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Novel anti-inflammatory agents targeting CXCR4: Design, synthesis, biological evaluation and preliminary pharmacokinetic study

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Abstract

CXCR4 plays a crucial role in the inflammatory disease process, providing an attractive means for drug targeting. A series of novel amide-sulfamide derivatives were designed, synthesized and comprehensively evaluated. This new scaffold exhibited much more potent CXCR4 inhibitory activity, with more than 70% of the compounds showed notably better binding affinity than the reference drug AMD3100 in the binding assay. Additionally, in the Matrigel invasion assay, most of our compounds significantly blocked the tumor cell invasion, demonstrating superior efficacy compared to AMD3100. Furthermore, compound **IIj** blocked mice ear inflammation by 75% and attenuated ear edema and damage substantially in an *in vivo* model of inflammation. Western blot analyses revealed that CXCR4 modulator **IIj** significantly blocked CXCR4/CXCL12-mediated phosphorylation of Akt. Moreover, compound **IIj** had no observable cytotoxicity and displayed a favourable plasma stability in our preliminary pharmacokinetic study. The preliminary structure-activity relationships were also summarized. In short, this novel amide-sulfamide scaffold exhibited potent CXCR4 inhibitory activity both *in vitro* and *in vivo*. These results also confirmed that developing modulators targeting CXCR4 provides an exciting avenue for treatment of inflammation.

Graphical Abstract

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Keywords

CXCR4; Amide-sulfamide; Inflammation; Structure–activity relationship; Pharmacokinetic study

1. Introduction

Inflammation is a fundamental protective response of the immune system against pathogens or harmful irritants. The classical signs of inflammation are redness, swelling, heat, and pain, which may cause tissue damage and lead to a host of diseases including cancer [1]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are some of the most widely prescribed medications around the world [2]. However, since 1938, the gastrointestinal (GI) damaging effects of NSAIDs have been very well characterized and recognized as the major limitation for the use of this class of drugs for long term treatment of inflammatory conditions [3]. Cyclooxygenase (COX) enzymes are the main target of most NSAIDs and exist in two isoforms, namely COX-1 and COX-2. These side effects are related primarily to suppression of COX-1-derived prostaglandin (PG) synthesis in the stomach by non-selective NSAIDs [4]. To overcome the gastrointestinal toxicity, selective COX-2 inhibitors, such as celecoxib and rofecoxib, were introduced for clinical use. However, while selective COX-2 inhibitors reduce gastroduodenal damage, they do not eliminate it. Furthermore, COX-2 inhibitors have also been associated with adverse cardiovascular effects [2]. Despite many efforts for the development of NSAIDs, GI bleeding and ulceration remain major clinical concerns. Therefore, the search for safer anti-inflammatory drugs targeting novel pathways remains a feasible and promising strategy.

C-X-C chemokine receptor type 4 (CXCR4) is a 7-transmembrane chemokine receptor in the chemokine family [5]. The ligand interacting with CXCR4 is stromal-derived-factor-1 (SDF-1) or C-X-C chemokine ligand 12 (CXCL12) [6]. The CXCL12/CXCR4 axis has been shown to be involved in a number of pathological conditions, including cancer and inflammation [7]. Although the exact mechanisms are not well understood, it has been proved that CXCR4 plays crucial roles in the pathophysiology of inflammatory diseases, including autoimmune diseases, rheumatoid arthritis, inflammatory bowel disease, ischemic injuries and lung diseases [8]. Taking CXCR4 as a new target, CXCR4 modulators may present a new avenue for the development of novel and safe anti-inflammatory drugs.

The first small molecule CXCR4 antagonist to enter clinical trials was AMD3100 (1) (Fig. 1), which was originally used for treatment of HIV[9]. However, AMD3100 was not approved for this purpose due to poor oral bioavailability and serious cardiotoxicity [10–13]. In particular, AMD3100, a complete CXCR4 inhibitor, acting as an effective stem cell

mobilizer by dissociating CXCR4 from its ligand CXCL12, has been FDA-approved for the use in patients with multiple myeloma in order to mobilize and harvest stem cells [14, 15].

Our lab has been developing partial CXCR4 modulators without cell mobilizing capability, which can be safe and effective in long term use [16, 17]. We designed and synthesized a series of novel and potent CXCR4 modulators (Class I shown in Fig. 1) [18, 19]. Among the promising candidates, compound **2** exhibits very low toxicity and excellent activity both *in vitro* and *in vivo*, and has been under Phase II clinical trials.

Taking the symmetrical bis-secondary amines (Class I) as lead structures, here we attempted to design and synthesize a novel series of anti-CXCR4 compounds. Based on the principle of bioisosteres, one imine bound (–NH–) in the side chain was substituted with an amide group (–CONH–). In addition, our previous work found that a sulfamide scaffold (–SO₂NH–) is also an effective pharmacophore for CXCR4 inhibitory activity [20]. Therefore, the imine bound in the other side chain was then substituted with the sulfamide group, giving rise to the novel amide-sulfamide compounds. Preliminary docking study and biological evaluation had proved this amide-sulfamide structure successfully maintained potent CXCR4 inhibitory activity [21]. Herein, the amide side chain was comprehensively modified and optimized. Nearly forty compounds were prepared and afforded a systematic biological analysis. The preliminary structure-activity relationship (SAR) was also summarized.

2. Results and discussion

2.1. Chemistry

The synthetic route chosen to synthesize the targeted compounds was outlined in Scheme 1. The target compounds were prepared from 4-(Boc-aminomethyl)benzylamine (**3**). Compound **4** was synthesized by sulfonylation of the starting material **3** with the corresponding sulfonylchlorides in dichloromethane (DCM). The protective group Boc was subsequently removed in the presence of trifluoroacetic acid (TFA) producing the benzylamine intermediate **5**. The final compounds **Ia-r** and **IIa-r** were synthesized by the acylation of intermediate **5** with corresponding benzoyl chloride derivatives.

2.2. Primary binding affinity screening

All of the prepared compounds were first screened with a binding affinity assay as described in our previous publications [19, 20, 22]. This is a competitive binding assay between a potent CXCR4 peptidic inhibitor, biotinylated TN14003, and the target compounds **Ia-r** and **IIa-r** at concentrations of 1, 10, 100, and 1000 nM, for binding to the CXCR4 receptors. 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 control (Fig. 2, without CXCR4 modulators). Thus, this initial screening is a semi-quantitative, primary screening of the level of activity, which is different from IC₅₀.

When the two –NH– groups were substituted with –CONH– and –SO₂NH–, respectively, the obtained amide-sulfamide parent structure successfully maintained significant CXCR4 binding affinity and showed even greater activity. Surprisingly, among the 34 synthesized compounds, only three compounds (**If**, **IIa** and **IIq**) displayed weaker binding affinity

compared to the reference drug AMD3100. More than 70% of the compounds showed significant better binding effects (Table 1 and Fig. 2). Compounds **Ia**, **Ib**, **Ie**, **Iii** and **IIj** exhibited 1000-fold stronger potency than AMD3100, with an EC of only 1 nM. Generally, most of the derivatives substituted at the 2'-position of the benzamide group showed more potent activity. Incorporating a methyl group to the benzene sulfonamide side chain significantly enhanced the binding affinity. Further modification of the benzenesulfonamide group was needed to summarize and explain the structure-activity relationships (SARs).

2.3. Matrigel invasion assay

Activation of CXCR4 through its ligand CXCL12 mediates migration and invasion. Thus, we used the Matrigel invasion assay to probe whether the selected compounds from our primary binding affinity assay can block CXCR4/CXCR12 mediated chemotaxis and invasion [20, 22]. The target compounds (100 nM) and cells were added to in the upper chamber of a vessel and CXCL12 was added in the lower chamber as a chemoattractant in serum-free medium. The binding of CXCR4 in cell surface with the selected compounds would block CXCL12 chemotaxis to the cells. Therefore, the MDA-MB-231 human breast cancer cells in the top chamber treated with the compounds would be inhibited to migrate from the top chamber through the Matrigel-coated filter pores to the bottom of the filter. The inhibition of cell invasion with each tested compound was calculated by comparing to the cell invasion without treatment. The results of Matrigel invasion were summarized in Fig. 3 and Fig. 4.

