Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 221–228
BBRC
www.elsevier.com/locate/ybbrc

Identification of CXCR3 receptor agonists in combinatorial small-molecule libraries

Ilana L. Stroke *, Andrew G. Cole, Srilatha Simhadri, Marc-Raleigh Brescia, Madhuri Desai, Joan J. Zhang, J. Robert Merritt, Kenneth C. Appell, Ian Henderson, Maria L. Webb

Pharmacopeia Drug Discovery, Inc., P.O. Box 5350, Princeton, NJ 08543, USA

Received 25 July 2006
Available online 11 August 2006

Abstract

In a high-throughput screen of four million compounds from combinatorial libraries for small-molecule modulators of the chemokine receptor CXCR3, two classes of receptor agonists, based on tetrahydroisoquinoline and piperidinyl diazepanone templates, were identified. Several of these compounds stimulated calcium flux in HEK293 cells expressing the recombinant human CXCR3 receptor with efficacies and kinetics similar to those of native ligand CXCL11/I-TAC and stimulated chemotaxis of activated human T-cells. The agonist small molecules also inhibited binding of another CXCR3 ligand, CXCL10/IP-10, to the receptor. The response to small-molecule agonists was inhibited by a CXCR3-specific small-molecule antagonist previously identified within the same combinatorial compound collection but structurally unrelated to the agonists. Remarkably, while other, non-amino acid substituents were present in the majority of the library compounds screened, the agonists from both classes contained a positively charged amino acid component, with preference for Arg > Lys, as well as a hydrophobic component.

Keywords: CXCR3; Chemokine; Combinatorial chemistry; Calcium; G protein-coupled receptor; High-throughput screening; Small molecule; Agonist; Chemotaxis

The chemokine receptor CXCR3 is a member of the seven transmembrane-spanning G protein-coupled receptor (GPCR) superfamily. The natural chemokine ligands for CXCR3, CXCL9/Mig (monokine induced by interferon-γ), CXCL10/IP-10 (interferon-inducible protein 10), and CXCL11/I-TAC (interferon-inducible T cell α chemoattractant) are thought to play a key role in directing activated T cells and other cell types (such as NK cells) to sites of inflammation (a fourth ligand, BCA-1, or B-lymphocyte chemoattractant, CXCL13, has also been reported to bind to CXCR3 [1]). CXCR3 is one of the most abundant chemokine receptors on Th1 cells, and this receptor has been implicated in Th1 cell-mediated inflammation [2]. Activation of CXCR3 has been demonstrated in a number of diseases involving T cells, including inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and diabetes, as well as in allograft rejection. CXCR3 also functions in angiogenesis, but its role has been reported to be either angiogenic or angiostatic. Postischemic neovascularization is decreased in CXCR3-deficient mice [3]. However, activation of the receptor has more often been observed to have an angiostatic effect [4–7] and expression of the receptor in endothelial cells is cell cycle-regulated [8]. The angiostatic effect is likely mediated by activation of an alternatively spliced variant of CXCR3, CXCR3-B, and signaling through a different cellular pathway [9].

High-throughput screening of encoded combinatorial libraries synthesized using Encoded Combinatorial Libraries on Polymeric Support (ECLiPS™) technology [10,11] in functional assays for small-molecule CXCR3 agonists or antagonists resulted in the discovery not only of antagonists [12] but also two classes of receptor agonists. While the compounds tested in the screen were non-peptid-

* Corresponding author. Fax: +1 609 655 4187.
E-mail address: ilana@pcop.com (I.L. Stroke).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.019

ic, the screen for agonists selected for a very limited number of compounds, each containing one basic amino acid in the context of a small-molecule scaffold. Representative compounds of the two chemotypes were evaluated for receptor binding and receptor activation in a calcium flux assay and a T-cell chemotaxis assay. The compounds were shown to be specific for CXCR3 in that they did not induce calcium flux in a cell line expressing the same chimeric G protein but lacking the recombinant receptor, they inhibited binding of CXCL10 to the receptor, they did not inhibit ligand–receptor binding in a panel of GPCR selectivity assays, and their functional activity was blocked by a selective small-molecule CXCR3 antagonist. While several small-molecule CXCR3 antagonists have been identified [12–15], the compounds described here are, to our knowledge, the first reported non-peptide agonists. Along with a recently identified agonist of CCR8 [16] they may be the only reported non-peptide chemokine receptor agonists as well.

Materials and methods

Combinatorial compound libraries. Library generation was conducted utilizing ECLiPS™ technology [17,18], in which compounds are synthesized on polymeric beads via a split and mix protocol. This provides all combinations of the combinatorial variants employed in library synthesis, with libraries ranging from three to five combinatorial steps (resulting in the generation of approximately 20,000 to 100,000 compounds per library). During library synthesis, each combinatorial variant is encoded using a unique combination of polyhalogenated phenolic ether-based tagging molecules in an elaborated binary sequence [17] with the exception of the final combinatorial step, which is termed a sub-library. Each sub-library is defined by the specific fragment incorporated in the final combinatorial step. Encoding molecules (tags) are covalently attached to the polymeric support through rhodium (II) catalyzed C–H insertion of a carbene, generated from the corresponding diazoketone. Encoding molecules are released from the polymeric support via an oxidative process employing ceric ammonium nitrate (CAN), allowing determination of the chemical history of specific library members. The identity of the encoding molecules is determined by electron capture gas chromatography, thus allowing identification of the library member.

