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Development of CXCR4 modulators by virtual HTS of a novel amide-sulfamide compound library

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Abstract

CXCR4 plays a crucial role in recruitment of inflammatory cells to inflammation sites at the beginning of the disease process. Modulating CXCR4 functions presents a new avenue for anti-inflammatory strategies. However, using CXCR4 antagonists for a long term usage presents potential serious side effect due to their stem cell mobilizing property. We have been developing partial CXCR4 antagonists without such property. A new computer-aided drug design program, the FRESH workflow, was used for anti-CXCR4 lead compound discovery and optimization, which coupled both compound library building and CXCR4 docking screens in one campaign. Based on the designed parent framework, 30 prioritized amide-sulfamide structures were obtained after systemic filtering and docking screening. Twelve compounds were prepared from the top-30 list. Most synthesized compounds exhibited good to excellent binding affinity to CXCR4. Compounds **Ig** and **Im** demonstrated notable *in vivo* suppressive activity against xylene-induced mouse ear inflammation (with 56% and 54% inhibition). Western blot analyses revealed that **Ig** significantly blocked CXCR4/CXCL12-mediated phosphorylation of Akt. Moreover, **Ig** attenuated the amount of TNF- α secreted by pathogenic *E. coli*-infected macrophages. More importantly, **Ig** had no observable cytotoxicity. Our results demonstrated that FRESH virtual high throughput screening program of targeted chemical class could successfully find potent lead compounds, and

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the amide-sulfamide pharmacophore was a novel and effective framework blocking CXCR4 function.

Graphical abstract

Keywords

CXCR4; Amide-sulfamide; virtual HTS; compound library building; Anti-inflammatory activity

1. Introduction

Chemokines are a superfamily of small cytokines which induce cytoskeletal rearrangements and directional migration of some cell types through their interaction with G-protein-coupled receptors [1, 2]. C-X-C chemokine receptor type 4 (CXCR4) is a 7-transmembrane chemokine receptor within the chemokine family [3, 4]. The ligand interacting with CXCR4 is stromal-derived-factor-1 (SDF-1) or C-X-C chemokine ligand 12 (CXCL12) [5]. Recently CXCR4 receptor has been proved to be involved in multiple pathological conditions, such as cancer and inflammation [6].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the widely used medications for treatment of a variety of inflammatory conditions [7]. By inhibiting the activity of cyclooxygenase (COX), NSAIDs prevent the formation of prostaglandin (PG) [8]. However, the long term use of NSAIDs is associated with potential serious side effects such as kidney failure, ulcers, and prolonged bleeding after an injury or surgery [9]. Several NSAIDs were withdrawn from the market (bromfenac, benoxaprofen, and lumiracoxib) because of the relevance of postmarketing monitoring to detect severe cases of liver injury. The development of cyclooxygenase-2 (COX-2) inhibitors, which have decreased gastrointestinal side effects, opened a promising era for NSAIDs. But the evidence of severe cardiovascular events led to the withdrawal of rofecoxib in 2004 [10].

CXCR4 and CXCL12 are involved in tissue infiltration of neutrophils and lymphocytes after inflammatory stimuli [11]. CXCR4 also plays crucial roles in neutrophil homeostasis, and is associated with the pathophysiology of inflammatory diseases, including autoimmune diseases, rheumatoid arthritis, inflammatory bowel disease, ischemic injuries, and lung diseases [12]. Based on these findings, development of CXCR4 modulators presents a new avenue for the investigation of novel and safe anti-inflammatory drugs.

AMD3100 (**1**) was the first small molecule CXCR4 antagonist to enter clinical trials [13]. However, AMD3100 is a metal-chelating bicyclam that has been shown to block calcium

flux, which can be linked to cardiotoxicity [14]. In particular, AMD3100 is an effective stem cell mobilizer by dissociating CXCR4 from its ligand CXCL12, and has been FDA-approved for this indication. While complete CXCR4 inhibitor, such as AMD3100, mobilizes and, at the same time, blocks homing of CXCR4-positive inflammatory cells, a partial CXCR4 modulator, without stem cell mobilizing capability, can be safe and effective in long term use [15]. Our laboratory has been developing such CXCR4 modulators, leading to the design and synthesis of a series of novel and potent CXCR4 modulators (Class I & Class II, shown in Fig. 1) [16-18]. Among the promising candidates, compound **2** shows excellent activity both in vitro and in vivo. In addition to its potency, compound **2** exhibits very low toxicity, and is now under clinical trial (Phase II).

The path to a drug discovery begins with identification of a molecular target, followed by finding the lead compound, and the lead modification and optimization usually accounts for the dominant preclinical expense [19]. The long journey to synthesize numerous analogues of the hit compounds aims to improve their potency and favorable in vivo pharmacokinetics, but success is not guaranteed [19].