24 compounds showing greater binding affinity than AMD3100 were selected for the Matrigel invasion assay. Most of the corresponding compounds displayed exceptional inhibition of CXCR4, except **Id**, **In** and **Iib**, with less than 50% inhibition. Compounds **Io**, **Ilg**, **Iii**, **Iio**, **Iip** and **Iir** showed favorable blocking effect comparable to AMD3100 (55%), while **Ia**, **Ii**, **Ij**, **Ik**, **Ip**, **Iq**, **Ir**, **IIj**, **IIm** and **IIn** performed quite well in the Matrigel assay with more than 60% inhibition. Compounds **Ib**, **Ie**, **Ile**, **IIf** and **IIk** exhibited the most potent anti-invasion effect, preventing more than 90% cells from invading through the Matrigel gel. These invasion results give eloquent proof that the designed amide-sulfamide scaffold is a novel structure to block CXCR4 function.

2.4. *In vivo* suppression against xylene-induced ear edema

To evaluate the *in vivo* anti-inflammatory activity of the amide-sulfamide compounds, we performed a xylene-induced ear edema experiment [23]. This xylene-induced ear edema model is widely used in the evaluation of inflammatory activity. The application of xylene induces neurogenous edema, which is partially associated with substance P. In the periphery, release of substance P from sensory neurons leads to vasodilatation and plasma extravasations, which causes ear swelling in mice [24].

Compounds achieving greater than 65% inhibition in the Matrigel assay were evaluated in the ear edema test. Although AMD3100 is the best investigated small molecule CXCR4 antagonist, its bicyclam structure leads to serious toxicity in this animal model. Therefore, AMD3100 was not selected as the reference drug in this test[16].

Most of the selected compounds displayed moderate to significant anti-inflammatory activity. Interestingly, compounds **Ib**, **Ie**, **Ik**, **Ip** and **Ile** demonstrated excellent anti-CXCR4 activity in the Matrigel invasion assay, but were ineffective in suppressing the mice ear inflammation (Fig. 5). Compounds **IIf** and **IIk** also exhibited weak activity (28% and 26% inhibition). Substances **Ij** and **Ir** had moderate anti-inflammatory effect with ~40% inhibition. Meanwhile, compounds **Iq**, **IIm** and **IIn** suppressed inflammation by 53%, 57% and 50%, respectively. Surprisingly, compounds **Ia** and **IIj** showed excellent suppressive activity with 62% and 75% inhibition, respectively.

Histological analysis was then performed to further investigate the significant anti-inflammatory activity of compound **IIj**. As shown in Fig. 6, a histological assay of compound **IIj** showed remarkable attenuation of ear inflammation and damage with decreases in ear thickness, edema volume, and the number of inflammatory cells (C1–2). These results confirm that the amide-sulfamide compound **IIj** targeting CXCR4 can inhibit inflammation as anticipated. In addition, developing anti-CXCR4 agents for inflammation provides an exciting avenue to target different pathways from COX.

2.5. Evaluation of anti-CXCR4 activity of compound **IIj** at the molecular level

Phosphoinositide 3-kinase (PI3K) activates Akt, a serine threonine kinase which plays a key role in tumor cell survival and possibly proliferation. PI3K/Akt pathways are independently involved in the proliferative signal mediated by CXCL12 [25]. Our previous results demonstrated that CXCR4/CXCL12 induced Akt phosphorylation, which resulted in tumor angiogenesis and progression of tumors by increasing expression of vascular endothelial growth factor (VEGF) through the activation of the PI3K/Akt pathway [26]. The activity of **IIj** in blocking the PI3K/Akt pathway was investigated by Western blot analysis. As shown in Fig. 7, CXCR4 modulator **IIj** blocked the CXCR4/CXCL12-mediated phosphorylation of Akt in a dose-dependent manner, and significantly inhibited phosphorylation at concentrations of 10 and 100 nM.

2.6. Preliminary cytotoxicity evaluation of compound **IIj**

In addition to their therapeutic effects, cytotoxic agents have the potential of causing serious destruction to healthy and normal cells. To preliminarily evaluate the safety of compound **IIj**, its cytotoxicity was evaluated on two representative human breast cancer cell lines, MDA-MB-231 (CXCR4-positive) and MCF-10A (CXCR4-negative) by a cell viability (MTT) assay. Of note, blocking CXCR4 does not impact adherent cell proliferation [27]. Compound **IIj** displayed potent CXCR4 binding affinity at only 1 nM, however, it did not inhibit the proliferation of MDA-MB-231 and MCF-10A cells even at concentrations as high as 10 μ M (Fig. 8), a 1000-fold increase to the working concentrations. Generally, **IIj** had no observable cytotoxicity.

2.7. Preliminary pharmacokinetic study of compound **IIj**

The plasma stability of a drug has a significant influence on the concentration of drug available in circulation. To determine the preliminary pharmacokinetics of these amide-sulfamide compounds, the concentration and stability of compound **IIj** in plasma were evaluated in a mouse model. The peak area of **IIj** in plasma was detected by high-

performance liquid chromatography (HPLC) at three time points: 5, 20 and 50 minutes. Generally, compound **IIj** demonstrated a satisfying plasma stability. When normalizing the concentration of compound **IIj** to the peak area at 5 minutes (100%), the concentration remained 65% and 45% at 20 and 50 minutes, respectively (Fig. 9). The approximate half-life ($t_{1/2}$) was calculated to be ~40 minutes. However, the basal metabolic rate per gram of body weight (the mass-specific rate) is seven times greater in mice than in humans [28]. Therefore, we inferred that $t_{1/2}$ of **IIj** in human would be reasonable. Compared to a previously reported candidate belonging to Class I [19], which was nearly undetectable by HPLC within 1 hour, **IIj** exhibited favorable plasma stability. The amide-sulfamide structure is not only effective both *in vitro* and *in vivo*, but is also a metabolically tolerated pharmacophore.

2.8. Discussion of Structure–activity relationships (SARs) discussion

Based on the results of binding affinity screening, Matrigel invasion test and *in vivo* anti-inflammatory evaluation, the preliminary SARs were then characterized (Fig. 10).

In terms of binding affinity, the benzamide side chain showed favorable tolerance. The influence of electron-withdrawing and electron-donating groups on the binding activity did not demonstrate a notable difference; both could maintain or improve the binding affinity. When the 2'-position of the benzamide side chain was substituted, most of the derivatives exhibited better affinity, which was basically stronger than the 3' or 4'-position substituted compounds. A direct comparison between the 3' and 4'-position substituted derivatives showed no obvious difference in binding affinity. Moreover, when an electron-donating substituent, such as a methyl group, was introduced to the 4'-position of the benzene sulfonamide side chain, binding affinity increased significantly. Further modification on the benzene sulfonamide structure is needed.

In terms of the Matrigel invasion test, the benzamide scaffold was still tolerated. Both electron-withdrawing and electron-donating groups enhanced the inhibitory potency. When the 2'-position was substituted with methyl, methoxyl or chlorine groups, these corresponding compounds displayed the most potent activity, better than 3' or 4'-position modified compounds. In contrast, when substituted with fluorine, the 3' and 4'-position substituted compounds demonstrated a greater effect.

In the *in vivo* anti-inflammatory evaluation, when the 4'-position of benzamide side chain was substituted, the melting point increased significantly, especially with strong electron-withdrawing groups. These changes decreased the solubility of the target compounds, and may also impact the bioavailability. Although compound **IIj** (4'-position substituted with fluorine) showed the strongest anti-inflammatory activity, its solubility was not favourable. Taking **IIj** as the lead compound, introducing groups containing nitrogen atoms to the benzene sulfonamide structure will be an advisable strategy in the future optimization. The obtained compounds can be converted into salts to address the solubility issues.

All in all, compared to the bis-secondary amine lead structure, this novel amide-sulfamide scaffold successfully maintained the potent CXCR4 blocking effect, and showed even stronger activity both *in vitro* and *in vivo*. Based on the summarized SARs, further

modification and optimization will be performed, especially to the benzene sulfonamide side chain.