Materials and cell lines. All reagents were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise specified. Chemoattractants were obtained from R&D Systems (Minneapolis, MN; CXCL11/I-TAC) or Peprotech (Rocky Hill, NJ; CXCL9/Mig, CXCL10/IP-10, and CCL21/SLC). Cell lines were kindly provided by C. Haskell (Berlex Biosciences, Richmond, CA).

Calcium mobilization assay. Functional CXCR3 activity was determined using an HEK293/CXCR3 G_{qi5} or HEK293/G_{qi5}E cell calcium flux assay on FLIPR^{384} (Molecular Devices, Sunnyvale, CA), with recombinant human CXCL11/I-TAC (EC_{50}–EC_{70}) at 40 nM as control, as described in [12].

Receptor-binding assays. Membranes were prepared from HEK293/CXCR3 G_{qi5} cells by rinsing culture flasks twice with cation-free phosphate-buffered saline, scraping into ice-cold lysis buffer (10 mM Hepes, pH 7.4, 2.5 mM EDTA), Dounce-homogenizing, centrifuging for 15 min at 4 °C at 27,000*g*, and finally, resuspending in lysis buffer. Four micrograms membrane preparation was incubated with agitation for 1 h at 25 °C with 25 pM [^{125}I] CXCL10/IP-10 (New England Nuclear/ Perkin-Elmer, Boston, MA) in 100 μL of 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% bovine serum albumin (BSA), and 1% dimethyl sulfoxide (DMSO). Unlabeled CXCL9/Mig was used at a final concentration of 1 μM to define nonspecific binding. Following incubation, binding reaction mixtures were filtered through MAFCNOB 96-well filter plates (Millipore, Billerica, MA) that had been pretreated with 0.33% polyethyleneimine for 30 min, drained, and washed once with ice-cold wash buffer (50 mM Hepes, pH 7.5, 0.5 M NaCl, and 0.1% BSA) prior to the application of samples. Filter plates were washed three times, dried, and filled with 4 μL OptiPhase Super Mix (Perkin-Elmer, Boston, MA) per well. Bound radioactivity was counted in the TriLux (Perkin-Elmer, Boston, MA). The panel of 10 G protein-coupled receptor radioligand binding assays was carried out by MDS Pharma Services (Taipei, Taiwan) using membrane preparations from CHO-K1 cells expressing recombinant human receptors. Compounds were tested in duplicate at a single concentration of 10 μM. Binding conditions were as follows: adrenergic α_{2B}, 2.5 nM [^{3}H]rauwolscine in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 12.5 mM $MgCl_2$, 0.2% BSA, and 1% DMSO, for 1 h at 25 °C; CCR1, 20 pM [^{125}I]CCL3/MIP-1α in 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA, and 1% DMSO, for 3 h at 25 °C; CCR2B, 100 pM [^{125}I]CCL2/MCP-1α in 25 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, 0.1% NaN_3, and 1% DMSO, for 1 h at 25 °C; CCR4, 100 pM [^{125}I]CCL17/TARC in 50 mM Hepes, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, 0.1% NaN_3, and 1% DMSO, for 2 h at 25 °C; CCR5, 100 pM [^{125}I]CCL4/MIP-1β in 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, and 1% DMSO, for 2 h at 25 °C; CX3CR1, 20 pM [^{125}I]CX3CL1/fractalkine in 50 mM Hepes, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, 0.1% NaN_3, and 1% DMSO, for 1.5 h at 25 °C; CXCR2, 15 pM [^{125}I]CXCL8/IL-8, 25 mM Hepes, pH 7.4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% BSA, and 1% DMSO, for 1 h at 25 °C; dopamine D_{2S}, 160 pM [^{3}H]spiperone, 50 mM Tris–HCl, pH 7.4, 1.4 mM ascorbic acid, 150 mM NaCl, 0.001% BSA, and 1% DMSO, for 2 h at 25 °C; muscarinic M_1, 800 pM [^{3}H]*N*-methyl scopolamine 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$, and 1% DMSO, for 2 h at 25 °C; serotonin (5-hydroxytryptamine) 5-HT_{1A}, 1.5 nM [^{3}H] 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin), 50 mM Tris–HCl, pH 7.4, 6 mM ascorbic acid, 0.5 mM EDTA, 10 mM $MgSO_4$, and 1% DMSO, for 1 h at 25 °C.

Chemotaxis assay. Chemotaxis assays were performed essentially as described in [12]. Human peripheral blood mononuclear cells (AllCells, Berkeley, CA) were cultured for 3 days with 10 μg/mL phytohemagglutinin, then for 4–7 days with 100 U/mL interleukin-2 (Peprotech, Rocky Hill, NJ). Cells were washed once and resuspended in assay buffer: Hanks, Buffered Saline Solution (Gibco, Carlsbad, CA), 10 mM Hepes, and 0.1% bovine serum albumin, pH 7.4. 500,000 cells per well were added in 100 μL to the upper chamber of a 96-well chemotaxis plate (Costar Transwell, 3 μM pore size; Corning, NY). Chemoattractants were added in 150 μL assay buffer with 0.1% DMSO to the lower chamber. After 1.5 h at 37 °C/CO_2, 100 μL containing migrated cells was transferred from the lower chamber to a white 96-well microtiter plate, with detection using CellTiter Glo (Promega, Madison, WI). Luminescence was measured for 5 s at fast speed and high gain in the ViewLux (Perkin-Elmer, Boston, MA).

