (Figure 6b).

Superposition of all the docked active analogs suggests that they reside in a similar location and form either a salt bridge or a hydrogen bond with Asp97 or Glu288, which play key roles in CXCL12 binding and signal transduction^[13a] (Figure 6c). For compounds classified as binding inactive (EC > 1000 nM), the unscaled binding free energies are in the range of −10 to −20 kcal/mol except for **5h**, **5i** and **5l**, which fall between −40 and −42 kcal/mol (Table 3). No polar interactions or hydrogen bonds are formed between the two key CXCR4 residues Asp187 or Glu288 for the latter agents as well as inactive **7**. For selected compounds the IC₅₀ values vs. docking scores (Table 4) show a trend similar to the EC values vs. docking scores (Table 3). Superposition of **5h**, **5i** and **5l**, all with predicted binding free energies in the highly active range (−40 to −42 kcal/mol), suggests that these agents associate with CXCR4 in poses that place the structures in a distinct part of the binding pocket near Asp97. The nitrogens of the pyrrolidine groups are protonated and form salt bridges (2.7 Å) to Asp97 (Figure 7). None show overlap with the peptide **12** binding geometry (Figures 5a and 5d), unlike the actives displayed in Figure 6. Thus, it is suggested that these three compounds bind efficiently to the CXCR4/**12** complex, but are unable to compete with peptide **2**. This explains the inconsistency between the high experimental 1000

nM concentrations and the substantial calculated binding free energies for **5h**, **5i** and **5l**, since peptide **2** and **12** occupy essentially the same but different sector of the binding pocket (Figure 5c). It may also explain the activities of **3b**, **5h** and **5i** in the Matrigel invasion assay, which incorporates full-length CXCL12 and overlaps with both sectors of CXCR4 (Figure 5).

A further observation provides additional support for this interpretation, while taking into account a unique structural factor for **5h**, **5i** and **5l** in terms of the para-F or para-OMe located within the benzyl substituent. That is, **5h** and its congeners are predicted to adopt alternative poses as depicted in Figure 7b and 7c. The protonated amine forms a salt bridge with Asp97 consistent with the same interaction for all the active analogs as monitored by the EC binding affinities. In addition, the SO₂ moiety engages in a bifurcated H-bond with Ser285, one face of the fluorophenyl group resides in a hydrophobic environment and the para-fluorine atom is closely associated with cationic Lys38. (Figure 7d) It is well known that organic fluorides enjoy a strong electrostatic interaction with cationic amines sufficient to alter the classic axial/equatorial rules of conformational analysis.^[15] While the corresponding para-OMe analog **5l** can be expected to experience a similar productive interaction with Lys38, the t-Bu analogues **5j** and **5k** do not do so for both steric and electrostatic reasons, adopting the poses of Figure 6 instead. In sum, this analysis suggests that the polar para-substituents provide an unexpected anchor site altering the binding pose while directing the compounds away from bound peptide **2**.

As a test of the hypothesis regarding the role of aromatic fluorine in reshaping the binding pose, we prepared **5m** and **5n** (Table 1), analogs of **5i** in which the para-F substituent was separately moved to the ortho and meta positions. According to the model of Figure 7d, Arg183 hydrogen bonded to Asp97 is perfectly poised to anchor the aromatic fluoride at these centers analogous to the action of Lys38 at the para-position of **5h**. Accordingly, when **5m** and **5n** were subjected to the Matrigel assay at 100 nM, they blocked invasion at 88 and 98%, respectively. This compares with 82 and 99% at the same concentration for **5h** and **5i**, respectively. Such stabilization is not unprecedented. As part of a mechanistic enzyme study, a conformational partition of a CF₃-methionine between two closely spaced arginines in bacteriophage lambda lysozyme has been observed by NMR spectroscopy.^[16]

Conclusion

We have synthesized a novel class of benzenesulfonamides that inhibit CXCR4 as evidenced by the displacement of antagonist TN14003 (**2**) from the receptor. Compounds **5a** and **5b** were among the most potent compounds, exhibit an IC₅₀ of less than 10 nM in the binding affinity assay and show more than 90% inhibition of invasion in the Matrigel invasion assay as compared to control. Computer modeling reveals that the potent analogs interact with key residues Asp97 and Arg188 in the CXCR4 binding pocket, mutations of which interfere with receptor action. In addition, the modeling provides a satisfying explanation for compounds that perform poorly in the binding affinity assay against peptide **2**, but deliver favorable blockade of Matrigel invasion and MM-GBSA binding free energies reflecting good to excellent potency. The binding/Matrigel inconsistency is resolved by the observation that these compounds are predicted to dock in the CXCL12 binding region but, in the expansive binding site, do not overlap with peptide **2** and, thus, do not compete with it. It is unusual for two drug-sized molecules to bind simultaneously to the same binding site. However, the muscarinic M2 receptor^[17] and the sweet receptor^[18] may be two examples of the phenomenon exemplified by Figure 7b. Unfortunately, these results suggest that the utility of the EC binding site assay would appear to be compromised as a primary screen for CXCR4 antagonists, and, at the very least, needs to be complemented by a second functional assay.