3. Conclusion

Taking the symmetrical bis-secondary amines (Class I) as lead structures, a series of novel amide-sulfamide derivatives were designed, synthesized and comprehensively evaluated. This novel amide-sulfamide scaffold not only maintained significant CXCR4 modulating effect, but also exhibited more potent anti-CXCR4 activity. In the *in vitro* assays, more than 70% of compounds showed notably better binding affinity than the reference drug AMD3100. Compounds **Ia**, **Ib**, **Ie**, **Iii** and **Ij** exhibited 1000-fold stronger potency than AMD3100, with an EC of only 1 nM. In the Matrigel invasion assay, most of the compounds significantly blocked tumor cell invasion, demonstrating superior inhibition compared to AMD3100. For the *in vivo* evaluation, compounds **Ia** and **Ij** showed excellent mice ear inflammation suppressive activity (62% and 75%, respectively). Histological analysis proved that compound **Ij** attenuated ear edema and damage substantially, with ear thickness, edema volume, and the number of inflammatory cells all decreasing by a wide margin. Western blot analyses revealed that CXCR4 modulator **Ij** blocked the CXCR4/CXCL12-mediated phosphorylation of Akt in a dose-dependent manner. Compound **Ij** also significantly attenuated the amount of TNF- α by 59% in bacterial-infected J774A.1 macrophages. In the preliminary pharmacokinetic study, compound **Ij** also displayed a favourable plasma stability. In the cytotoxicity screening, **Ij** did not inhibit the proliferation of MDA-MB-231 and MCF-10A cells even at 10 μ M, and showed no observable cytotoxicity.

Based on the results of binding affinity screening, Matrigel invasion test, *in vivo* anti-inflammatory evaluation and preliminary pharmacokinetic study, the preliminary SARs were summarized. Future directions for this work will include further optimization and modification of the benzene sulfonamide scaffold, especially the introduction of hydrophilic structures or groups containing nitrogen atom(s) to improve the solubility and bioavailability.

In summary, the novel amide-sulfamide scaffold exhibited potent CXCR4 inhibitory activity both *in vitro* and *in vivo*, and showed reasonable metabolic stability. These results also confirmed that compound **Ij** targeting CXCR4 can inhibit inflammation as anticipated. Developing inhibitors targeting CXCR4 provides an exciting strategy for treatment of inflammation.

4. Experimental section

4.1. Chemistry

4.1.1. General information—Proton and carbon NMR spectra were recorded on INOVA-400 (400 MHz), INOVA-600 (600 MHz) or VNMR-400 spectrometers at Emory NMR Research Center. The spectra obtained in CDCl₃ and DMSO-*d*₆ were 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. Mass spectra were recorded on a JEOL spectrometer at Emory University Mass Spectrometry Center. Analytical thin layer

chromatography (TLC) was performed on precoated glass backed plates from Scientific Adsorbents Incorporated (Silica Gel 60 F254; 0.25 mm thickness).

4.1.2. General procedure for synthesis of intermediate 4—A solution of 4-(Boc-aminomethyl)benzylamine (**3**) (1.0 mmol) and TEA (3.0 mmol) in anhydrous DCM (8 mL) was cooled with an ice bath, then the corresponding sulfochloride (1.1 mmol, dissolved in 2 mL anhydrous DCM) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 1 h. After removing the cooling bath, the resulting mixture was stirred for 5 h at room temperature, then diluted with saturated aqueous NaHCO₃ and extracted with DCM (10 mL) for three times. The combined organic layer was sequentially washed with water and brine, dried with anhydrous Na₂SO₄, and concentrated in vacuo. The crude was purified by column chromatography with DCM/methanol (150:1, v/v) to give the product as a white solid.

4.1.3. General procedure for synthesis of intermediate 5—A solution of intermediate **4** (1.0 mmol) in DCM (10 mL) was treated with trifluoroacetic acid (4 mmol) at room temperature. The resulting mixture was stirred for 8 h. The solvent was removed under reduced pressure. The residue was dissolved in saturated aqueous NaHCO₃ (2 mL) followed by adding more saturated aqueous NaHCO₃ to adjust to pH = 10. Then the mixture was filtered and the intermediate **5** was obtained as the filter cake without further purification.

4.1.4. General procedure for synthesis of target compounds Ia-r and IIa-r—A solution of intermediate **5** (1.0 mmol) and TEA (3.0 mmol) in anhydrous DCM (8 mL) was cooled with an ice bath, then the corresponding benzoyl chloride derivatives (1.1 mmol, dissolved in 2 mL anhydrous DCM) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 1 h. After removing the cooling bath, the resulting mixture was stirred for 5 h at room temperature, then diluted with saturated aqueous NaHCO₃ and extracted with DCM (10 mL) three times. The combined organic layer was sequentially washed with water and brine, dried with anhydrous Na₂SO₄, and concentrated in vacuo. The crude was purified by column chromatography with DCM/methanol to give the product as a white solid.

4.1.4.1. *N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ia): White solid, yield 85%, m.p. 139–141 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.86–7.86 (m, 2H), 7.76–7.79 (m, 2H), 7.56–7.61 (m, 1H), 7.49–7.54 (m, 3H), 7.41–7.46 (m, 2H), 7.25–7.28 (m, 2H), 7.16–7.20 (m, 2H), 6.42 (s, 1H), 4.72 (s, 1H), 4.59 (d, *J* = 5.8 Hz, 2H), 4.13 (d, *J* = 6.2 Hz, 2H). ¹³C NMR (100 MHz, Methanol-*d*₄) δ 170.22, 142.31, 139.69, 137.69, 135.77, 133.61, 132.86, 130.25, 129.73, 129.25, 128.77, 128.44, 128.10, 47.79, 44.31. HRMS calcd for C₂₁H₂₁N₂O₃S 381.12674 [M + H]⁺, found 381.12651.

4.1.4.2. 2-Methyl-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ib): White solid, yield 79%, m.p. 129–131 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.78 (t, *J* = 6.1 Hz, 1H), 8.15 (t, *J* = 6.3 Hz, 1H), 7.79–7.82 (m, 2H), 7.56–7.66 (m, 3H), 7.31–7.35 (m, 2H), 7.19–7.26 (m, 6H), 4.39 (d, *J* = 6.1 Hz, 2H), 3.96 (d, *J* = 6.2 Hz, 2H), 2.32 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 168.97, 140.68, 138.60, 136.98, 136.04, 135.14, 132.29, 130.39, 129.23, 129.15, 127.53, 127.03, 126.96, 126.41, 125.47, 45.87, 42.01, 19.40. HRMS calcd for C₂₂H₂₂O₃N₂SNa 417.12433 [M + Na]⁺, found 417.12427.

4.1.4.3. 3-Methyl-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ic): White solid, yield 81%, m.p. 112–114 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.97 (t, *J* = 6.0 Hz, 1H), 8.13 (t, *J* = 6.2 Hz, 1H), 7.78–7.80 (m, 2H), 7.70 (qd, *J* = 1.3, 0.7 Hz, 1H), 7.65–7.68 (m, 1H), 7.55–7.63 (m, 3H), 7.34–7.35 (m, 2H), 7.16–7.23 (m, 4H), 4.41 (d, *J* = 6.0 Hz, 2H), 3.94 (d, *J* = 6.2 Hz, 2H), 2.35 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 166.20, 140.66, 138.68, 137.52, 136.01, 134.33, 132.27, 131.72, 129.14, 128.16, 127.75, 127.51, 127.11, 126.41, 124.32, 45.89, 42.28, 20.93. HRMS calcd for C₂₂H₂₃O₃N₂S 395.14239 [M + H]⁺, found 395.14241.

4.1.4.4. 4-Methyl-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Id): White solid, yield 87%, m.p. 188–190 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.94 (t, *J* = 6.0 Hz, 1H), 8.13 (t, *J* = 6.3 Hz, 1H), 7.77–7.80 (m, 4H), 7.55–7.63 (m, 3H), 7.26–7.28 (m, 2H), 7.15–7.20 (m, 4H), 4.41 (d, *J* = 6.0 Hz, 2H), 3.94 (d, *J* = 6.2 Hz, 2H), 2.35 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 165.96, 141.02, 140.66, 138.74, 135.98, 132.26, 131.53, 129.13, 128.79, 127.50, 127.20, 127.10, 126.40, 45.88, 42.24, 20.92. HRMS calcd for C₂₂H₂₃N₂O₃S 395.14239 [M + H]⁺, found 395.14219.