Data analysis. Curve fitting was performed using nonlinear regression analysis (sigmoidal dose–response with variable slope) with GraphPad Prism® software (GraphPad Software, San Diego, CA).

Results

High-throughput compound screening for modulators of CXCR3

Over four million small molecules were evaluated in a dual screen designed to identify both agonists and antagonists at the human CXCR3 receptor. The screen measured calcium mobilization in the HEK293/CXCR3 G_{qi5} cell line in response to test compounds, as well as inhibition of the response to CXCL11 by test compounds. Cells were seeded in microtiter plate wells and cultured for 24–48 h, followed by loading with fluorescent calcium-sensitive dye. Fluorescence was monitored over time as test compounds were added to the cells in a FLIPR^{384} detector, to identify ago-

nists among the test compounds. After 30 min of incubation with test compounds, cells were challenged with a CXCR3 agonist (CXCL11) at a concentration giving 80% of the maximal calcium flux response, in order to detect antagonist activity among the test compounds. In the first phase of screening, 80 combinatorial libraries were surveyed at multiple (up to 20) compounds per well, at concentrations of 1–5 μM for each compound. Active libraries were identified and the most active sub-library (as defined by the final combinatorial component) in each library was selected for follow-up testing at a single compound per well. The structures of active compounds were identified and compounds were subsequently resynthesized on a multi-milligram scale to confirm activity.

The initial multiple-compound-per-well survey screen identified two libraries (#1 and #2) with agonist activity, but no antagonists were found in these two libraries (the identification of specific antagonists in other libraries is described in [12]). In order to identify individual compounds with agonist activity, library #1/sub-library #8 and library #2/sub-library #23 were rescreened at a single compound per well at a concentration of approximately 4 μM, in three library equivalents. As the distribution of compounds in the screening plates is random, the screening of multiple library equivalents maximizes the coverage of compounds within the screen. Theoretically, coverage of three library equivalents assures that >95% of the compounds are evaluated within the screen at least once, with a high probability that the active compounds will be detected more than once. In this way, it is possible to obtain a set of hit structures that are considered non-random [18]. Activity was reproduced in a screen at the single-compound-per-well level, indicating the presence of efficacious agonists in the library. Examination of the response to library #1 and #2 test compounds suggested that the increase in fluorescence resulted from activation of the CXCR3 receptor (or some other receptor) and not from test compound fluorescence, as both CXCL11 and the test compounds elicited a transient response with similar kinetics (Fig. 1). All of the other active compounds from agonist screening assays in libraries #1 and #2 exhibited a similar response with respect to time, although the amplitude of the signal varied. Similarly, a single-compound-per-well rescreen of library #2 reproduced activity observed in the initial screen. To test for specificity for CXCR3, putative agonists were tested for activity on null cell line HEK293/G_{qi5}E. Replicate compound arrays of the active sub-libraries were added to the HEK293/G_{qi5}E control cells but no calcium response occurred, suggesting that the compounds were specific agonists for the CXCR3 receptor; in contrast, cells in positive control wells responded robustly to 500 μM ATP acting at endogenous purinergic receptors (data not shown).

Library #1 is an ~30,000-membered library consisting of compounds with a tetrahydroisoquinoline core and three combinatorially varied substituents (R^1–R^3), but agonist activity was only detected in one of its twelve 2520-membered sub-libraries (#8, in which R^3 was a 4-oxo-4-phenylbutyramide; Fig. 2). Seven structures were identified multiple times in a screen of library #1/sub-library #8 at a single compound per well. Selected among a set of 16 possible naturally occurring and 47 other amino acids, the R^2 substituent in these structures was always a positively charged amino acid residue. Arginine was the most common (6/7) and less frequently lysine (1/7 structures). Furthermore, whereas (L) and (D) amino acids were both incorporated into the library, only (L) enantiomers were represented among these seven structures. At a screening concentration of approximately 4 μM, the compounds exhibited activities between 16 and 55% of the 40 nM CXCL11 control (Suppl. Table 1 online). At the R^1 position, structure-activity relationship was also apparent, with only 7 of 40 possible R^1 synthons eliciting putative agonist activity. Structures identified within the screen showed a preference for hydrophobic functionality, as exemplified by the cyclohexylmethyl and diphenylethyl moieties present in compounds **1** and **2** (Fig. 3). While agonist activity at a single test concentration permits an estimate of a minimum value for its efficacy at the receptor, this measurement does not distinguish the relative contributions of potency and efficacy. In order to address this question, two compounds from library #1, compounds **1** and **2**, were resynthesized on a larger scale (>10 mg; structures in Fig. 3). Compounds **1**

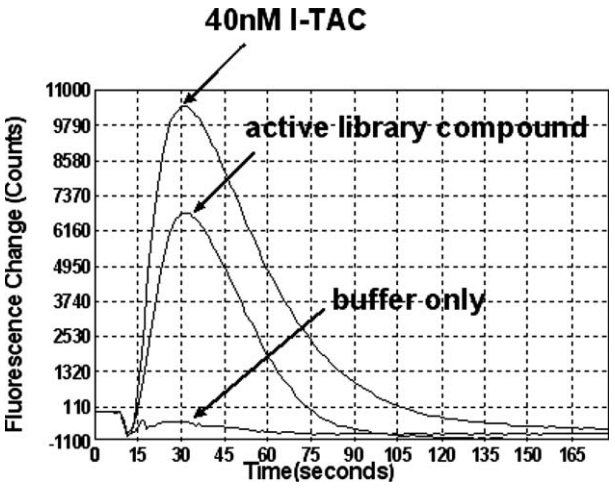

Fig. 1. Comparison of the calcium flux response triggered by a representative library #1 active compound to that of 40 nM CXCL11/I-TAC, or no agonist, in three wells of a 384-well FLIPR screening assay plate.