Experimental

Initial Screening of Anti-CXCR4 Small Molecules Based on a Binding Affinity Assay

Binding affinity and cell invasion assays are basic assay tools that apply to the initial screening. MDA-MB-231 cells cultured in an 8-well slide chamber were pre-incubated with the test compounds at 1, 10, 100, and 1000 nM. The cells were fixed with 4% formaldehyde and incubated with 50 nM biotinylated **2** followed by rhodamine staining.

IC₅₀ Measurement for Select Sulfonamide Analogs—IC₅₀ values of selected compounds were tested at 1, 4, 10, 40, 100, 400, and 1000 nM, or at 0.1, 0.4, 1, 4, 10, 40, and 100 μ M, based the results of initial screening. 2×10^4 MDA-MB-231 cells were cultured in an 8-well slide chamber for two days. The cells were pre-incubated with the testing compounds for 15 min, and then the cells were fixed with 4% formaldehyde. The fixed cells were subsequently incubated for 45 min with 50 ng/ml biotinylated **2**.^[10b, 11] Cells were incubated for 30 min in streptavidin-rhodamine at a 1:150 dilution (Jackson Immuno Research Laboratories, West Grove, PA) after washing three times with PBS. Finally, the slides were washed with PBS and mounted in an antifade mounting solution (Molecular Probes, Eugene, OR). Five pictures of stained cells for each treatment were taken on a Nikon Eclipse E800 microscope. Pictures were analyzed quantitatively with ImageJ, and IC₅₀ values for each compound were fitted with GraphPad 4.

Matrigel Cell Invasion Assay

Matrigel invasion chambers from BD Biocoat Cellware (San Jose, CA) were used for invasion assays. MDA-MB-231 cells were cultured on a layer of Matrigel in the upper chamber with testing compounds at 10 or 100 nM, while 200 ng/mL CXCL12 was added in the lower chamber as a chemo-attractant. Detailed procedures for the binding affinity and invasion assays have been described in previous publications.^[2c, 10b, 11]

Computational Protein-Ligand Docking

The acid dissociation constant (pK_a) of the benzene- sulfonamide derivatives was predicted by ACD software.^[19] Based on these values, nitrogen sites in these compounds were protonated when the pK_a was estimated to be greater than 7. All prepared benzenesulfonamide derivatives with the appropriate N-site protonated were docked flexibly into the cavity region of the human chemokine receptor CXCR4 crystallographic structure (PDB code: 3ODU) devoid of the small-molecule antagonist IT1t using Glide with standard precision (Schrödinger, LLC).^[20] This methodology regards the protein structure as a rigid body, but treats the ligand as a conformationally flexible molecule. The resulting CXCR4/ benzenesulfonamide complexes were subsequently sorted energetically with the MM-GBSA scoring algorithm, which provides an estimate of relative binding free energies.^[21] The volume of the CXCR4 cavity was obtained by the web-based CASTp package.^[22]

Chemistry: General—Proton NMR spectra were recorded on INOVA-400 (400 MHz) spectrometers. The spectra obtained in CDCl₃ or DMSO-d₆ was referenced to the residual solvent peak. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s = single; d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, br = broad. Mass spectra were recorded on a JEOL spectrometer at Emory University Mass Spectrometry Center. Elemental analyses were performed by Atlantic Microlab, Inc. Norcross, GA. HPLC was performed on a Beckman HPLC system using Nova Pak C18 4 μ m 3.9 mm $\times$ 150 mm column (Waters) and acetonitrile/water (0.1% TEA) (40:60) as an eluent.

General Procedure for Synthesis of 3: To a solution of xylelenediamine (1equiv.) in DCM was added DIPEA (2 equiv) and the sulfonyl chloride (2 equiv.). The reaction mixture was stirred at room temperature for 3 hours. The white precipitate was filtered and washed with DCM to give product as white crystals.