4.1.4.5. 2-Methoxy-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ie): White solid, yield 83%, m.p. 123–125 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.66 (t, *J* = 6.1 Hz, 1H), 8.12 (t, *J* = 6.3 Hz, 1H), 7.79–7.80 (m, 2H), 7.72 (dd, *J* = 7.7, 1.9 Hz, 1H), 7.56–7.63 (m, 3H), 7.47 (ddd, *J* = 9.2, 7.3, 1.9 Hz, 1H), 7.23 (d, *J* = 7.9 Hz, 2H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.14 (d, *J* = 8.3 Hz, 1H), 7.03 (t, *J* = 7.4 Hz, 1H), 4.44 (d, *J* = 6.1 Hz, 2H), 3.94 (d, *J* = 6.3 Hz, 2H), 3.88 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 140.66, 138.67, 135.91, 132.28, 132.10, 130.29, 129.14, 127.51, 126.94, 126.41, 123.19, 120.43, 111.96, 55.84, 45.90, 42.24. HRMS calcd for C₂₂H₂₃N₂O₄S 411.13730 [M + H]⁺, found 411.13739.

4.1.4.6. 3-Methoxy-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (If): White solid, yield 78%, m.p. 120–122 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.01 (t, *J* = 6.1 Hz, 1H), 8.12 (t, *J* = 6.2 Hz, 1H), 7.79 (d, *J* = 7.2 Hz, 2H), 7.56–7.63 (m, 3H), 7.37–7.47 (m, 3H), 7.22 (d, *J* = 8.0 Hz, 2H), 7.17 (d, *J* = 8.0 Hz, 2H), 7.09 (dd, *J* = 8.1, 2.7 Hz, 1H), 4.42 (d, *J* = 5.9 Hz, 2H), 3.94 (d, *J* = 6.3 Hz, 2H), 3.80 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 165.80, 159.14, 140.65, 138.60, 136.04, 135.72, 132.27, 129.41, 129.14, 127.52, 127.12, 126.40, 119.41, 117.08, 112.33, 55.24, 45.88, 42.33. HRMS calcd for C₂₂H₂₃N₂O₄S 411.13730 [M + H]⁺, found 411.13721.

4.1.4.7. 4-Methoxy-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ig): White solid, yield 85%, m.p. 164–166 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.88 (t, *J* = 6.0 Hz, 1H), 8.13 (t, *J* = 6.3 Hz, 1H), 7.84–7.88 (m, 2H), 7.78–7.80 (m, 2H), 7.55–7.63 (m, 3H), 7.15–7.22 (m, 4H), 6.98–7.02 (m, 2H), 4.41 (d, *J* = 6.0 Hz, 2H), 3.94 (d, *J* = 6.3 Hz, 2H), 3.81 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 165.56, 161.52, 138.85, 135.95, 132.27, 129.13, 129.00, 127.49, 127.08, 126.53, 126.40, 113.47, 55.32, 45.89, 42.22. HRMS calcd for C₂₂H₂₃N₂O₄S 411.13730 [M + H]⁺, found 411.13708.

4.1.4.8. 2-Fluoro-N-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ih): White solid, yield 70%, m.p. 122–124 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.10–8.14 (m, 1H), 7.86–7.88 (m, 2H), 7.45–7.60 (m, 4H), 7.09–7.30 (m, 6H), 7.03 (s, 1H), 4.78 (t, *J* = 6.2 Hz, 1H), 4.62

(d, $J = 5.6$ Hz, 2H), 4.14 (d, $J = 6.2$ Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 164.26, 160.66, 158.18, 140.83, 138.50, 136.46, 132.94, 132.86, 132.76, 130.36, 130.33, 129.57, 127.91, 127.42, 126.76, 124.95, 124.92, 124.21, 124.07, 116.59, 116.37, 46.18, 42.65. HRMS calcd for $\text{C}_{21}\text{H}_{19}\text{O}_3\text{N}_2\text{SFNa}$ 421.09926 $[\text{M} + \text{Na}]^+$, found 421.09950.

4.1.4.9. 3-Fluoro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ii): White solid, yield 74%, m.p. 132–134 °C. ^1H NMR (600 MHz, Chloroform- d) δ 7.87 (d, $J = 7.7$ Hz, 2H), 7.59 (t, $J = 7.4$ Hz, 1H), 7.49 – 7.53 (m, 4H), 7.40 (td, $J = 8.0, 5.5$ Hz, 1H), 7.17 – 7.22 (m, 5H), 6.45 (s, 1H), 4.76 (t, $J = 6.2$ Hz, 1H), 4.58 (d, $J = 5.8$ Hz, 2H), 4.13 (d, $J = 6.3$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.74, 163.16, 160.73, 140.66, 138.32, 136.69, 136.63, 136.14, 132.27, 130.52, 130.44, 129.14, 127.54, 127.16, 126.40, 123.37, 118.22, 118.01, 114.12, 113.89, 45.86, 42.41. HRMS calcd for $\text{C}_{21}\text{H}_{20}\text{O}_3\text{N}_2\text{SF}$ 399.11732 $[\text{M} + \text{H}]^+$, found 399.11710.

4.1.4.10. 4-Fluoro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ij): White solid, yield 72%, m.p. 162–164 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.06 (t, $J = 6.0$ Hz, 1H), 8.13 (t, $J = 6.3$ Hz, 1H), 7.93 – 7.98 (m, 2H), 7.78 – 7.81 (m, 2H), 7.55 – 7.63 (m, 3H), 7.28 – 7.34 (m, 2H), 7.16 – 7.23 (m, 4H), 4.42 (d, $J = 6.0$ Hz, 2H), 3.94 (d, $J = 6.3$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 165.08, 165.04, 162.60, 140.66, 138.52, 136.08, 132.27, 130.79, 129.88, 129.79, 129.14, 127.53, 127.13, 126.40, 115.31, 115.09, 45.88, 42.36. HRMS calcd for $\text{C}_{21}\text{H}_{20}\text{O}_3\text{N}_2\text{SF}$ 399.11732 $[\text{M} + \text{H}]^+$, found 399.11710.

4.1.4.11. 2-Chloro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ik): White solid, yield 76%, m.p. 116–118 °C. ^1H NMR (600 MHz, DMSO- d_6) δ 8.96 (t, $J = 5.6$ Hz, 1H), 8.14 (t, $J = 6.5$ Hz, 1H), 7.81 (d, $J = 7.7$ Hz, 2H), 7.63 – 7.65 (m, 3H), 7.38 – 7.51 (m, 4H), 7.27 (d, $J = 7.6$ Hz, 2H), 7.20 (d, $J = 7.6$ Hz, 2H), 4.40 (d, $J = 6.0$ Hz, 2H), 3.96 (d, $J = 6.2$ Hz, 2H). ^{13}C NMR (100 MHz, Chloroform- d) δ 166.58, 140.13, 137.83, 135.88, 134.95, 132.96, 131.69, 130.83, 130.53, 130.49, 129.38, 128.49, 128.40, 127.38, 127.32, 47.19, 44.00. HRMS calcd for $\text{C}_{21}\text{H}_{20}\text{O}_3\text{N}_2\text{SCl}$ 415.08777 $[\text{M} + \text{H}]^+$, found 415.08759.

4.1.4.12. 3-Chloro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Im): White solid, yield 80%, m.p. 114–116 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.16 (t, $J = 6.0$ Hz, 1H), 8.13 (t, $J = 6.3$ Hz, 1H), 7.92 (t, $J = 1.9$ Hz, 1H), 7.84 (ddd, $J = 7.7, 1.7, 1.1$ Hz, 1H), 7.78 – 7.80 (m, 2H), 7.50 – 7.63 (m, 5H), 7.22 (d, $J = 8.3$ Hz, 2H), 7.18 (d, $J = 8.3$ Hz, 2H), 4.42 (d, $J = 5.9$ Hz, 2H), 3.94 (d, $J = 6.3$ Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 164.70, 140.65, 138.33, 136.28, 136.18, 133.21, 132.34, 131.13, 130.40, 129.20, 127.59, 127.22, 127.06, 126.45, 126.04, 45.90, 42.46. HRMS calcd for $\text{C}_{21}\text{H}_{20}\text{O}_3\text{N}_2\text{SCl}$ 415.08777 $[\text{M} + \text{H}]^+$, found 415.08771.