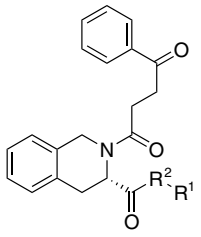

Fig. 2. Library #1/sub-library #8 compound structure. Variable positions include R^1 (40 possible synthons) and R^2 (63 possible synthons).

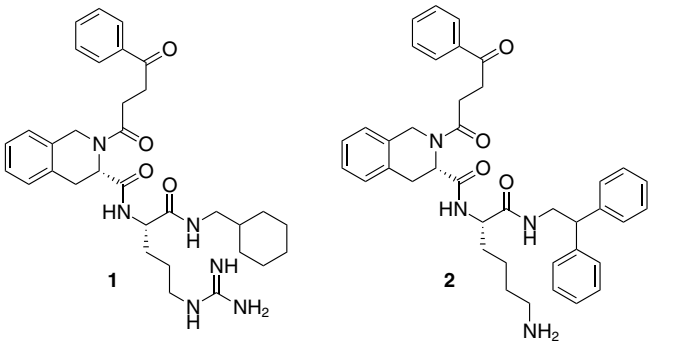

Fig. 3. Structures of compounds **1** and **2**, two agonists identified in library #1.

and **2** were tested for CXCR3 agonist activity at a range of concentrations from 500 pM to 30 μM. Both compounds showed efficacy approximately 20% greater than that of CXCL11, with potencies of 1.1 ± 0.2 μM ($n = 7$) and 3.3 ± 0.6 μM ($n = 13$), respectively (Fig. 4). The compounds elicited no response in the null cell line (Suppl. Fig. 1 online), indicating that the agonist response was dependent on the expression of CXCR3.

A more limited set of compounds were active in library #2. Library #2 is an ~80,000-membered library, consisting of fifty-one 1575-compound sub-libraries, based on a fused piperidinyl diazepanone structural motif and incorporating four combinatorially varied substituents (R^1–R^4). CXCR3 agonist activity was identified in only one of these sub-libraries (sub-library #23). The generic structure of sub-library #23 compounds (corresponding to R^4 functionalization as the *ortho*-methoxybenzyl tertiary amine) is shown in Fig. 5. Two compounds were identified

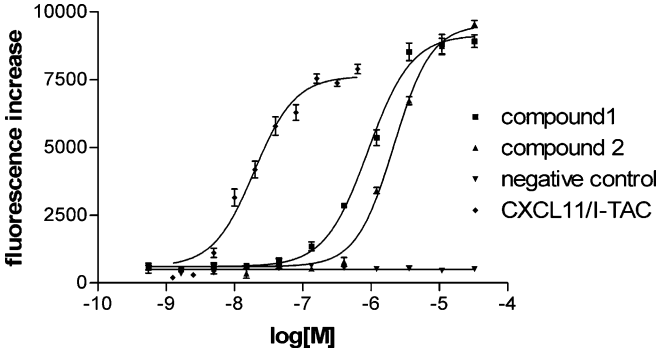

Fig. 4. Calcium flux induced by compound **1**, compound **2**, buffer alone (negative control), and CXCL11 in HEK293/CXCR3 G_{qi5} cells.

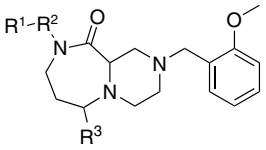

Fig. 5. Structure of library #2/sub-library #23 compounds, containing 15 possible R^1, 15 possible R^2, and 7 possible R^3 substituents.

multiple times when library #2/sub-library #23 was screened in three library equivalents at a concentration of 4 μM, with average activities of 33% and 10% relative to the positive control (CXCL11 at a sub-saturating concentration, 40 nM). Lysine (but not arginine) and several acidic or uncharged amino acids were present in this library. Both compounds from library #2 contained a (D)-Lys substituent at the R^2 position. Compound **3** was resynthesized on a multi-milligram scale for full dose–response analysis in the calcium flux assay (Fig. 6). Compound **3** exhibited an EC_{50} of 1.7 ± 0.6 μM ($n = 6$) and efficacy of approximately 100% that of CXCL11 (Fig. 7) but it had no agonist activity on the control null cell line (Suppl. Fig. 2 online).

Antagonism of small-molecule CXCR3 agonists by a small-molecule antagonist

We have previously described the identification of aryl-[1,4]diazepane ureas as CXCR3 antagonists [12]. Representative compounds from this class specifically inhibit CXCR3-mediated functional responses. Compound **4** (Suppl. Fig. 3 online) is a functional antagonist, with an IC_{50} value of 60 nM, of CXCL11 (at 40 nM) and a 20 nM antagonist of CXCL10 (at 100 nM) in calcium flux assays, inhibits binding of [^{125}I] CXCL10/IP-10 to the CXCR3 receptor, and has been shown to specifically antagonize CXCL11-induced T cell chemotaxis *in vitro* [12].