N,N-(1,4-Phenylenebis(methylene))dibenzenesulfonamide (**3a**): White crystal, 34% (¹H NMR (400 MHz, CDCl₃) 8.22 (2H, s), 7.84 (2H, d, *J* = 8Hz), 7.62 (6H, m), 7.13(4H, s), 3.89 (4H, s). HRMS calcd for C₂₀H₂₀N₂O₄S₂, 415.07917; found, 415.07879 [M + H]⁺. CHN

N,N-(1,4-Phenylenebis(methylene))bis(4-methoxybenzenesulfonamide) (**3b**): White crystal, 66%. ¹H NMR (400 MHz, DMSO-d₆) 7.94 (2H, s, br), 7.73 (4H, d, *J* = 8.8 Hz), 7.14 (s, 4H), 7.10 (4H, d, *J* = 9.2 Hz), 3.88 (4H, s), 3.83 (6H, s). HRMS calcd for C₂₂H₂₄N₂O₆S₂, 477.11486; found, 477.11457 [M + H]⁺. CHN

General Procedure for Synthesis of 4: To a solution of 4-(bromomethyl)benzene-1-sulfonyl chloride (1 equiv) in DCM (0.1M) was added the amine (2 equiv). The reaction mixture was allowed to stir at room temperature 2 hours to overnight. The reaction mixture was washed with water and brine and the combined organic layers dried over MgSO₄ and concentrated to give an off-white solid.

N-benzyl-4-(bromomethyl)-*N*-methylbenzenesulfonamide (**4a**): Off-white solid, 20%. ¹H NMR(400 MHz, CDCl₃) 7.84 (2H, d, *J* = 8 Hz), 7.59 (2H, d, *J* = 8 Hz), 7.34 – 7.31 (5H, m), 4.65 (2H, s), 4.16 (2H, s), 2.61 (3H, s). HRMS calcd for C₁₅H₁₅BrN₂O₂SNa 376.9930; found, 376.9932 [M + H]⁺. CHN

4-(bromomethyl)-*N*-(4-fluorobenzyl)-*N*-methylbenzenesulfonamide (**4b**): White powder, 32%. ¹H NMR (400 MHz, CDCl₃) 7.81 (2H, d, *J* = 8.4 Hz), 7.58 (2H, d, *J* = 8.4 Hz), 7.27 (2H, d, *J* = 8.4 Hz), 7.03 (2H, d, *J* = 8.4 Hz), 4.53 (2H, s), 4.13 (2H, s), 2.61 (3H, s). HRMS calcd for C₁₅H₁₇N₂O₂F₁S₁ 372.0065; found, 372.0064 [M + H]⁺. CHN

4-(bromomethyl)-*N*-(4-(*tert*-butyl)benzyl)-*N*-methylbenzenesulfonamide (**4c**): White powder, 90%. ¹H NMR (400 MHz, CDCl₃) 7.81 (2H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.4 Hz), 7.35 (2H, d, *J* = 8.8 Hz), 7.21 (2H, d, *J* = 8.4 Hz), 4.53 (2H, s), 4.13 (2H, s), 2.62 (3H, s), 1.31 (9H, s) HRMS calcd for C₁₉H₂₄NO₂BrNaS 432.0603; found, 432.0607 [M + H]⁺, CHN

4-(bromomethyl)-*N*-(4-methoxybenzyl)-*N*-methylbenzenesulfonamide (**4d**): Off-white powder, 81%. ¹H NMR (400 MHz, CDCl₃) 7.81 (2H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.8 Hz), 7.20 (2H, d, *J* = 8.8 Hz), 6.86 (2H, d, *J* = 8.4 Hz), 4.53 (2H, s), 4.10 (2H, s), 3.80 (3H, s), 2.59 (3H, s) HRMS calcd for C₁₆H₁₈NO₃BrNaS 406.0083; found, 406.0093 [M + Na]⁺. CHN

General Procedure for Synthesis of 5: To a solution of **4** (1 equiv) in acetonitrile was added K₂CO₃ (2 equiv) and the amine (1 equiv). The reaction mixture was allowed to stir at room temperature overnight. The organic solvent was removed by rotary evaporation and the residue was dissolved in DCM and washed with water and brine, dried over MgSO₄ and concentrated. The crude product was purified by column chromatography.