4.1.4.13. 4-Chloro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (In): White solid, yield 78%, m.p. 182–184 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.12 (t, $J = 5.9$ Hz, 1H), 8.14 (t, $J = 6.3$ Hz, 1H), 7.88 – 7.92 (m, 2H), 7.78 – 7.81 (m, 2H), 7.53 – 7.63 (m, 5H), 7.15 – 7.23 (m, 4H), 4.42 (d, $J = 4.4$ Hz, 2H), 3.94 (d, $J = 6.1$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.98, 140.66, 138.40, 136.12, 136.03, 133.00, 132.29, 129.15, 128.38, 127.72, 127.54, 127.15, 126.42, 45.87, 42.27. HRMS calcd for $\text{C}_{21}\text{H}_{19}\text{O}_3\text{N}_2\text{SClNa}$ 437.06971 $[\text{M} + \text{Na}]^+$, found 437.07017.

4.1.4.14. 4-Nitro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Io): White solid, yield 70%, m.p. 201–203 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.37 (t, J = 6.0 Hz, 1H), 8.33 (d, J = 8.8 Hz, 2H), 8.10 – 8.15 (m, 3H), 7.79 (d, J = 6.6 Hz, 2H), 7.66 – 7.52 (m, 3H), 7.24 (d, J = 7.9 Hz, 2H), 7.19 (d, J = 7.9 Hz, 2H), 4.45 (d, J = 5.9 Hz, 2H), 3.94 (d, J = 6.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.51, 149.01, 140.66, 139.94, 138.07, 136.25, 132.28, 129.14, 128.73, 127.58, 127.23, 126.40, 123.53, 45.85, 42.56. HRMS calcd for $\text{C}_{21}\text{H}_{20}\text{O}_5\text{N}_3\text{S}$ 426.11182 $[\text{M} + \text{H}]^+$, found 426.11192.

4.1.4.15. 2,4,6-Trichloro-*N*-(4-(phenylsulfonamidomethyl)benzyl)benzamide (Ip): White solid, yield 79%, m.p. 158–160 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.19 (t, J = 6.0 Hz, 1H), 8.15 (t, J = 6.3 Hz, 1H), 7.80 – 7.82 (m, 2H), 7.76 (s, 2H), 7.57 – 7.66 (m, 3H), 7.28 (d, J = 8.2 Hz, 2H), 7.20 (d, J = 8.2 Hz, 2H), 4.43 (d, J = 6.0 Hz, 2H), 3.96 (d, J = 6.3 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 162.88, 140.66, 137.42, 136.34, 135.49, 134.29, 132.31, 132.03, 129.16, 127.93, 127.48, 127.30, 126.42, 45.84, 42.13. HRMS calcd for $\text{C}_{21}\text{H}_{18}\text{O}_3\text{N}_2\text{SCl}_3$ 483.00982 $[\text{M} + \text{H}]^+$, found 483.00996.

4.1.4.16. *N*-(4-(phenylsulfonamidomethyl)benzyl)nicotinamide (Iq): White solid, yield 60%, m.p. 109–111 °C. ^1H NMR (400 MHz, CDCl_3) δ 8.83 (dd, J = 2.3, 0.9 Hz, 1H), 8.69 (dd, J = 4.9, 1.7 Hz, 1H), 8.11 (ddd, J = 7.9, 2.3, 1.7 Hz, 1H), 7.86–7.90 (m, 2H), 7.57–7.62 (m, 1H), 7.50–7.54 (m, 2H), 7.36 (ddd, J = 8.0, 4.9, 0.9 Hz, 1H), 7.12–7.25 (m, 4H), 6.79 (t, J = 5.6 Hz, 1H), 5.24 (t, J = 6.1 Hz, 1H), 4.57 (d, J = 5.7 Hz, 2H), 4.11 (d, J = 6.1 Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 165.82, 152.08, 148.20, 140.05, 137.79, 136.05, 135.49, 132.89, 130.11, 129.36, 128.58, 128.30, 127.20, 123.69, 47.09, 43.83. HRMS calcd for $\text{C}_{20}\text{H}_{20}\text{O}_3\text{N}_3\text{S}$ 382.12199 $[\text{M} + \text{H}]^+$, found 382.12154.

4.1.4.17. *N*-(4-(phenylsulfonamidomethyl)benzyl)isonicotinamide (Ir): White solid, yield 63%, m.p. 154–156 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.33 (t, J = 6.0 Hz, 1H), 8.73 – 8.74 (m, 2H), 8.14 (t, J = 6.3 Hz, 1H), 7.78 – 7.81 (m, 4H), 7.55 – 7.64 (m, 3H), 7.17 – 7.25 (m, 4H), 4.45 (d, J = 6.0 Hz, 2H), 3.95 (d, J = 6.3 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.58, 150.25, 141.21, 140.65, 138.03, 136.25, 132.28, 129.14, 127.57, 127.19, 126.40, 121.21, 45.85, 42.41. HRMS calcd for $\text{C}_{20}\text{H}_{20}\text{O}_3\text{N}_3\text{S}$ 382.12199 $[\text{M} + \text{H}]^+$, found 382.12164.

4.1.4.18. *N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide (IIa): White solid, yield 89%, m.p. 162–164 °C. ^1H NMR (400 MHz, CDCl_3) δ 7.75–7.79 (m, 4H), 7.49–7.53 (m, 1H), 7.41–7.46 (m, 2H), 7.27–7.33 (m, 4H), 7.18–7.20 (m, 2H), 6.41 (s, 1H), 4.62 (s, 1H), 4.60 (d, J = 5.7 Hz, 2H), 4.11 (d, J = 6.2 Hz, 2H), 2.44 (s, 3H). ^{13}C NMR (100 MHz, Methanol- d_4) δ 170.22, 144.69, 139.66, 139.25, 137.76, 135.75, 132.84, 130.78, 129.72, 129.24, 128.74, 128.45, 128.19, 47.77, 44.32, 21.57. HRMS calcd for $\text{C}_{22}\text{H}_{22}\text{O}_3\text{N}_2\text{SNa}$ 417.12433 $[\text{M} + \text{Na}]^+$, found 417.12417.

4.1.4.19. 2-Methyl-*N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide (IIb): White solid, yield 87%, m.p. 147–149 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 8.79 (t, J = 6.1 Hz, 1H), 8.05 (t, J = 6.3 Hz, 1H), 7.68 – 7.71 (m, 2H), 7.38 – 7.40 (m, 2H), 7.30 – 7.36 (m, 2H), 7.19 – 7.26 (m, 6H), 4.39 (d, J = 6.1 Hz, 2H), 3.92 (d, J = 6.3 Hz, 2H), 2.38 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 168.98, 142.54, 138.59, 137.78,

136.99, 136.10, 135.15, 130.40, 129.57, 129.24, 127.53, 127.02, 126.97, 126.51, 125.47, 45.87, 42.03, 20.94, 19.39. HRMS calcd for $C_{23}H_{25}O_3N_2S$ 409.15804 $[M + H]^+$, found 409.15808.

4.1.4.20. 3-Methyl-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIc): White solid, yield 82%, m.p. 123–125 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.98 (t, J = 6.0 Hz, 1H), 8.03 (t, J = 6.3 Hz, 1H), 7.65 – 7.71 (m, 4H), 7.34 – 7.39 (m, 4H), 7.17 – 7.24 (m, 4H), 4.42 (d, J = 6.0 Hz, 2H), 3.90 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 166.21, 142.52, 138.66, 137.76, 137.52, 136.07, 134.34, 131.72, 129.56, 128.16, 127.75, 127.51, 127.10, 126.50, 124.33, 45.88, 42.28, 20.92. HRMS calcd for $C_{23}H_{25}O_3N_2S$ 409.15804 $[M + H]^+$, found 409.15771.