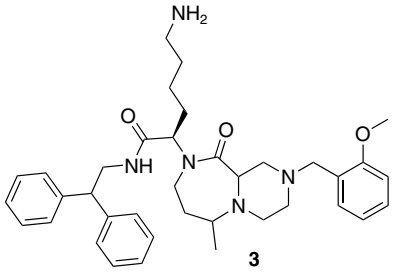

Fig. 6. CXCR3 agonist from library #2 (compound **3**). Activity in the CXCR3 calcium flux screen was 33 ± 7% of the response to 40 nM CXCL11.

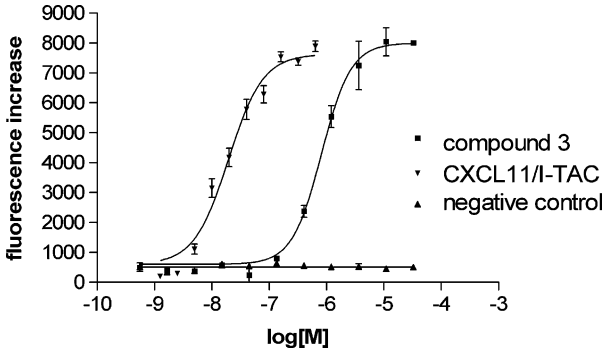

Fig. 7. Response of HEK293/CXCR3 G_{qi5} cells to library #2 compound **3**, CXCL11, or buffer alone (negative control) in calcium flux assay. EC_{50} values were 800 nM and 20 nM, respectively, for compound **3** and CXCL11.

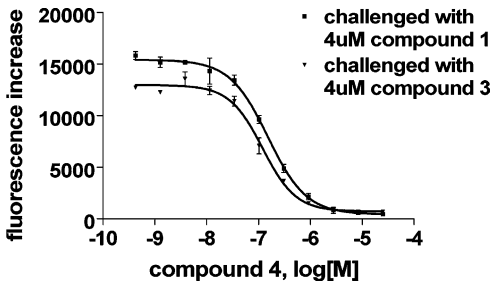

Fig. 8. Antagonism of 4 μM small-molecule CXCR3 agonists compounds **1** and **3** in the HEK293/CXCR3 G_{qi5} cell calcium flux assay by small-molecule antagonist compound **4**.

Compound **4** was inactive when tested in radioligand binding assays for a panel of fourteen GPCRs (with less than 50% inhibition of binding at a concentration of 10 μM [12]), suggesting that it binds specifically to CXCR3. We therefore asked whether this small molecule could inhibit calcium flux induced in HEK293/CXCR3 G_{qi5} cells by small-molecule agonists from libraries #1 and #2. After preincubation with the antagonist, agonist compound **1** (library #1) or compound **3** (library #2) was added to a final concentration of 4 μM (approximately EC_{70}). Fig. 8 shows that compound **4** was able to antagonize the response to both agonists with IC_{50} values of 140 nM and 110 nM, respectively, consistent with the antagonist potency of 60 nM observed when using 40 nM CXCL11 to activate the receptor [12].

Selective inhibition of binding to CXCR3

The two representative CXCR3 agonists identified in libraries #1 and #2 were also tested to determine whether they could inhibit the binding of CXCR3 ligand CXCL10 to the receptor. Compounds **1** and **3** competed for binding of radiolabeled CXCL10 to membranes prepared from HEK293/CXCR3 G_{qi5} cells, with IC_{50} values of 42 ± 21 nM and 65 ± 25 nM, respectively ($n = 2$; Fig. 9). These binding IC_{50} values were approximately 30-fold lower than the EC_{50} values measured for induction of calcium flux. A similar shift between potencies for binding and functional activity was observed for CXCL11; CXLC11 has been reported to bind the receptor with a high affinity binding component K_d of approximately 100–300 pM

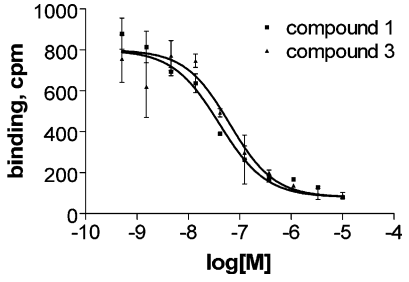

Fig. 9. Competition of [^{125}I] CXCL10 (25 pM) binding to HEK293/CXCR3 G_{qi5} cell membranes by compounds **1** and **3**.

[19,20] yet its EC_{50} in the calcium flux assay is 20 nM (Figs. 4 and 7). This may be due in part to the fact that the local concentration of agonist experienced by the cells in the functional assay depends on mixing conditions in the assay well, with the response occurring prior to establishment of equilibrium. More importantly, signaling is likely to depend on receptor expression levels and G protein coupling and thus to require a threshold level of ligand binding.