Pipeline Pilot is a scientific visual and dataflow programming language, which is useful in building compound library, absorption, distribution, metabolism, excretion and toxicity (ADMET) screening, virtual High-Throughput Screening (vHTS) and QSAR study. Schrodinger Maestro is also frequently-used software for computational modeling and docking in drug development. These two software programs are both commercially available and usually used separately. To find the lead compounds and drug candidates efficiently, we combined the technology of Pipeline Pilot and Schrodinger Maestro, and developed a pipeline program named FRESH (FRagment-based Exploitation of modular Synthesis by vHTS) [20]. This strategy couples the functions of Pipeline Pilot and Schrodinger Maestro in one campaign to optimize the potency and ADMET characteristics of the compounds. Three retrospective case studies and one prospective study have been reported to validate the application of this new method, which proved that the combination of Pipeline Pilot and Schrodinger Maestro was able to capture potent lead compound successfully [20]. We built the compound library and screened for drug-like properties in Pipeline Pilot, and then we further filtered the remaining candidates by docking with CXCR4 by Schrodinger Maestro. After combining the different functions, the comprehensive lead compound discovery process included a new compound library generation, quantitative structure—activity relationship construction, fragment processing, ADMET probability calculation, vHTS and potency estimation.

To develop novel CXCR4 modulators, herein we designed a unique amide-sulfamide structural framework (Fig. 1) based on our experience [16, 17, 21]. The FRESH program was further customized and applied to find promising drug-like CXCR4 modulators with amide-sulfamide pharmacophores and the selected compounds were synthesized and evaluated for their biological activities.

2. Results and discussion

2.1. Development of novel CXCR4 inhibitory candidates by FRESH workflow

Pipeline Pilot was first used to build the compound library based on the designed amide-sulfamide scaffold. Then all the compounds within the library were screened for drug-like properties and ADMET factors by Pipeline Pilot. After obtaining the minimized number of compounds, Schrodinger Maestro was finally applied to further filter remaining compounds by docking with CXCR4. The specific process is described as follows.

The FRESH workflow was constructed according to the synthetic route shown in Scheme 1. The building block library was queried for two building blocks, sulfochlorides and carboxylic acids. Initially, there were ~100 hits identified as sulfochlorides and ~800 hits as carboxylic acids. As illustrated in Fig. 2, these building blocks were then marked as fragments and filtered by several criteria (like no bridge-head or spiral carbon, no silicon, no permanent charges, etc.). The remaining ~50 sulfochlorides and ~360 carboxylic acids were linked to the core structure. About 19000 molecule structures were obtained by combinatorial library enumeration. Then a series of calculations on their physical/ADMET properties were performed based on Lipinski's "Rule of Five", Jorgensen's "Rule of Three", Morelli, Bourgeois, and Roche's "Rule of Four" [22-24]. 19000 molecules were reduced to only 364 compounds with drug-like properties. Consequently, after docking with CXCR4 receptor, the preliminary docking scores were obtained. The top 30 docking score ranked amide-sulfamide structures were obtained as the priority list for further evaluation.

Based on the comprehensive consideration of the diversity of the fragment, starting material availability, ease of synthesis, and yields of the reaction, finally twelve representative amide-sulfamide molecules were prepared from the top-30 list to evaluate whether this designed amide-sulfamide structure was a promising framework for further investigation.

2.2. Chemistry

The synthetic route chosen to synthesize the title compounds is 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-m** were synthesized by the acylation of intermediate **5** with corresponding acids. Herein we used a mild and convenient one-pot protocol for the conversion of carboxylic acids into amides through carboxyl activation by the reagent combination of trimethyl phosphate and iodine [25]. Compared with existing methods, the reaction time of the present method is only 3-4 hours, and operations permit the synthesis under less restrictive conditions.

2.3. Primary binding affinity screening

The selected twelve compounds were screened with a binding affinity assay as described in our previous publications [17, 21]. This assay involves competition against a potent CXCR4 peptidic inhibitor, biotinylated TN14003, with the target compounds **Ia-m**. MDA-MB-231

cells were pre-incubated with compounds at concentrations of 1, 10, 100, and 1000 nM, followed by paraformalin fixation. The fixed cells were then incubated with biotinylated TN14003 and subsequent streptavidin-conjugated rhodamine to determine the binding affinity of the new compounds to the TN14003 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. Thus, this screening is a semi-quantitative, primary screening of the level of activity.

Just as anticipated, the amide-sulfamide structural framework displayed potent CXCR4 binding affinity. As illustrated in Table 1 and Fig. 3, most of the compounds (>80%) exhibited moderate to powerful binding affinity (< 1000 nM) in binding assay. Only compounds **Ie** and **Ii** failed to block the binding of biotinylated peptide TN14003 to CXCR4, with an EC more than 1000 nM. The binding affinity of compounds **Ia**, **Ic**, **Id**, **If** and **Ih** was comparable to the reference drug AMD3100 (1000 nM), while **Ij**, **Ik** and **Im** showed better EC from 10 to 100 nM. Surprisingly, compounds **Ib** and **Ig** exhibited an EC of only 1 nM, and experienced 1000-fold improvement compared with AMD3100. The above data confirmed that the amide-sulfamide structure is a novel and effective chemical class blocking CXCR4. And more importantly, the results demonstrated that the FRESH program could successfully find potent lead compounds from a large number of structure candidates, which saved time and effort compared with the structural modification and optimization in traditional medicinal chemistry methods.