4.1.4.21. 4-Methyl-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIId): White solid, yield 80%, m.p. 180–182 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.95 (t, J = 6.0 Hz, 1H), 8.03 (t, J = 6.3 Hz, 1H), 7.79 (d, J = 8.2 Hz, 2H), 7.68 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 7.6 Hz, 2H), 7.27 (d, J = 7.6 Hz, 2H), 7.22 (d, J = 8.2 Hz, 2H), 7.17 (d, J = 8.3 Hz, 2H), 4.42 (d, J = 6.0 Hz, 2H), 3.90 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H), 2.35 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 165.98, 142.53, 141.03, 138.73, 136.05, 131.55, 129.56, 128.79, 127.51, 127.21, 127.09, 126.50, 45.88, 42.25, 20.92. HRMS calcd for $C_{23}H_{25}N_2O_3S$ 409.15804 $[M + H]^+$, found 409.15776.

4.1.4.22. 2-Methoxy-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIe): White solid, yield 79%, m.p. 154–156 °C. 1H NMR (600 MHz, DMSO- d_6) δ 8.66 (t, J = 5.9 Hz, 1H), 8.03 (t, J = 6.3 Hz, 1H), 7.71–7.74 (m, 1H), 7.68 (dd, J = 8.1, 1.9 Hz, 2H), 7.47 (ddd, J = 8.2, 7.2, 1.8 Hz, 1H), 7.38 (dd, J = 8.6, 1.0 Hz, 2H), 7.24 (d, J = 8.4 Hz, 2H), 7.18 (d, J = 6.7 Hz, 2H), 7.14 (d, J = 8.4 Hz, 1H), 7.03 (td, J = 7.4, 1.1 Hz, 1H), 4.45 (d, J = 6.1 Hz, 2H), 3.91 (d, J = 6.3 Hz, 2H), 3.88 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 165.10, 156.93, 142.60, 138.70, 137.74, 135.99, 132.16, 130.33, 129.62, 127.56, 126.97, 126.56, 123.21, 120.46, 111.97, 55.85, 45.93, 42.28, 20.98. HRMS calcd for $C_{23}H_{25}N_2O_4S$ 425.15295 $[M + H]^+$, found 425.15280.

4.1.4.23. 3-Methoxy-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIIf): White solid, yield 82%, m.p. 130–132 °C. 1H NMR (600 MHz, DMSO- d_6) δ 9.02 (t, J = 6.1 Hz, 1H), 8.02 (t, J = 6.3 Hz, 1H), 7.68 (d, J = 8.3 Hz, 2H), 7.36 – 7.47 (m, 5H), 7.22 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.2 Hz, 2H), 7.09 (dd, J = 8.0, 3.0 Hz, 1H), 4.43 (d, J = 6.1 Hz, 2H), 3.90 (d, J = 6.4 Hz, 2H), 3.80 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 165.81, 159.15, 142.53, 138.58, 137.75, 136.11, 135.73, 129.56, 129.40, 127.52, 127.11, 126.50, 119.42, 117.08, 112.34, 55.24, 45.87, 42.33, 20.92. HRMS calcd for $C_{23}H_{25}N_2O_4S$ 425.15295 $[M + H]^+$, found 425.15283.

4.1.4.24. 4-Methoxy-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIg): White solid, yield 75%, m.p. 147–149 °C. 1H NMR (600 MHz, DMSO- d_6) δ 8.87 (t, J = 6.0 Hz, 1H), 8.02 (t, J = 6.3 Hz, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.68 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 8.0 Hz, 2H), 7.21 (d, J = 8.0 Hz, 2H), 7.17 (d, J = 8.0 Hz, 2H), 6.99 (d, J = 8.8 Hz, 2H), 4.41 (d, J = 6.0 Hz, 2H), 3.90 (d, J = 6.2 Hz, 2H), 3.80 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 165.57, 161.53, 142.52, 138.83, 137.74, 136.02, 129.55,

129.01, 127.49, 127.07, 126.53, 126.49, 113.46, 55.32, 45.88, 42.23, 20.92. HRMS calcd for $C_{23}H_{25}O_4N_2S$ 425.15295 $[M + H]^+$, found 425.15334.

4.1.4.25. 2-Fluoro-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIh): White solid, yield 70%, m.p. 138–140 °C. 1H NMR (400 MHz, DMSO- d_6) δ 8.04 (t, J = 6.3 Hz, 1H), 7.67 – 7.69 (m, 2H), 7.62 (td, J = 7.4, 1.7 Hz, 1H), 7.50 – 7.56 (m, 1H), 7.37 – 7.39 (m, 2H), 7.18 – 7.32 (m, 7H), 4.41 (s, 2H), 3.91 (d, J = 6.3 Hz, 2H), 2.38 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 163.56, 160.32, 142.53, 138.18, 136.15, 132.40, 132.31, 130.03, 130.00, 129.56, 127.71, 127.54, 127.27, 127.01, 126.52, 126.50, 124.48, 124.44, 116.18, 115.96, 45.86, 42.18, 20.93. HRMS calcd for $C_{22}H_{22}O_3N_2SF$ 413.13297 $[M + H]^+$, found 413.13295.

4.1.4.26. 3-Fluoro-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIi): White solid, yield 72%, m.p. 137–139 °C. 1H NMR (600 MHz, Chloroform- d) δ 7.75 (d, J = 8.4 Hz, 2H), 7.49 – 7.53 (m, 2H), 7.37 – 7.41 (m, 1H), 7.31 (d, J = 7.8 Hz, 2H), 7.25 – 7.26 (m, 2H), 7.17 – 7.21 (m, 3H), 6.51 (t, J = 5.5 Hz, 1H), 4.75 (t, J = 6.2 Hz, 1H), 4.57 (d, J = 5.7 Hz, 2H), 4.09 (d, J = 6.4 Hz, 2H), 2.44 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.77, 163.16, 160.73, 142.53, 138.31, 137.75, 136.69, 136.63, 136.20, 130.52, 130.44, 129.56, 127.55, 127.14, 126.50, 123.38, 118.22, 118.01, 114.12, 113.90, 45.86, 42.42, 20.92. HRMS calcd for $C_{22}H_{22}O_3N_2SF$ 413.13297 $[M + H]^+$, found 413.13274.

4.1.4.27. 4-Fluoro-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIj): White solid, yield 67%, m.p. 187–189 °C. 1H NMR (400 MHz, DMSO- d_6) δ 9.06 (t, J = 6.0 Hz, 1H), 8.03 (t, J = 6.3 Hz, 1H), 7.93 – 8.00 (m, 2H), 7.66 – 7.69 (m, 2H), 7.36 – 7.37 (m, 2H), 7.28 – 7.34 (m, 2H), 7.17 – 7.24 (m, 4H), 4.43 (d, J = 6.0 Hz, 2H), 3.90 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 165.04, 162.61, 142.53, 138.51, 137.76, 136.14, 132.01, 130.79, 129.88, 129.79, 129.56, 127.53, 127.11, 126.50, 115.30, 115.09, 109.54, 45.87, 42.36, 20.92. HRMS calcd for $C_{22}H_{22}O_3N_2SF$ 413.13297 $[M + H]^+$, found 413.13303.

4.1.4.28. 2-Chloro-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIk): White solid, yield 80%, m.p. 140–142 °C. 1H NMR (600 MHz, DMSO- d_6) δ 8.97 (t, J = 6.1 Hz, 1H), 8.05 (t, J = 6.3 Hz, 1H), 7.70 (d, J = 8.0 Hz, 2H), 7.50 – 7.51 (m, 1H), 7.38 – 7.46 (m, 5H), 7.27 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 8.1 Hz, 2H), 4.41 (d, J = 6.0 Hz, 2H), 3.92 (d, J = 6.3 Hz, 2H), 2.39 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 166.30, 142.55, 138.06, 137.78, 136.88, 136.20, 130.72, 129.82, 129.58, 128.82, 127.52, 127.09, 127.05, 126.51, 109.53, 45.85, 42.11, 20.95. HRMS calcd for $C_{22}H_{22}O_3N_2SCl$ 429.10342 $[M + H]^+$, found 429.10349.