The specificity of compounds **1** and **3** was evaluated in radioligand binding assays for a panel of ten human GPCRs, including six chemokine receptors. No significant inhibition (i.e., greater than 50%) was observed for either compound at a test concentration of 10 μM against the adrenergic α_{2B}, dopamine D_{2S}, muscarinic M_1, serotonin 5-HT_{1A}, CCR1, CCR2B, CCR4, CCR5, CX3CR1, and CXCR2 receptors.

Stimulation of chemotaxis

While these small-molecule agonists induced calcium flux in a recombinant CXCR3 cell line expressing the chimeric G protein G_{qi5}, we sought to determine whether they could elicit a functional response in a more physiological assay. Compounds **1** and **3** were therefore evaluated for their ability to stimulate the chemotaxis of activated human T-cells *in vitro*. Interleukin-2 (IL-2)-treated T-cells isolated from two different blood donors migrated towards compound **1**, compound **3**, and CXCL11 (Fig. 10 and Suppl. Fig. 4 online). T-cells from the second blood donor were additionally evaluated for their migration toward CXCL10 and CCL21 (Suppl. Fig. 4 online). The chemotactic response to compounds **1** and **3** exhibited the bell-shaped concentration-response curve characteristic of chemotaxis to natural peptide chemoattractants, as observed for CXCL11 ([21], Fig. 10). T-cell chemotaxis to compounds **1** and **3** was inhibited by the small-molecule CXCR3 antagonist, compound **4**, as was migration to CXCL10 and CXCL11. In contrast, in the control experiment, migration induced by CCL21 via a different chemokine receptor, CCR7, was unaffected by the CXCR3-specific antagonist (Fig. 11).

Discussion

Our work highlights the importance of basic amino acids in CXCR3 activation, as does previously published work. An extensive mutational study of mouse CXCL10 was carried out in which 13 of the 16 basic amino acid residues were substituted with Ala, though Arg38 could not be successfully replaced and therefore was not tested [19], and NMR structures for CXCL10 [20] and CXL11 [21] have been determined. Both the mutation and NMR studies of CXL10 indicated that Arg8 may play a significant role in receptor activation. While CXCR3 ligands do not contain the Glu-Leu-Arg (ELR) sequence that is critical for agonist activity in a number of other CXC chemokines [22], Arg8 corresponds to the Arg in "ELR" in sequence alignments

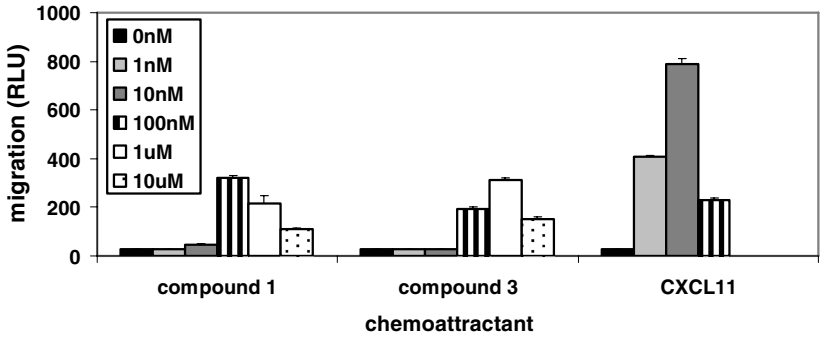

Fig. 10. Chemotaxis of activated human T-cells from one of two blood donors in response to small-molecule CXCR3 agonists (compounds **1** and **3**) and chemokine CXCL11. Small-molecule agonists were tested at concentrations of $\leqslant$10 μM and the chemokine CXCL11 at $\leqslant$100 nM.