2.4. Matrigel invasion assay

To model *in Vitro* chemotaxis/invasion, a Matrigel invasion assay was used as a functional assay [17, 21]. This assay was performed for those compounds with an EC value no more than 1000 nM to test whether they could block the CXCR4/CXCL12-mediated chemotaxis and invasion at 100 nM. The compounds and cells are added on the upper chamber of a vessel and CXCL12 is added in the lower chamber as a chemoattractant in serum-free medium. A Matrigel membrane separates the upper and lower chambers. If the compounds demonstrate strong CXCR4 inhibitory effect, very few cells are able to invade through the Matrigel membrane. The results of Matrigel invasion were summarized in Fig. 4 and Fig. 5.

All the ten selected compounds behaved well in the blocking of Matrigel invasion assay, showing moderate to potent activity. Compounds **Ib**, **Ic**, **Id**, **Ih** and **Ij** **Im** demonstrated around 40% anti-invasion effect. Compounds **If** and **Im** displayed a comparable inhibitory rate to the reference drug AMD3100 (55%). There were three compounds, **Ia**, **Ig** and **Ik**, that showed much better activity than AMD3100, with 60, 74 and 68% inhibition, respectively. Both the binding assay and invasion estimate give eloquent proof that the FRESH workflow can discover promising lead candidates from a huge number of compounds belonging to a certain chemical class. This unique program provides a novel and useful strategy in lead compound discovery.

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

The 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 the substance P, and acts as a neurotransmitter or neuromodulator in several physiological processes. In the periphery, release of substance P from sensory neurons leads to vasodilatation and plasma extravasations, which causes ear swelling in the mice [26].

On the basis of the *in vitro* assay results, six of the best compounds (**Ia**, **Ic**, **If**, **Ig**, **Ik** and **Im**) were selected for further investigation. A separate experiment was carried out to evaluate the anti-inflammatory activity by xylene-induced ear edema in mice model and the results are listed in Fig. 6 [27]. Although AMD3100 is the only well investigated small molecule CXCR4 antagonist, its bicyclam structure leads to toxicity in the animal model. Therefore, AMD3100 was not selected as the reference drug in this test. Most of the selected compounds displayed significant anti-inflammatory activity. Although compounds **Ia** and **Ik** demonstrated excellent CXCR4 antagonistic activity (> 60% in invasion assay), they showed very weak *in vivo* potency or were even inactive, with 10% and 0 inhibition, respectively. Compounds **Ic** and **If** exhibited moderate anti-inflammatory effect (38 and 35%). Surprisingly, compounds **Ig** and **Im** showed excellent suppressive activity, with 56 and 54% inhibition on inflammation, respectively. COX-2 inhibitor celecoxib was reported to show 47% inhibition in xylene-induced ear edema in a mouse model at a dose of 30 mg/kg [9]. By inhibiting CXCR4 instead of COX, reaching such obvious anti-inflammatory effect is really notable.

After being treated with **Ig** and **Im**, the inflammation induced in the ear was clearly suppressed. As shown in Fig. 7, the mouse ear inflammation and damage were significantly attenuated by compounds **Ig** and **Im** in histological assay. The ear thickness, edema volume, and the number of inflammatory cells decreased observably (C1-2 and D1-2). These results confirm that the selected candidates targeting CXCR4 can inhibit inflammation as anticipated.

Developing anti-CXCR4 drugs for inflammation provides an exciting avenue to target different pathways from COX. By combining drugs targeting different pathways, the efficacy will be enhanced without increasing dosage of a drug associated with unwanted side effects.

Previous reports on drug testing against inflammation were geared toward prevention by treating animals 30 minutes before the administration of xylene. In order to better evaluate the anti-inflammatory activity of our compounds, here we optimized the xylene -induced ear edema model. The compounds were administrated 30 minutes after xylene treated in our evaluation.

2.6. Molecular modeling (docking) studies

To better understanding the possible interaction between the three best ligands (**Ig**, **Ik** and **Im**) and CXCR4, further molecular docking study was performed based on the available crystal structure of CXCR4 (PDB code: 3ODU [28]). Fig. 8 illustrates the binding pose with the best docking score. Two hydrogen bonds were formed on ligand **Ig** with Asp97 and Arg188. One pyridine ring of **Ig** showed a π - π stacking with Trp94. Ligand **Ik** also demonstrated a π - π stacking with Trp94 and two hydrogen bonds between Asn33 and Ser285. For ligand **Im**, a hydrogen bond was observed with Asp97. The terminal phenyl ring

showed a π - π stacking with Trp94 and a π -cation interaction was present between Arg183 and the heterocyclic ring.

2.7. Evaluation of anti-CXCR4 activity of compound **Ig** at the molecular level

2.7.1. Compound **Ig** suppressed CXCL12 induced phosphorylation of Akt—

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 [29]. 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 [30]. The activity of **Ig** in blocking the PI3K/Akt pathway was investigated by Western blot analysis. As shown in Fig. 9, CXCR4 antagonist **Ig** blocked the CXCR4/CXCL12-mediated phosphorylation of Akt in a dose-dependent manner, and significantly inhibited phosphorylation at concentrations of 10 and 100 nM.