N-Benzyl-*N*-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (**5a**): Off-white solid, 41%. ¹H NMR(400 MHz, CDCl₃) 7.81 (2H, d, *J* = 8.4 Hz), 7.58 (2H, d, *J* = 8.4 Hz), 7.31

(5H, m), 4.15 (2H, s), 3.74 (2H, s), 2.60 (7H, s), 1.85 (4H, m). HRMS calcd for $C_{19}H_{25}N_2O_2S$ 345.16313; found, 345.16296 $[M + H]^+$. CHN

N-Benzyl-N-methyl-4-(piperidin-1-ylmethyl)benzenesulfonamide (5b): 1H NMR(400 MHz, $CDCl_3$) 7.76 (2H, d, $J = 8.8$ Hz), 7.50 (2H, d, $J = 8.4$ Hz), 7.29 (5H, m), 4.13 (2H, s), 3.53 (2H, s), 2.58 (3H, s), 2.38 (4H, s), 1.58 (4H, q, $J = 5.4$ Hz), 1.42 (2H, m). HRMS calcd for $C_{20}H_{27}N_2O_2S$ 359.17878; found, 359.17856 $[M + H]^+$. Off white solid, 30% CHN

N-Benzyl-N-methyl-4-(morpholinomethyl)benzenesulfonamide (5c): White solid, 57%. 1H NMR(400 MHz, $CDCl_3$) 7.78 (2H, d, $J = 8.4$ Hz), 7.52 (2H, d, $J = 8.4$ Hz), 7.31 – 7.24 (5H, m), 4.13 (2H, s), 3.71 (4H, t, $J = 4.4$ Hz), 3.59 (2H, s), 2.59 (3H, s), 2.46 (4H, t, $J = 4.8$ Hz). HRMS calcd for $C_{19}H_{25}N_2O_3S$ 361.1580; found, 361.1581 $[M + H]^+$.

N-Benzyl-4-((diethylamino)methyl)-N-methylbenzenesulfonamide (5d): White solid, 60%. 1H NMR(400 MHz, $CDCl_3$) 7.77 (2H, d, $J = 8.4$ Hz), 7.58 (2H, d, $J = 8.4$ Hz), 7.28 (5H, m), 4.13 (2H, s), 3.70 (2H, s), 2.59 (7H, m), 1.09 (6H, t, $J = 7$ Hz). HRMS calcd for $C_{19}H_{27}N_2O_2S$ 347.1788; found, 347.1787 $[M + H]^+$. CHN

N-Benzyl-N-methyl-4-(piperazin-1-ylmethyl)benzenesulfonamide (5e): White solid, 46%. 1H NMR(400 MHz, $CDCl_3$) d 7.72 (2H, d, $J = 8.4$ Hz), 7.46 (2H, d, $J = 8.8$ Hz), 7.28 – 7.19 (5H, m), 4.08 (2H, s), 3.51 (2H, s), 2.86 (4H, t, $J = 4.8$ Hz), 2.54 (3H, s), 2.39 (4H, s, br); HRMS calcd for $C_{19}H_{26}N_3O_2S$ 360. 1740; found, 360.1741 $[M + H]^+$. HPLC

4-((1H-Pyrrol-1-yl)methyl)-N-benzyl-N-methylbenzenesulfonamide (5f): White powder, 53%. 1H NMR (400 MHz, $CDCl_3$) 7.77 (2H, dd, $J = 2, 8$ Hz), 7.32 – 7.21 (7H, m), 6.70 (2H, t, $J = 2.0$ Hz), 6.23 (2H, t, $J = 2.4$ Hz), 5.16 (2H, s), 4.23 (2H, s), 2.56 (3H, s); HRMS calcd for $C_{19}H_{21}N_2O_2S$ 341.1318; found, 341.1316 $[M + H]^+$. CHN

N-Benzyl-N-methyl-4-(((pyridin-2-ylmethyl)amino)methyl)benzenesulfonamide (5g): Yellow solid, 21%. 1H NMR(400 MHz, $CDCl_3$) 8.56 (1H, d, $J = 4$ Hz), 7.79 (2H, d, $J = 8$ Hz), 7.66 (1H, td, $J = 8, 1.6$ Hz), 7.34 – 7.24 (6H, m), 7.20 - 7.17 (1H, m), 4.12 (2H, s), 3.96 (2H, s), 3.95 (2H, s), 2.57 (3H, s); HRMS calcd for $C_{21}H_{24}N_3O_2S$ 382.1584; found, 382.1582 $[M + H]^+$. CHN