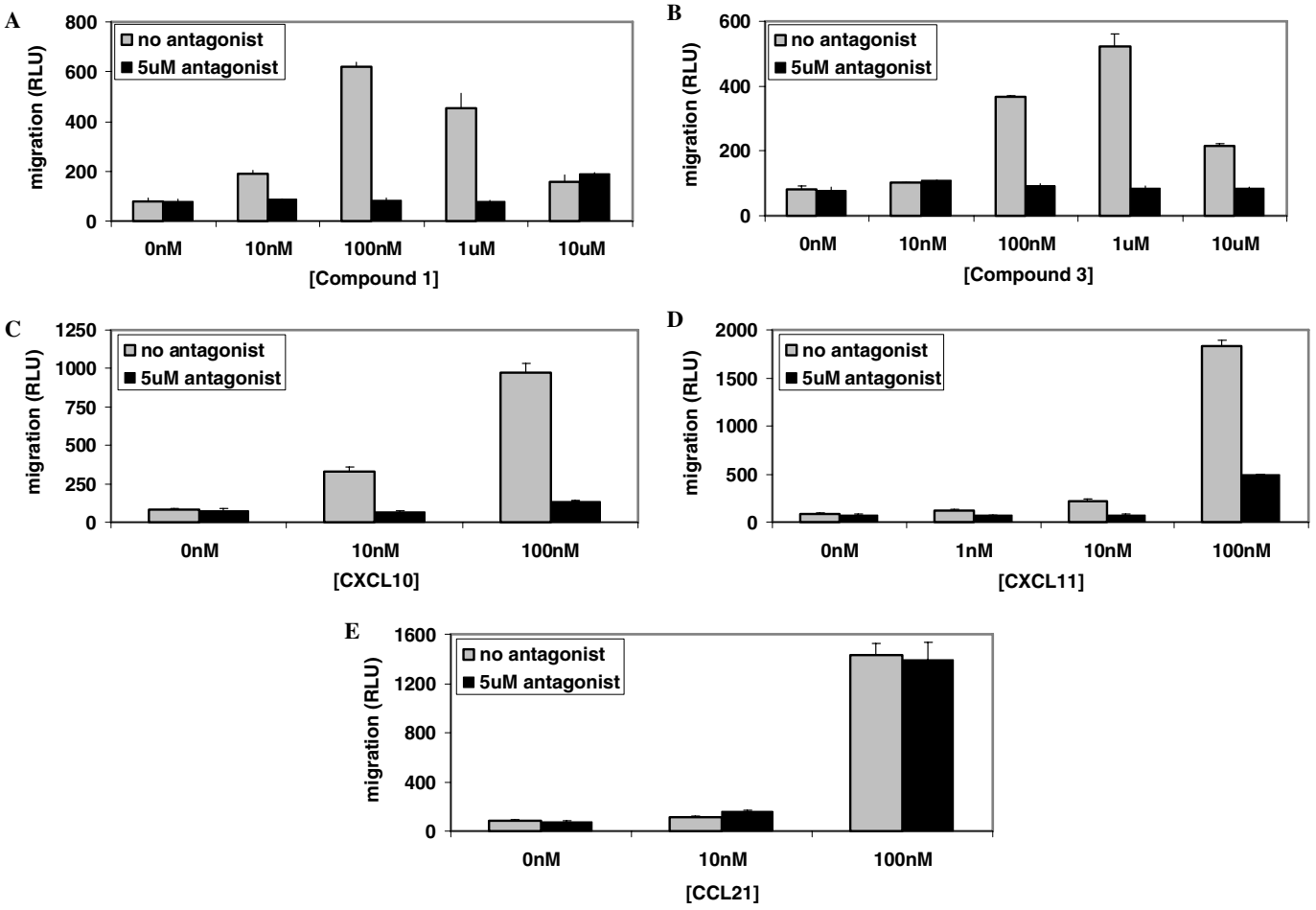

Fig. 11. Inhibition by small-molecule CXCR3 antagonist of small-molecule agonist- or chemokine-induced T-cell chemotaxis. Chemoattractants added to the lower chamber of the Transwell were: compound **1** (A); compound **3** (B); CXCL10 (C); CXCL11 (D); CCL21 (E). Cells were preincubated for 10 min with assay buffer or with antagonist compound **4** prior to addition to the upper Transwell chambers. Data for the five chemoattractants were collected from the same experiment but plotted separately.

of CXC chemokines [23] and is conserved in CXCR3 ligands. In the CXCL10 NMR structure, Arg38 is in very close proximity to Arg8, and both Arg residues experience conformational change upon receptor binding [20].

The small molecules described here are able to at least partially replace chemokines as agonists, albeit with lower potencies. It is likely that the two-step binding model for chemokine binding (in the "N-loop") and activation by the NH_2 terminus, reviewed in [24], does not have to apply in the case of small-molecule agonists, as long as the surrogate agonist can induce a conformational change in the receptor. Therefore, the small molecules may not mimic the N-termini of the chemokines and rather point out other important interactions between chemokine and receptor.

Conclusion

In a screen of four million compounds from a collection of diverse combinatorial libraries, we have identified novel small-molecule agonists of the human CXCR3 receptor from two different chemical classes, tetrahydroisoquinolines and fused piperidinyl diazepanones. These compounds were shown to specifically activate CXCR3 in functional assays (calcium mobilization and chemotaxis), to inhibit binding of CXCR3 ligand CXCL10/IP-10 to the receptor *in vitro*, and to be antagonized by a specific small-molecule antagonist. These compounds can activate the receptor with full efficacy in the calcium flux assay. The fact that the small molecules function as agonists not only on a recombinant cell line but in primary cells, and that their chemotactic activity can be inhibited by a compound that has been demonstrated to specifically antagonize CXCR3 [12], suggests that these compounds are true agonists of this receptor. Regardless of the scaffold, and while the majority of the compounds screened were non-peptidic, the preferred chemotype acting as a functional agonist contains a single basic amino acid, as well as a hydrophobic substituent. Additional studies will be required to determine the precise mechanisms by which these two classes of small molecules activate CXCR3.

Acknowledgments

We thank Christopher Haskell (Berlex Biosciences) and Bojing Wang (Pharmacopeia Drug Discovery) for technical advice, and Anthony Klon and David Diller (Pharmacopeia Drug Discovery) for comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.019.

References

[1] C.-H. Jenh, M.A. Cox, W. Hipkin, T. Lu, C. Pugliese-Sivo, W. Gonsiorek, C.-C. Chou, S.K. Narula, P.J. Zavodny, Human B cell-attracting chemokine 1 (BCA-1; CXCL13) is an agonist for the human CXCR3 receptor, Cytokine 15 (2001) 113–121.

[2] F. Annunziato, G. Galli, L. Cosmi, P. Romagnani, R. Manetti, E. Maggi, S. Romagnani, Molecules associated with human Th1 or Th2 cells, Eur. Cytokine Netw. 9 (suppl. 3) (1998) 12–16.