2.7.2. Compounds **Ig** attenuated the level of TNF- α —

Increase level of tumor necrosis factor- α (TNF- α) is associated with various inflammatory processes [31]. Our previous study has shown that inhibition of CXCR4 attenuated induction of TNF- α by macrophages in response to infection with pathogenic *E. coli* strain [18]. To determine whether compound **Ig** could attenuate the level of TNF- α , J774A.1 macrophages were infected with Crohn's disease (CD)-associated *E. coli* strain 13I or non-pathogenic EFC-1 as a control in the absence or presence of compound **Ig** at 200 nM [32]. The CD isolates 13I greatly induced TNF- α secretion by macrophages compare with EFC-1. The addition of compound **Ig** to the culture media during the bacterial infection, the TNF- α level was attenuated by 67 ± 8 % at 200 nM, suggesting that **Ig** can effectively suppress the host response to CD-associated invasive *E. coli*.

2.8. Preliminary cytotoxicity evaluation of compound **Ig**

In addition to their therapeutic effects, cytotoxic agents have the potential of causing serious destruction to healthy and normal cells. To determine the cytotoxicity of compound **Ig**, two representative human breast cancer cell lines, MDA-MB-231(CXCR4-positive) and A1N4 (CXCR4-negative), were treated with different concentrations of **Ig** and the effects on proliferation was determine by a cell viability (MTT) assay. Previously, we reported that some CXCR4 antagonists do not exhibit any cytotoxic effect on adherent cell proliferation. [33]. Compound **Ig** displayed potent CXCR4 binding affinity at only 1 nM, while it did not inhibit cell proliferation even at 10 μ M. Thus, it is unlikely that **Ig** reduces inflammation because of its cytotoxic effect.

3. Conclusion

Traditional lead compound discovery and optimization are time-intensive and associated with significant costs. Numerous derivatives and analogues usually need to be synthesized in order to find the potent candidates.

With the assistance of our novel FRESH program, the top-30 ranked amide-sulfamide structures were obtained after systemic filtering and docking screening based on the designed parent framework. Most of the synthesized compounds exhibited moderate to excellent binding affinity in binding assay. Several compounds even displayed an EC of only 1 nM, which experienced 1000-fold improvement compared with AMD3100. In the Matrigel invasion assay, compounds **If** and **Im** exhibited a comparable inhibition to the reference drug AMD3100, while compounds **Ia**, **Ig** and **Ik** showed better activity than AMD3100. In the *in vivo* anti-inflammatory test, compounds **Ig** and **Im** demonstrated notable suppressive activity on inflammation, with 56 and 54% inhibition, respectively. Besides clearly inhibiting the mouse ear edema, **Ig** and **Im** also significantly attenuated the ear damage and decreased the number of inflammatory cells in histological analysis. Western blot analyses revealed that CXCR4 modulator **Ig** blocked the CXCR4/CXCL12-mediated Akt phosphorylation in a dose-dependent manner. Compound **Ig** also significantly suppressed TNF- α secretion by bacterial-infected J774A.1 macrophages.

These promising results demonstrated that FRESH is a useful program to catch potent lead compounds from a compound library. In addition, the amide-sulfamide framework is a novel and effective pharmacophore blocking CXCR4.

Although this amide-sulfamide framework is an effective key structure to block CXCR4, we need more studies to get the detailed structure-activity relationships (SARs). Further modification based on the amide and sulfamide side chains will be performed to summarize the SARs of this novel parent structure as the next step. The docking score does not necessarily correlate well with the biological activity *in vivo*, which results in inaccurate to predict the potency by computer-aided drug design (CADD). The key advantage of FRESH program is to discover some novel promising key structures like the amide-sulfamide framework for further research. After rapid confirmation by this program, they can be selected as promising lead structures for further systematic modification and optimization using traditional medicinal chemistry method. The strategy of combining the traditional medicinal chemistry method with the CADD technique can be a faster and more effective method in drug development.

4. Experimental section

4.1 Development of novel CXCR4 inhibitory candidates by FRESH workflow

The FRESH workflow was constructed primarily on the Pipeline Pilot (Version 7.0) platform. The compound library used to query building blocks was obtained from Maybridge. The criteria used for filtering fragments were “no chiral atom, no positive atom, no negative atom, no bridgehead atom, no spiro atom, no Si atom, the total number of N and O ≤ 3 , molecular weight < 301 , the number of hydrogen bond donors ≤ 3 , the number of hydrogen bond acceptors ≤ 3 and AlogP ≤ 3 ”. Fragments containing any substructure in the attached files were also removed. After obtaining 19080 molecule structures, the following filters were applied in Pipeline Pilot to select drug-like candidates: “the total number of N and O ≤ 10 , the number of hydrogen bond donors ≤ 5 , the number of hydrogen bond acceptors ≤ 5 , the number of rotatable bonds ≤ 10 , molecular weight < 501 , AlogP ≤ 5 , polar surface area ≤ 120 , the number of metabolites ≤ 6 , logS ≥ -6 (S, solubility)”. With ADMET

filtering, 364 compounds remained that were rank-ordered for the top 30 compounds after docking with CXCR4 receptor.