4-((Diethylamino)methyl)-N-(4-fluorobenzyl)-N-methylbenzenesulfonamide (5h): White solid, 59%. 1H NMR(400 MHz, $CDCl_3$) 7.77 (2H, d, $J = 8.4$ Hz), 7.60 (2H, d, $J = 8.4$ Hz), 7.26 (2H, t, $J = 7.4$ Hz), 7.02 (2H, t, $J = 7.0$ Hz), 4.12 (2H, s), 3.65 (2H, s), 2.60 – 2.55 (7H, m), 1.07 (6H, t, $J = 7.2$ Hz). HRMS calcd for $C_{19}H_{26}N_3FO_2S$ 365.1694; found, 382.1690 $[M + H]^+$. CHN

N-(4-Fluorobenzyl)-N-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (5i): Yellow solid, 57%. 1H NMR(400 MHz, $CDCl_3$) 7.76 (2H, d, $J = 8$ Hz), 7.52 (2H, d, $J = 8$ Hz), 7.25 (2H, t, $J = 8.4$), 7.00 (2H, d, $J = 8.4$ Hz), 4.09 (2H, s), 3.69 (2H, s), 2.57 – 2.51 (7H, m), 1.80 (4H, sb) HRMS calcd for $C_{19}H_{24}N_2FO_2S$ 363.1537; found, 363.1534 $[M + H]^+$. CHN

N-(4-(tert-butyl)benzyl)-4-((diethylamino)methyl)-N-methylbenzenesulfonamide (5j): White solid, 56%. NMR(400 MHz, $CDCl_3$) 7.80 (2H, d, $J = 8.4$ Hz), 7.57 (2H, d, $J = 8.4$ Hz), 7.37 (2H, d, $J = 8$ Hz), 7.25 (2H, d, $J = 8.4$ Hz), 4.15 (2H, s), 3.67 (2H, s), 2.63 (3H, s), 2.58 (4H, q, $J = 7.2$ Hz), 1.34 (9H, s), 1.09 (6H, t, $J = 7.2$ Hz). HRMS calcd for $C_{23}H_{35}N_2O_2S$ 403.2414; found, 403.2418 $[M + H]^+$. CHN

***N*-(4-(*tert*-butyl)benzyl)-*N*-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (5k).** ¹H NMR(400 MHz, CDCl₃) 7.77 (2H, d, *J* = 8.4 Hz), 7.54 (2H, d, *J* = 8.4 Hz), 7.32 (2H, s, *J* = 8.4 Hz), 7.19 (2H, d, *J* = 8.0 Hz), 4.10 (2H, s), 3.72 (2H, s), 2.58 (7H, s), 1.83 (4H, s), 1.24 (9H, s). HRMS calcd for C₂₃H₃₃N₂O₂S 403.2257; found, 403.2261 [M + H]⁺. CHN

***N*-(4-methoxybenzyl)-*N*-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (5l):** Yellow powder, 77%. ¹H NMR(400 MHz, CDCl₃) 7.81 (2H, d, *J* = 8.4 Hz), 7.53 (2H, d, *J* = 8.4 Hz), 7.20 (2H, d, *J* = 8.4 Hz), 6.85 (2H, d, *J* = 8.4 Hz), 4.07 (2H, s), 3.79 (2H, s), 3.70 (3H, s), 2.56 – 2.52 (7H, m), 1.83 – 1.80 (4H, m). HRMS calcd for C₂₀H₂₇N₂O₃S 375.1737; found, 375.1737 [M + H]⁺ CHN

***N*-(2-fluorobenzyl)-*N*-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (5m):** White powder, ¹H NMR(400 MHz, CDCl₃) 7.71 (2H, d, *J* = 6.8 Hz), 7.49 (2H, d, *J* = 8.4 Hz), 7.23 – 7.21 (1H, m), 7.02 – 6.91 (3H, m), 4.06 (2H, s), 3.66 (2H, s), 2.55 (3H, s), 2.58 (4H, s, br), 1.76 (4H, t, *J* = 6.8 Hz). HRMS calcd for C₁₉H₂₃O₂N₂F 363.1537; found, 363.1536 [M + H]⁺

***N*-(3-fluorobenzyl)-*N*-methyl-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (5n):** White powder, ¹H NMR(400 MHz, CDCl₃) 7.71 (2H, d, *J* = 6.8 Hz), 7.49 (2H, d, *J* = 8.4 Hz), 7.23 – 7.21 (1H, m), 7.02 – 6.91 (3H, m), 4.06 (2H, s), 3.66 (2H, s), 2.55 (3H, s), 2.58 (4H, s, br), 1.76 (4H, t, *J* = 6.8 Hz). HRMS calcd for C₁₉H₂₃O₂N₂F 363.1537; found, 363.1536 [M + H]⁺