4.1.4.29. 3-Chloro-N-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(IIl): White solid, yield 71%, m.p. 110–112 °C. 1H NMR (400 MHz, DMSO- d_6) δ 9.16 (t, J = 6.0 Hz, 1H), 8.03 (t, J = 6.3 Hz, 1H), 7.92 (td, J = 1.7, 0.9 Hz, 1H), 7.84 (ddd, J = 7.7, 1.7, 1.1 Hz, 1H), 7.66 – 7.69 (m, 2H), 7.61 (ddd, J = 8.0, 2.2, 1.1 Hz, 1H), 7.49 – 7.53 (m, 1H), 7.36 – 7.39 (m, 2H), 7.17 – 7.24 (m, 4H), 4.43 (d, J = 5.9 Hz, 2H), 3.90 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.66, 142.52, 138.26, 137.79,

136.26, 136.20, 133.16, 131.05, 130.32, 129.55, 127.54, 127.16, 127.01, 126.49, 125.98, 45.86, 42.44, 20.92. HRMS calcd for $C_{22}H_{22}O_4N_3S$ 429.10342 $[M + H]^+$, found 429.10327.

4.1.4.30. 4-Chloro-*N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(II_n): White solid, yield 71%, m.p. 201–203 °C. 1H NMR (400 MHz, $CDCl_3$) δ 7.71–7.77 (m, 4H), 7.19–7.41 (m, 8H), 6.38 (s, 1H), 4.62 (s, 1H), 4.59 (d, J = 5.8 Hz, 2H), 4.10 (d, J = 6.2 Hz, 2H), 2.44 (s, 3H). ^{13}C NMR (101 MHz, $DMSO-d_6$) δ 165.75, 143.08, 138.67, 137.90, 136.52, 133.27, 131.49, 129.98, 129.48, 128.81, 127.91, 127.53, 126.84, 46.16, 42.73, 21.26. HRMS calcd for $C_{22}H_{21}O_3N_2SClNa$ 451.08536 $[M + Na]^+$, found 451.08588.

4.1.4.31. *N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)-4-nitrobenzamide

(II_o): White solid, yield 66%, m.p. 207–209 °C. 1H NMR (400 MHz, $DMSO-d_6$) δ 9.37 (t, J = 6.0 Hz, 1H), 8.31–8.34 (m, 2H), 8.02–8.12 (m, 2H), 8.04 (t, J = 6.3 Hz, 1H), 7.68 (d, J = 8.2 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 7.19 (d, J = 8.0 Hz, 2H), 4.46 (d, J = 5.9 Hz, 2H), 3.91 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, $DMSO-d_6$) δ 164.51, 149.00, 142.53, 139.94, 138.05, 137.73, 136.31, 129.56, 128.73, 127.58, 127.21, 126.49, 123.53, 45.84, 42.57, 20.93. HRMS calcd for $C_{22}H_{22}N_3O_5S$ 440.12747 $[M + H]^+$, found 440.12768.

4.1.4.32. 2,4,6-Trichloro-*N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)benzamide

(II_p): White solid, yield 77%, m.p. 188–190 °C. 1H NMR (400 MHz, $DMSO-d_6$) δ 9.19 (t, J = 6.0 Hz, 1H), 8.05 (t, J = 6.3 Hz, 1H), 7.76 (s, 2H), 7.68–7.70 (m, 2H), 7.38–7.40 (m, 2H), 7.29 (d, J = 8.2 Hz, 2H), 7.20 (d, J = 8.2 Hz, 2H), 4.43 (d, J = 6.0 Hz, 2H), 3.92 (d, J = 6.3 Hz, 2H), 2.39 (s, 3H). ^{13}C NMR (100 MHz, $DMSO-d_6$) δ 162.88, 142.56, 137.76, 137.40, 136.39, 135.49, 134.29, 132.03, 129.58, 127.93, 127.48, 127.28, 126.51, 45.84, 42.12, 20.95. HRMS calcd for $C_{22}H_{20}N_2O_3SCl_3$ 497.02547 $[M + H]^+$, found 497.02593.

4.1.4.33. *N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)nicotinamide (II_q): White solid, yield 65%, m.p. 120–122 °C. 1H NMR (400 MHz, Chloroform-*d*) δ 8.86 (d, J = 1.8 Hz, 1H), 8.70 (dd, J = 4.9, 1.8 Hz, 1H), 8.11 (dt, J = 7.9, 2.0 Hz, 1H), 7.75–7.78 (m, 2H), 7.35–7.38 (m, 1H), 7.27–7.33 (m, 3H), 7.18–7.25 (m, 3H), 6.75 (t, J = 5.9 Hz, 1H), 5.07 (t, J = 6.1 Hz, 1H), 4.59 (d, J = 5.7 Hz, 2H), 4.09 (d, J = 6.1 Hz, 2H), 2.44 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.86, 152.19, 148.30, 143.70, 137.82, 137.10, 136.16, 135.35, 130.08, 129.96, 128.56, 128.30, 128.27, 123.62, 47.07, 43.84, 21.72. HRMS calcd for $C_{21}H_{22}O_3N_6S$ 396.13764 $[M + H]^+$, found 396.13734.

4.1.4.34. *N*-(4-((4-methylphenylsulfonamido)methyl)benzyl)isonicotinamide (II_r): White solid, yield 63%, m.p. 163–165 °C. 1H NMR (400 MHz, $DMSO-d_6$) δ 9.33 (t, J = 6.0 Hz, 1H), 8.72–8.74 (m, 2H), 8.04 (t, J = 6.3 Hz, 1H), 7.78–7.79 (m, 2H), 7.67–7.70 (m, 2H), 7.37–7.39 (m, 2H), 7.16–7.25 (m, 4H), 4.45 (d, J = 6.0 Hz, 2H), 3.91 (d, J = 6.3 Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (100 MHz, $DMSO-d_6$) δ 164.58, 150.24, 142.53, 141.21, 138.01, 137.75, 136.31, 129.56, 127.58, 127.18, 126.50, 121.22, 45.84, 42.42, 20.92. HRMS calcd for $C_{21}H_{22}O_3N_3S$ 396.13764 $[M + H]^+$, found 396.13739.

4.2. Primary binding affinity screening

For binding affinity assay, 2×10^4 MDA-MB-231 cells in 300 μ L of cell culture medium were seeded in an 8-well slide chamber 2 days before the experiments were conducted. Various concentrations of different compounds (1, 10, 100, or 1000 nM) were added to the separate wells and incubated for 10 minutes at room temperature, and then the cells were fixed in 4% ice-cold paraformaldehyde. The cells were rehydrated in phosphate-buffered saline (PBS). The slides were subsequently incubated for 30 minutes at room temperature with 0.05 μ g/mL biotinylated TN14003, washed three times with PBS, and incubated in streptavidin-rhodamine (1:150 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes at room temperature. Finally, the slides were washed with PBS and mounted in an anti-fade mounting solution (Molecular Probes, Eugene, OR), and the samples were analyzed on a Nikon Eclipse E800 microscope [19, 20, 22].

4.3. Matrigel invasion assay

Matrigel invasion assay was performed by using a Matrigel invasion chamber from Corning Biocoat (Bedford, MA). CXCL12 α (200 ng/mL; R & D Systems, Minneapolis, MN) was added to the bottom chamber to induce the invasion of MDA-MB-231 cells through the Matrigel. The selected compounds (100 nM) or AMD3100 were added to the cells before the cells were seeded in the top chamber. The Matrigel invasion chamber was incubated for 22 hours in a humidified cell culture incubator. First, non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells on the filter at the bottom of the Matrigel were fixed in methanol and stained with hematoxylin and eosin (H & E). The percent of invasion was determined by counting the H&E stained cells [20, 22].