4.2. Chemistry

4.2.1. General information—Proton and carbon NMR spectra were recorded on INOVA-400 (400 MHz) or VNMR-400 spectrometers at Emory NMR Research Center. The spectra obtained in CDCl₃, Methanol-*d*₄ 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. 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. Analytical thin layer chromatography (TLC) was performed on precoated glass backed plates from Scientific Adsorbents Incorporated (Silica Gel 60 F254; 0.25 mm thickness). Plates were visualized using ultraviolet.

4.2.2. General procedure for synthesis of intermediate 4—A solution of 4-(Boc-aminomethyl)benzylamine (1.0 mmol) in anhydrous DCM (8 mL) was cooled with an ice bath, then the corresponding sulfochloride (1.1 mmol, dissolved in 2 mL 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 4 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 (150:1, v/v) to give the product as a white solid.

4.2.3. 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 added 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.

4.2.4. General procedure for synthesis of target compounds la-m—A solution of P(OMe)₃ (1.5 mmol) in DCM (10 mL) was cooled with an ice bath, then I₂ (1.5 mmol) was added. After the solid iodine was completely dissolved, corresponding acid (1.2 mmol) and Et₃N (3.0 mmol) were added in sequential order, and the solution was stirred for 15 min in a cooling bath. Intermediate 5 (1.0 mmol) was added and the mixture was stirred for 15 min. After removing the cooling bath, the reaction mixture was stirred for 3.5 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 (100:1 to 50:1, v/v) to give the product as a white solid.

4.2.4.1. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)nicotinamide

(Ia): White solid, yield 75%, m.p. 137–139 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.86 (s, 1H), 8.69 (dt, *J* = 4.8, 1.7 Hz, 1H), 8.11 (dt, *J* = 8.0, 1.9 Hz, 1H), 7.64–7.68 (m, 2H), 7.35–7.38 (m, 1H), 7.17–7.27 (m, 4H), 6.81–6.84 (m, 2H), 5.09 (s, 1H), 4.68 (t, *J* = 8.8 Hz, 2H), 4.58 (d, *J* = 5.7 Hz, 2H), 4.07 (d, *J* = 6.5 Hz, 2H), 3.25 (t, *J* = 8.8 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 165.76, 164.04, 152.48, 148.18, 137.75, 136.25, 135.35, 131.4, 130.09, 128.93, 128.62, 128.39, 124.67, 123.72, 110.23, 109.75, 72.52, 47.11, 43.94, 29.29. HRMS calcd for C₂₂H₂₂O₄N₃S 424.13255 [M + H]⁺, found 424.13204.

4.2.4.2. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide (Ib):

White solid, yield 82%, m.p. 180–182 °C. ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.57 (dd, *J* = 7.1, 1.1 Hz, 1H), 7.70 (dt, *J* = 9.1, 1.2 Hz, 1H), 7.56–7.59 (m, 2H), 7.23–7.29 (m, 5H), 7.03 (d, *J* = 1.0 Hz, 1H), 6.98 (td, *J* = 6.9, 1.4 Hz, 1H), 6.78–6.80 (m, 1H), 4.61 (t, *J* = 8.8 Hz, 2H), 4.57 (s, 2H), 4.02 (s, 2H), 3.21 (t, *J* = 8.8 Hz, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 162.78, 161.36, 147.92, 147.88, 140.64, 138.54, 136.12, 128.75, 128.63, 127.78, 127.56, 127.16, 124.30, 123.96, 119.03, 114.06, 108.94, 97.43, 72.09, 45.93, 41.85, 28.46. HRMS calcd for C₂₄H₂₃O₄N₄S 463.14345 [M + H]⁺, found 463.14297.

4.2.4.3. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)-1-methyl-1*H*-pyrazole-5-carboxamide (Ic):

White solid, yield 63%, m.p. 143–145 °C. ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.57–7.61 (m, 2H), 7.45 (d, *J* = 2.1 Hz, 1H), 7.17–7.24 (m, 4H), 6.78–6.80 (m, 2H), 4.62 (t, *J* = 8.8 Hz, 2H), 4.47 (s, 2H), 4.10 (s, 3H), 4.01 (s, 2H), 3.21 (t, *J* = 8.8 Hz, 2H). ¹³C NMR (100 MHz, Methanol-*d*₄) δ 165.15, 161.99, 139.30, 138.81, 137.97, 137.19, 133.64, 130.09, 129.60, 129.32, 128.71, 125.56, 110.23, 108.45, 73.56, 47.77, 43.76, 39.46, 30.05. HRMS calcd for C₂₁H₂₃O₄N₄S 427.14345 [M + H]⁺, found 427.14292.