Synthesis of 4-(Bromomethyl)-*N*-methyl-*N*-(pyridin-2-ylmethyl)benzenesulfonamide (6): Follows same general procedure as for synthesis of **4**. The crude product was used without further purification (1.24 g, 94%). ¹H NMR(400 MHz, DMSO-d₆) 8.52 (1H, d, *J* = 4.4 Hz), 7.90 – 7.82 (3H, m), 7.71 (2H, d, *J* = 8.4 Hz), 7.45 (1H, d, *J* = 8.0 Hz), 7.38 – 7.34 (1H, m), 4.86 (2H, s), 4.30 (2H, s), 2.70 (3H, s) HRMS calcd for C₁₄H₁₅N₂O₂BrNaS 376.9930; found, 376.9928 [M + Na]⁺. CHN

Synthesis of *N*-methyl-*N*-(pyridin-2-ylmethyl)-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (7): Follows general procedure for synthesis of **5**. Crude product purified by column chromatography (20:1 DCM/MeOH) to give final compound as a brown oil (72.5 mg, 50%). ¹H NMR(400 MHz, CDCl₃) 8.47 (1H, m), 7.77 (2H, d, *J* = 8.4 Hz), 7.71 (2H, td, *J* = 8.0 Hz, 2.0 Hz), 7.56 – 7.52 (2H, m), 7.20 – 7.18 (2H, m), 4.29 (2H, s), 3.73 (2H, s), 2.69 (3H, s), 2.57 (4H, s), 1.84 (4H, s) HRMS calcd for C₁₈H₂₄N₃O₂S 346.1584; found, 346.1584 [M + H]⁺. HPLC

Synthesis of *tert*-butyl 4-(2-(diethylamino)ethoxy)phenylcarbamate 8: To a solution of *tert*-butyl 4-hydroxyphenylcarbamate (2g, 9.56 mmol) in DMF (20 ml) was added 2-chloro-*N,N*-diethylethanamine (1.97 g, 11.48 mmol) and NaOH(0.96g, 23.9 mmol). The reaction mixture was stirred at room temperature for 3 hours. The reaction mixture was filtered and the filtrate diluted with DCM (20 ml). The organic layer was washed with brine (2×100mL), dried over MgSO₄ and concentrated. The crude product was purified by column chromatography, silica gel, DCM/MeOH (9:1) to give a brown solid (1.99 g, 60%). ¹H NMR(400 MHz, CDCl₃) 7.22 (2H, d, *J* = 8.9 Hz), 6.75 (2H, d, *J* = 9.2 Hz), 4.14 (2H, t, *J* = 5.6 Hz), 3.09 (2H, t, *J* = 5.2 Hz), 2.88 (4H, q, *J* = 7.2 Hz), 1.44 (9H, s), 6.08 (6H, t, *J* = 7.2 Hz).

4-(2-(diethylamino)ethoxy)benzenamine (9): To a solution of **8** (1.99g, mmol) in dioxane (10 mL) in an ice bath was added 4M HCl in dioxane (3 mL). The reaction was allowed to

stir overnight and warmed to room temperature. Ether was added to the reaction mixture and the white precipitate was filtered to give product (1.49g, 51%). ^1H NMR (400 MHz, CDCl_3) 6.74 (2H, d, J = 8.8 Hz), 6.63 (2H, d, J = 8.8 Hz), 4.00 (2H, t, J = 6.4 Hz), 2.87 (2H, t, J = 6.4 Hz), 2.66 (4H, q, J = 7.2 Hz), 1.08 (6H, t, J = 6.8 Hz). CHN

4-(bromomethyl)-*N*-(4-(2-(diethylamino)ethoxy)phenyl)benzenesulfonamide (10): To a solution of 4-(bromomethyl)benzene-1-sulfonyl chloride (823mg, 3.0 mmol) in DMF (15 ml) was added **9** (635 mg, 3.0 mmol) and allowed to stir at room temperature overnight. The reaction mixture was diluted with DCM (20 ml), washed with water ($2 \times 100\text{ml}$) and brine ($1 \times 100\text{ ml}$), dried over MgSO_4 and concentrated. The crude product was purified by column chromatography, silica gel, DCM/MeOH (15:1 to 10:1 to 5:1) to give an off-white solid (1.27 g, 90%). ^1H NMR (400MHz, CDCl_3) 7.70 (2H, d, J = 8.4 Hz), 7.41 (2H, d, J = 8.0 Hz), 6.98 (2H, d, J = 8.8 Hz), 6.65 (2H, d, J = 9.2 Hz), 4.55 (2H, d, J = 6Hz), 4.36 (2H, t, J = 4.6 Hz), 3.47 (2H, t, J = 4.4 Hz), 3.26 (4H, s, br), 1.39 (6H, t, J = 7.2 Hz). HRMS calcd for $\text{C}_{19}\text{H}_{26}\text{N}_2\text{O}_3\text{SBr}$ 441.0842 found, 441.0847 $[\text{M} + \text{H}]^+$.