4.4. Xylene-induced ear inflammation suppression test

Five mice per group were used to determine the effect of the CXCR4 modulators. The inner and outer surfaces of the right ear of each mouse were treated with a total 30 μ L of xylene for the induction of ear edema, whereas the left ear was treated with 30 μ L of saline, which was used as a non-inflammation control. The selected compounds were dissolved in 10% DMSO and 90% of 45% (2-hydroxypropyl)- β -cyclodextrin (CD) in PBS. 30 minutes after the application of xylene, 14 selected compounds were administered intraperitoneally (i.p.) at 10 mg/kg. Control animals received corresponding i.p. injections of the vehicle. The animals were sacrificed 2 hours later, and two ear plugs (7 mm in diameter) were removed from both the treated ear and the untreated ears. Weights of treated and untreated ear plugs were measured. The difference in weight of the two ear plugs was taken as a measure of edematous response. The inflammation-suppression percentage was calculated by comparing the drug-treated group to the control group [23].

4.5. Western blot analysis

Forty micrograms of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 30 min in a blocking solution (5 % milk in Trisbuffered saline containing 0.1% Tween-20) and incubated overnight at 4°C using monoclonal rabbit anti-phospho-Akt (Ser473) antibody (Cat No., 9271)/monoclonal rabbit anti-Akt (pan) antibody (cat No., 4691) or monoclonal rabbit anti-

phospho-p44/42 MAPK (Thr202/Tyr204) antibody (cat No., 4376)/monoclonal rabbit anti-p44/42 MAPK antibody (cat No., 4695) at 1:500 in blocking solution. All antibodies were purchased from Cell Signaling Technology (Danvers, USA). The membrane was incubated for 1 hour with goat anti-rabbit IgG (H+L)-HRP conjugated secondary antibody at 1:10000 (Cat No. 1706515; Bio-rad, Hercules, USA) after washing. Enzyme-linked chemiluminescence was performed to detect hybridized protein bands.

4.6. Preliminary cytotoxicity study of compound IIj (MTT assay)

The antiproliferative activity of the compounds was determined using MTT assay. Human breast cancer MDA-MB-231 or MCF-10A Cells were seeded in 96-well micro culture plates at 3000 cells/well in 100 μ L of medium and incubated for 24 h at 37 °C in CO₂ incubator. Following the incubation for 24 h, these cells were treated with Compound IIj for 24 h at 37 °C. Finally, 20 μ L of CellTiter 96AQ reagent (Promega, Madison, WI) was added into each well and incubated for an additional 2 h, and the absorbance at 490 nm was measured.

4.7. Preliminary pharmacokinetic study of compound IIj

Nude mice with body weight about 20 g were used in the PK study. The mice were anaesthetized with Ketamine hydrochloride (90 mg/kg) and Xylazine (4.5 mg/kg). Compound IIj was dissolved in 10% DMSO and 90% of 45% (2-hydroxypropyl)- β -cyclodextrin (CD) in PBS. The mice were warmed by the heat lamp for 2 minutes and then received a single dose (30 mg/kg) of compound IIj via intravenous injection (i.v.). Blood samples (100 μ L) were collected at 0, 5, 20 and 50 minutes post dose from orbital venous sinus with heparinized capillary tubes (I.D. 1.1–1.2 mm). After collection, the blood sample was centrifuged at 13000 g for 4 min at 4 °C. The supernatant was then filtrated by microcon centrifugal filter (10000 NMWL) at 13000 g for 30 min at 4 °C. The filtrate was frozen at –80°C until HPLC analysis. The HPLC analysis of the blood sample was performed on a C18 column (250*4.6 mm). Acetonitrile (0.1%TFA)-Water (0.1%TFA) (50:50, V/V) was applied as the mobile phase and the detective wavelength was set at 254 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

A series of amide-sulfamide compounds targeting CXCR4 were designed and synthesized;

Most of the compounds showed potent *in vitro* CXCR4 inhibitory activity;

Compound **IIj** significantly attenuated mouse ear inflammation by 75%;

IIj significantly blocked CXCR4/CXCL12-mediated phosphorylation of Akt;

IIj displayed a favourable plasma stability in our primary pharmacokinetic study.

Fig. 1.
Strategy for the discovery of novel anti-inflammatory agents.

Fig. 2.

Representative immunofluorescence images of competition-binding affinity assay of three selected compounds compared to AMD3100. CXCR4 receptors on the cell surface are shown in red fluorescent color in this binding affinity assay using biotinylated TN14003 that binds to CXCR4. When our test compounds are preincubated with the cells and block the binding of biotinylated TN14003, the red fluorescent color gets reduced. The effective concentration (EC) of AMD3100 was 1000 nM, while compounds Ie, IIj and Iq showed much better EC of only 1, 1 and 10 nM, respectively.

Fig. 3.

Summary of Matrigel invasion assay results induced by CXCR4/CXCL12-mediated interaction using MDA-MB-231 cells in the presence of CXCR4 modulators (all compounds, $P < 0.05$). CXCL12 α was added to the bottom chamber, and the compounds were added to the top chamber. After incubating for 22 hours, the invading cells were fixed in methanol and stained with hematoxylin and eosin. The percent of invasion was determined by counting the stained cells. 0% inhibition was determined with CXCL12 α added to the bottom chamber, but without any CXCR4 inhibitor. 100% was defined by counting the stained cells without adding CXCL12 α nor compound. Compounds achieving greater than 65% inhibition (over blue line) were evaluated in the ear edema test.

Fig. 4. Micrographs of Matrigel invasion assay induced by CXCR4/CXCL12-mediated interaction using MDA-MB-231 cells in the presence of CXCR4 modulators. The number of invading cells to the bottom chamber decreased when cells were incubated with our compounds in the top chamber.

Fig. 5.

In vivo anti-inflammatory activity of 14 selected compounds (n=5, * P < 0.05). The right ear of each mouse was treated with a 30 μ L of xylene. The selected compounds were administered intraperitoneally (i.p.) at 10 mg/kg, 30 minutes later. Two hours later, one each ear plug was removed from both ears and weighed to calculate the inflammation-suppression percentage.

Fig. 6. Histological analysis of the anti-inflammatory activity of compound **IIj**. Whole tissue slices were scanned/digitized by NanoZoomer 2.0 HT. Software NDP.view 2 was used to zoom in.

Fig. 7.
Compound **IIj** blocked the phosphorylation of Akt mediated by CXCR4/CXCL12 axis.

Fig. 8. Cytotoxicity evaluation of compound IIj in MDA-MB-231 and MCF-10A cell lines. The antiproliferative activity of the compounds was determined using MTT assay. CXCR4-positive MDA-MB-231 or CXCR4-negative MCF-10A cells were treated with Compound IIj or vehicle control for 72 h. The results showed no statistically significant difference, which suggests that IIj does not have antiproliferative activity.

Fig. 9.

The amount of compound **IIj** in mouse serum at various time points following the compound administration. The nude mice were received a single dose (30 mg/kg) of compound **IIj** via intravenous injection (i.v.). Blood samples (100 μ L) were collected at 0, 5, 20 and 50 minutes. The blood sample was centrifuged and filtrated and analyzed by HPLC to estimate the amount of compound in serum.

Fig. 10.
Structure–activity relationships of amide-sulfamide compounds.

Scheme 1.

Reagents and conditions: (a) DCM, triethylamine (TEA), ice bath to r.t., 6 h, 75–90%; (b) i. DCM, TFA, r.t., 8 h; ii. NaHCO₃, 88–95%; (c) DCM, TEA, benzoyl chloride derivatives, ice bath to r.t., 6 h, 60–89%.

Table 1

Preliminary effective concentration (EC) of anti-CXCR4 compounds.

Compd	EC (nM)	Compd	EC (nM)	Compd	EC (nM)	Compd	EC (nM)
Ia	1	Ij	100	IIa	>1000	IIj	1
Ib	1	Ik	10	IIb	100	IIk	10
Ic	1000	Im	1000	IIc	1000	IIm	100
Id	100	In	100	IId	1000	IIn	10
Ie	1	Io	100	IIe	100	IIo	10
If	>1000	Ip	10	IIf	10	IIp	10
Ig	1000	Iq	10	IIg	100	IIq	>1000
Ih	1000	Ir	10	IIh	1000	IIr	10
II	100			IIi	1		
AMD3100	1000						