4.2.4.4. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)-1,3-dihydroisobenzofuran-4-carboxamide (Id):

White solid, yield 68%, m.p. 149–151 °C. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.25 (t, *J* = 6.1 Hz, 1H), 7.90 (t, *J* = 6.5 Hz, 1H), 7.60–7.62 (m, 1H), 7.55–7.57 (m, 1H), 7.17–7.26 (m, 4H), 7.08 (dd, *J* = 7.7, 1.2 Hz, 1H), 6.89–6.94 (m, 1H), 6.13 (s, 2H), 4.60–4.65 (m, 2H), 4.45 (d, *J* = 6.0 Hz, 2H), 3.90 (d, *J* = 6.4 Hz, 2H), 3.22 (t, *J* = 8.8 Hz, 2H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 163.01, 162.79, 147.69, 145.26, 138.25, 136.92, 136.29, 132.22, 128.65, 127.77, 127.62, 127.26, 127.13, 123.99, 123.94, 121.58, 121.20, 116.43, 110.93, 108.96, 101.60, 72.11, 45.91, 42.29, 28.47. HRMS calcd for C₂₄H₂₃O₆N₂S 467.12713 [M + H]⁺, found 467.12605.

4.2.4.5. *N*-(4-((2,4-difluorophenyl)sulfonamido)methyl)benzyl)-1*H*-imidazole-2-carboxamide (Ie):

White solid, yield 75%, m.p. 225–227 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 13.00 (s, 1H), 8.92 (t, *J* = 6.4 Hz, 1H), 8.51 (t, *J* = 6.2 Hz, 1H), 7.77 (td, *J* = 8.6, 6.4 Hz, 1H), 7.41–7.44 (m, 1H), 7.27 (dd, *J* = 2.3, 1.1 Hz, 1H), 7.12–7.22 (m, 5H), 7.04 (t, *J* = 1.3 Hz, 1H), 4.36 (d, *J* = 6.4 Hz, 2H), 4.06 (d, *J* = 6.3 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 158.67, 141.11, 138.69, 136.09, 131.86, 131.77, 127.77, 127.66, 127.41,

127.23, 112.40, 112.18, 109.87, 106.23, 105.98, 45.96, 41.94. HRMS calcd for $C_{18}H_{17}O_3N_4SF_2$ 407.09839 $[M + H]^+$, found 407.09849.

4.2.4.6. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)-1,3-dimethyl-1H-pyrazole-5-carboxamide (If): White solid, yield 62%, m.p. 176–178 °C. 1H NMR (400 MHz, Chloroform-*d*) δ 7.65–7.68 (m, 2H), 7.19–7.25 (m, 4H), 6.84 (d, J = 8.3 Hz, 1H), 6.27 (s, 1H), 6.27 (s, 1H), 4.69 (t, J = 8.8 Hz, 2H), 4.62 (t, J = 6.3 Hz, 1H), 4.53 (d, J = 5.8 Hz, 2H), 4.11 (s, 3H), 4.10 (d, J = 6.8 Hz, 2H), 3.26 (t, J = 8.8 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d*₆) δ 163.08, 159.75, 145.84, 138.39, 136.58, 135.95, 132.41, 128.98, 128.08, 127.89, 127.34, 124.20, 109.24, 106.75, 72.40, 46.13, 42.03, 38.62, 28.71, 13.24. HRMS calcd for $C_{22}H_{25}O_4N_4S$ 441.15910 $[M + H]^+$, found 441.15984.

4.2.4.7. *N*-(4-((2,4-difluorophenylsulfonamido)methyl)benzyl)pyrimidine-5-carboxamide (Ig): White solid, yield 59%, m.p. 173–175 °C. 1H NMR (400 MHz, Methanol-*d*₄) δ 9.28 (s, 1H), 9.17 (s, 2H), 7.80 (td, J = 8.5, 6.2 Hz, 1H), 7.17–7.25 (m, 4H), 6.99–7.08 (m, 2H), 4.52 (s, 2H), 4.16 (s, 2H). ^{13}C NMR (100 MHz, Methanol-*d*₄) δ 165.73, 161.28, 161.23, 157.31, 139.07, 137.76, 133.22, 133.12, 129.79, 129.35, 128.93, 112.84, 112.65, 106.74, 106.48, 106.22, 47.55, 44.42. HRMS calcd for $C_{19}H_{17}O_3N_4SF_2$ 419.09839 $[M + H]^+$, found 419.09835.

4.2.4.8. *N*-(4-((3-fluorophenylsulfonamido)methyl)benzyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide (Ih): White solid, yield 82%, m.p. 163–165 °C. 1H NMR (400 MHz, Methanol-*d*₄) δ 8.55 (d, J = 7.0 Hz, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.58 (dt, J = 8.0, 1.2 Hz, 1H), 7.46–7.51 (m, 2H), 7.15–7.30 (m, 6H), 7.02 (s, 1H), 6.96 (td, J = 6.9, 1.4 Hz, 1H), 4.55 (s, 2H), 4.08 (s, 2H). ^{13}C NMR (100 MHz, Methanol-*d*₄) δ 165.14, 164.70, 162.66, 148.86, 144.76, 144.69, 142.90, 139.56, 137.54, 132.37, 132.30, 129.94, 129.32, 128.86, 125.54, 124.09, 124.05, 120.58, 120.37, 120.34, 115.38, 115.27, 115.02, 98.66, 47.81, 43.74. HRMS calcd for $C_{22}H_{20}O_3N_4SF$ 439.12347 $[M + H]^+$, found 439.12221.