General Procedure for Synthesis of 11: A solution of **10** (1 equiv) and secondary amine (1 equiv) in DCM was stirred at room temperature overnight. The solvent was removed by rotary evaporation and the crude product was purified by column chromatography.

***N*-(4-(2-(diethylamino)ethoxy)benzyl)-4-(piperazin-1-ylmethyl)benzenesulfonamide (11a):** Brown oil, 20%. ^1H NMR (400 MHz, CDCl_3) 7.61 (2H, d, J = 8.4 Hz), 7.35 (2H, d, J = 8.0 Hz), 6.94 (2H, d, J = 8.8 Hz), 6.72 (2H, d, J = 9.2 Hz), 3.97 (2H, t, J = 6.0 Hz), 3.49 (2H, s), 2.92 (4H, q, J = 4.8 Hz), 2.84 (2H, t, J = 6.0 Hz), 2.64 (4H, m), 2.42 (4H, s, br), 1.07 (6H, t, J = 7.2 Hz). HRMS calcd for $\text{C}_{23}\text{H}_{34}\text{N}_4\text{O}_3\text{S}$ 447.2424; found, 447.2420 $[\text{M} + \text{H}]^+$. HPLC

***N*-(4-(2-(Diethylamino)ethoxy)phenyl)-4-(morpholinomethyl)benzenesulfonamide (11b):** Yellow solid, 10%. ^1H NMR(400 MHz, CDCl_3) 7.68 (2H, d, J = 7.6 Hz), 7.39 (2H, d, J = 7.6 Hz), 7.00 (2H, d, J = 8.0 Hz), 6.71 (2H, d, J = 8.4 Hz), 4.37(2H, br), 3.70 (4H, t, J = 4.8 Hz), 3.51 (2H, s), 3.20 (2H, s br), 2.94 (4H, q, J = 7.6 Hz), 2.42 (4H, t, J = 4.4 Hz), 1.23 (6H, t, J = 8.8 Hz). HRMS calcd for $\text{C}_{23}\text{H}_{34}\text{N}_3\text{O}_4\text{S}$ 448.2265; found, 448.2261 $[\text{M} + \text{H}]^+$. HPLC

***N*-(4-(2-(Diethylamino)ethoxy)phenyl)-4-(piperidin-1-ylmethyl)benzenesulfonamide (11c):** Yellow oil, 9%. ^1H NMR(400 MHz, CDCl_3) 7.64 (2H, d, J = 8.4 Hz), 7.39 (2H, d, J = 8.0 Hz), 6.98 (2H, dt, J = 8.8, 3.6 Hz), 6.72 (2H, dt, J = 9.2, 3.6), 4.15 (2H, t, J = 5.2 Hz), 3.55 (2H, s), 2.99 (2H, t, J = 8.0 Hz), 2.79 (4H, q, J = 6.8 Hz), 2.40 (4H, s, br), 1.60 (4H, m), 1.41 (2H, br), 1.10 (6H, t, J = 8.4 Hz). HRMS calcd for $\text{C}_{35}\text{H}_{36}\text{N}_3\text{O}_3\text{S}$ 448.2265; found, 448.2261 $[\text{M} + \text{H}]^+$. HPLC

***N*-(4-(2-(Diethylamino)ethoxy)phenyl)-4-(pyrrolidin-1-ylmethyl)benzenesulfonamide (11d):** Yellow semi-solid, 19%. ^1H NMR(400 MHz, CDCl_3) 7.65 (2H, d, J = 8.4 Hz), 7.37 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 9.2 Hz), 6.72 (2H, J = 8.8 Hz), 3.96 (2H, t, J = 6.4 Hz), 3.61 (2H, s), 3.25 (2H, s), 2.83 (2H, t, J = 6.0 Hz), 2.61 (4H, q, J = 7.2Hz), 2.48 (4H, t, J = 6.4 Hz), 1.77 (4H, s, br), 1.05 (6H, t, J = 6.8 Hz) HRMS calcd for $\text{C}_{23}\text{H}_{34}\text{N}_3\text{O}_4\text{S}$ 432.2315; found, 432.2317 $[\text{M} + \text{H}]^+$. HPLC

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Abbreviations

GPCRs	G protein-coupled receptors
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C chemokine ligand 12
SDF-1	stromal-derived factor-1
HIV	human immunodeficiency virus
EC	effective concentrations

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Figure 1.
Structures of CXCR4 blockers It1t, **1** and peptidic antagonists **2** and **12**.