4.2.4.9. *N*-(4-((3,4-difluorophenylsulfonamido)methyl)benzyl)imidazo[1,2-*a*]pyridine-3-carboxamide (Ii): White solid, yield 58%, m.p. 192–194 °C. 1H NMR (400 MHz, $CDCl_3$) δ 9.46 (d, J = 6.9 Hz, 1H), 8.00 (s, 1H), 7.61–7.67 (m, 3H), 7.35–7.40 (m, 1H), 7.26–7.30 (m, 1H), 7.22 (d, J = 7.9 Hz, 2H), 7.13 (d, J = 7.9 Hz, 2H), 6.98 (t, J = 6.9 Hz, 1H), 6.70 (s, 1H), 5.62 (s, 1H), 4.56 (d, J = 5.8 Hz, 2H), 4.13 (s, 2H). ^{13}C NMR (100 MHz, Methanol-*d*₄) δ 162.50, 139.92, 137.48, 137.06, 129.37, 129.32, 129.29, 128.83, 125.69, 125.67, 125.65, 125.62, 125.58, 120.08, 119.38, 119.19, 117.98, 117.93, 117.91, 117.79, 115.51, 47.79, 43.52. HRMS calcd for $C_{22}H_{19}O_3N_4SF_2$ 457.11404 $[M + H]^+$, found 457.11433.

4.2.4.10. *N*-(4-((2-fluorophenylsulfonamido)methyl)benzyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide (Ij): White solid, yield 79%, m.p. 138–140 °C. 1H NMR (400 MHz, $CDCl_3$) δ 8.34–8.36 (m, 1H), 7.86 (td, J = 7.6, 1.8 Hz, 1H), 7.60 (d, J = 9.0 Hz, 1H), 7.51–7.57 (m, 1H), 7.39 (s, 1H), 7.11–7.26 (m, 7H), 7.09 (s, 1H), 6.84–6.88 (m, 1H), 5.06 (t, J = 6.2 Hz, 1H), 4.61 (d, J = 6.0 Hz, 2H), 4.19 (d, J = 6.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.20, 160.09, 157.57, 147.74, 141.54, 138.22, 135.30, 135.16, 135.08, 130.47, 128.54, 128.42, 128.32, 124.62, 124.58, 124.01, 119.48, 117.13, 116.92, 113.95, 98.31, 47.30, 43.03. HRMS calcd for $C_{22}H_{20}O_3N_4SF$ 439.12347 $[M + H]^+$, found 439.12276.

4.2.4.11. *N*-(4-((2,3-dihydrobenzofuran-5-sulfonamido)methyl)benzyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine-5-carboxamide (Ik): White solid, yield 77%, m.p. 141–143 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.65–7.68 (m, 2H), 7.17–7.27 (m, 4H), 7.08 (s, 1H), 6.83 (d, *J* = 8.9 Hz, 1H), 6.57 (s, 1H), 4.67–4.74 (m, 3H), 4.57 (d, *J* = 6.0 Hz, 2H), 4.32–4.35 (m, 2H), 4.21–4.23 (m, 2H), 4.09 (d, *J* = 6.1 Hz, 2H), 3.25 (t, *J* = 8.8 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.14, 160.78, 141.83, 141.37, 138.72, 136.60, 132.41, 129.05, 128.15, 127.97, 127.56, 124.28, 112.87, 109.31, 105.12, 72.47, 65.78, 64.27, 46.20, 42.39, 28.75. HRMS calcd for C₂₃H₂₃O₆N₂S₂ 487.09920 [M + H]⁺, found 487.09970.

4.2.4.12. *N*-(4-((3,4-difluorophenyl)sulfonamido)methyl)benzyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (Im): White solid, yield 80%, m.p. 148–150 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.33 (t, *J* = 5.9 Hz, 1H), 8.31 (t, *J* = 6.3 Hz, 1H), 7.76–7.81 (m, 1H), 7.62–7.86 (m, 2H), 7.21 (q, 4H), 4.41 (d, *J* = 5.9 Hz, 2H), 4.01 (d, *J* = 6.2 Hz, 2H), 2.78 (s, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 158.84, 158.58, 144.00, 137.99, 137.55, 136.11, 127.77, 127.33, 124.34, 124.28, 118.63, 118.49, 116.43, 116.27, 45.88, 42.71, 13.25. HRMS calcd for C₁₈H₁₆O₃N₄S₂F₂Na 461.05241 [M + Na]⁺, found 461.05271.

4.3. Primary binding affinity screening

For binding affinity assay, 2×10^4 MDA-MB-231 cells in 300 μL 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 μg/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 [17, 21].

4.4. Matrigel invasion assay

Matrigel invasion assay was performed by using a Matrigel invasion chamber from Corning Biocoat (Bedford, MA). CXCL12α (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 or AMD3100 were added to the cells (100 nM) 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 [17, 21].

4.5. Xylene-induced ear inflammation suppression test

Five mice per group were used to determine the effect of the CXCR4 modulators. The inner and external surfaces of the right ear of each mouse were treated with a total 30 μL of xylene for the induction of ear edema, whereas the left ear was treated with 30 μL 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. Thirty minutes after the application of xylene, compounds **Ia**, **Ic**, **If**, **Ig**, **Ik** and **Im** 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 ear. 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 [27].

4.6. Molecular modeling (docking) studies

The Schrodinger Maestro package was employed to prepare all the 2D ligand molecules obtained in Pipeline Pilot, calculate some physical/ADMET properties and perform the preliminary docking 2D ligand structures were prepared using Lig-prep and consequently submitted to Qikprop for property calculation. For the docking study, the CXCR4 receptor structure was constructed from the available crystal structure of CXCR4 (PDB code: 3ODU [28]) following the established Protein Prepare Wizard workflow. All water molecules were deleted and only Chain A was used to prepare the receptor. All residues beyond 20 Å of the ligand were removed to accelerate the calculation speed. The binding site was selected using the default Receptor Grid Generation procedure and the docking grid was generated around the co-crystallized small molecule ligand IT1t. The prepared ligand was then flexibly docked onto the receptor using Glide and the default setting parameters with no constraints. 5 docking poses were obtained and the one with the best Glide score was chosen to be the docking score for that structure.

4.7. Akt phosphorylation

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) and monoclonal rabbit anti-Akt (pan) antibody (cat No., 4691) 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.

4.8 TNF- α assay

J774A.1 macrophages were infected with CD-associated E. coli strain 13I or non-pathogenic EFC-1 as a control in the absence or presence of compound **Ig** (200 nM). The CD isolates 13I greatly induced TNF- α secretion by macrophages compare with EFC-1.

4.9. Preliminary cytotoxicity study of compound **Ig** (MTT assay)

The antiproliferative activity of the compounds was determined using MTT assay. Human breast cancer MDA-MB-231 or A1N4 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 Ig 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

CXCR4 is a potential target for development of new anti-inflammatory drugs;

A novel amide-sulfamide compound library was built for vHTS and additional screening;

30 amide-sulfamide compounds were obtained after ADMET and silico docking filtering against CXCR4;

12 selected compounds were synthesized and evaluated as CXCR4 modulators;

Ig showed significantly anti-inflammatory activity both *in vitro* and *in vivo*.

Fig. 1.
Strategy for the development of novel CXCR4 modulators.

Fig. 2.
The discovery of novel CXCR4 antagonists by FRESH.

Fig. 3. Fluorescence micrographs of binding affinity assay results of four selected compounds compared to AMD3100. The effective concentration (EC) of AMD3100 is 1000 nM, while compounds **Ib**, **If**, **Ig** and **Im** show EC of 1, 1000, 1, and 10 nM, respectively.

Fig. 4.
Matrigel invasion inhibition of AMD3100 and anti-CXCR4 compounds

Fig. 5.

Anti-Matrigel invasion effect of AMD3100 and four selected compounds. Very few MDA-MB-231 cells are able to move through the Matrigel after treatment with compounds **Ia**, **Ic**, **If**, **Ig**, **Ik** and **Im**.

Fig. 6.
In vivo anti-inflammatory activity of compounds **Ia**, **Ic**, **If**, **Ig**, **Ik** and **Im**.

Fig. 7.

Histological analysis of the anti-inflammatory activity of compound **Ig** and **Im**. Compounds **Ig** and **Im** significantly attenuated the mouse ear inflammation and damage in vivo. The ear tissue sections were stained with H&E. The whole tissue slices were scanned/digitized by NanoZoomer 2.0 HT. Software NDP.view 2 was used to zoom in. Compared to the normal tissue (A1-2), xylene-treated ear exhibited intense edema, and a dense infiltration of inflammatory cells (B1-2). The mice treated with **Ig** and **Im** had the ear thickness, the edema volume, and the number of inflammatory cells (dark purple) decreased observably (C1-2 and D1-2).

Fig. 8.

Best binding pose for compounds **Ig**, **Ik** and **Im** in the CXCR4 X-ray structure (grey). The best docking pose is illustrated by two different visualizations: ribbon (left) and protein surface (right) representations.

Fig. 9.
Compound Ig blocked the phosphorylation of Akt mediated by CXCR4/CXCL12 axis.

Scheme 1.

Reagents and conditions: (a) DCM, TEA, ice bath to r.t., 5 h, 75–90%; (b) DCM, TFA, r.t., 8 h, 94–98%; (c) DCM, TEA, P(OMe)_3 , I_2 , ice bath to r.t., 4 h.

Table 1

Structure, docking score and binding affinity of the 12 synthesized compounds.

Compound Rankings in Top 30 list	Compd	Structure	Glide Score	Binding affinity (EC, nM)
1	Ia		-7.90	1000
3	Ib		-7.85	1
4	Ic		-7.84	1000
6	Id		-7.65	1000
7	Ie		-7.64	>1000
11	If		-7.60	1000
14	Ig		-7.43	1
17	Ih		-7.37	1000
18	Ii		-7.37	>1000
23	Ij		-7.32	100
24	Ik		-7.31	10
25	Im		-7.29	10
Reference drug	AMD3100	--	--	1000