Figure 2.

Design of new sulfonamide analogs based on the scaffold of compound **1**. (R_1 , R_2 and R_3 = Alkyl or Aryl substituents)

Figure 3.
Two regions (A and B) modified for initial structure-activity pattern.

Figure 4.
 IC_{50} determination of selected compounds. a) **5b**, b) **5h** and c) **11a**

Figure 5. Mapping the location of peptide ligands onto the CXCR4 binding cavity based on X-ray structure and point mutations sensitive to ligand binding. (a) CXCL12 N-terminus, (yellow circle); (b) peptide **2** (orange circle); (c) CVX15 (**12**) (cyan circle); (d) Superposition of CXCL12, **2** and **12** binding sites.

(a) 2D structure of **5a**; (b) Best docking pose for **5a** in the CXCR4 binding pocket; (c) Superposition of the GLIDE/MM-GBSA best binding poses in CXCR4 for all active analogs.

Figure 7. (a) 2D structure of **5h**; (b) Best docking pose of **5i** (Cyan) in CXCR4 with X-ray position of **12** (Yellow); (c) Superposition of best binding poses of protonated **5h** (Cyan), **5i** (magenta) and **5l** (Yellow) in CXCR4; (d) Close-up of protonated **5h** showing key interactions with residues on adjacent helices in the binding site. The conformation of Lys38 was established by conformational searching of its side-chain following docking of **5h**.

Scheme 1.
Synthesis of 3a and 3b

Scheme 2.
Synthesis of 5a – 5n and 7

Scheme 3.
Synthesis of 11a – 11d

Table 1Sulfonamide analogs **3** and **5** evaluated by binding affinity and matrigel invasion assays.

Compound	Structure	EC (nM)	Inhibition of invasion ^a (%)	
			10 nM	100 nM
3a		1000	N/A	N/A
3b		1000	95	100
5a		10	100	100
5b		1	94	100
5c		1000	0	76

Compound	Structure	EC (nM)	Inhibition of invasion ^a (%)	
			10 nM	100 nM
5d		10	71	84
5e		1	78	76
5f		>1000	32	61
5g		100	48	72
5h		1000	61	82
5i		1000	64	99

Compound	Structure	EC (nM)	Inhibition of invasion ^a (%)	
			10 nM	100 nM
5j		1	40	88
5k		1	20	79
5l		>1000	N/A	N/A
5m		1000	54	98
5n		1000	54	88
7		1	39	44

Compound	Structure	EC (nM)	Inhibition of invasion ^a (%)	
			10 nM	100 nM
11a		10	77	77
11b		1000	0	37
11c		100	17	25
11d		10	34	82

^aNA = not applicable

Table 2

Selected sulfonamide analogs evaluated by both single point binding affinity (EC, nM) and 7-point IC₅₀ values (nM).

Compound	Structure	EC (nM)	IC ₅₀ (nM)
5a		10	8.0
5b		1	8.9
5c		1000	5300
5g		100	113
5h		1000	11850
5i		1000	7300
5l		>1000	64900
11a		10	79
11c		100	21

Table 3
Effective concentration and predicted relative binding free energies for active and inactive compounds

Active Compound	EC (nM)	MM-GBSA (kcal/mol)	Inactive Compound	EC (nM)	MM-GBSA (kcal/mol)
5b	1	-37.4	3a	1000	-21.5
5j	1	-31.4	3b	1000	-20.7
5k	1	-29.7	3c	1000	-13.4
7	1	-40.3	5f	1000	-15.0
5e	1	-31.7	5h	1000	-42.1
5a	10	-36.4	5i	1000	-40.0
11a	10	-34.5	5l	1000	-40.3
5d	10	-33.5			

Table 4

IC₅₀ (affinity binding assay) determination of selected compounds and the corresponding docking score.

Compound	Structure	IC ₅₀ (nM)	MMGBSA (kcal/mol)
5j		4.6	-31.4
5a		8.0	-36.4
11c		21.1	-31.4
5c		5285	-13.4
5f		9849	-15.0

Compound	Structure	IC ₅₀ (nM)	MMGBSA (kcal/mol)
5i		30359	-40.0