[3] L. Waeckel, Z. Mallat, S. Potteaux, C. Combadiere, M. Clergue, M. Duriez, L. Bao, C. Gerard, B.J. Rollins, A. Tedgui, B.I. Levy, J.-S. Silvestre, Impairment in postischemic neovascularization in mice lacking the CXC chemokine receptor 3, Circ. Res. 96 (2005) 576–582.

[4] A.D. Luster, S.M. Greenberg, P. Leder, The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation, J. Exp. Med. 182 (1995) 219–231.

[5] R.M. Strieter, P.J. Polverini, S.L. Kunkel, D.A. Arenberg, M.D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, S.-Y. Chan, S. Roczniak, A.B. Shanafelt, The functional role of the ELR motif in CXC chemokine-mediated angiogenesis, J. Biol. Chem. 270 (1995) 27348–27357.

[6] D.A. Arenberg, P.J. Polverini, S.L. Kunkel, A. Shanafelt, J. Hesselgesser, R. Horuk, R. M Strieter, The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer, J. Leukoc. Biol. 62 (1997) 554–562.

[7] M.M. Rosenkilde, T.W. Schwartz, The chemokine system—a major regulator of angiogenesis in health and disease, APMIS 112 (2004) 481–495.

[8] P. Romagnani, F. Annunziato, L. Lasagni, E. Lazzeri, C. Beltrame, M. Francalanci, M. Uguccioni, G. Galli, L. Cosmi, L. Maurenzig, M. Baggiolini, E. Maggi, S. Romagnani, M. Serio, Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity, J. Clin. Invest. 107 (2001) 53–63.

[9] L. Lasagni, M. Francalanci, F. Annunziato, E. Lazzeri, S. Giannini, L. Cosmi, C. Sagrinati, B. Mazzinghi, C. Orlando, E. Maggi, F. Marra, S. Romagnani, M. Serio, P. Romagnani, An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as a functional receptor for platelet factor 4, J. Exp. Med. 197 (2003) 1537–1549.

[10] M.H. Ohlmeyer, R.N. Swanson, L.W. Dillard, J.C. Reader, G. Asouline, R. Kobayashi, M. Wigler, W.C. Still, Complex synthetic chemical libraries indexed with molecular tags, Proc. Natl. Acad. Sci. USA 90 (1993) 10922–10926.

[11] H.P. Nestler, P.A. Bartlett, W.C. Still, A general method for molecular tagging of encoded combinatorial chemistry libraries, J. Org. Chem. 59 (1994) 4723–4724.

[12] A.G. Cole, I.L. Stroke, M.-R. Brescia, S. Simhadri, J.J. Zhang, Z. Hussain, M. Snider, C. Haskell, S. Ribeiro, K.C. Appell, I. Henderson, M.L. Webb, Identification and initial evaluation of 4-*N*-aryl-[1,4]diazepane ureas as potent CXCR3 antagonists, Bioorg. Med. Chem. Lett. 16 (2006) 200–203.

[13] C.E. Heise, A. Pahuja, S.C. Hudson, M.S. Mistry, A.L. Putnam, M.M. Gross, P.A. Gottlieb, W.S. Wade, Pharmacological characterization of CXC chemokine receptor 3 ligands and a small-molecule antagonist, J. Pharmacol. Exp. Ther. 313 (2005) 1263–1271.

[14] J.G. Ondeyka, K.B. Herath, H. Jayasuriya, J.D. Polishook, G.F. Bills, A.W. Dombrowski, M. Mojena, G. Koch, J. DiSalvo, J. DeMartino, Z. Guan, W. Nanakorn, C.M. Morenberg, M.J. Balick, D.W. Stevenson, M. Slatter, R.P. Borris, S.B. Singh, Discovery of structurally diverse natural product antagonists of chemokine receptor CXCR3, Mol. Divers. 9 (2005) 123–129.

[15] S. Storelli, P. Verdijk, D. Verzijl, H. Timmerman, A.C. van de Stolpe, C.P. Tensen, M.J. Smit, I.J. De Esch, R. Leurs, Synthesis and structure-activity relationship of 3-phenyl-3H-quinazolin-4-one derivatives as CXCR3 chemokine receptor antagonists, Bioorg. Med. Chem. Lett. 15 (2005) 2910–2913.

[16] C.A. Haskell, R. Horuk, M. Liang, M. Rosser, L. Dunning, I. Islam, L. Kremer, J. Gutierrez, G. Marquez, C. Martinez-A, M.J. Biscone, R.W. Doms, S. Ribeiro, Identification and characterization of a potent, selective nonpeptide agonist of the CC chemokine receptor CCR8, Mol. Pharmacol. 69 (2006) 309–316.

[17] R.E. Dolle, J. Guo, W. Li, N. Zhao, J.A. Connelly, Analysis of libraries encoded with GC tags: compound elution, tag decode analysis, and statistical sampling analysis, Mol. Divers. 5 (2000) 35–49.

[18] D.J. Diller, D.W. Hobbs, Deriving knowledge through data mining high-throughput screening data, J. Med. Chem. 47 (2004) 6373–6383.

[19] G.S.V. Campanella, E.M.J. Lee, J. Sun, A.D. Luster, CXCR3 and heparin binding sites of the chemokine IP-10 (CXCL10), J. Biol. Chem. 278 (2003) 17066–17074.

[20] V. Booth, D.W. Keizer, M.B. Kamphuis, I. Clark-Lewis, B.D. Sykes, The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions, Biochemistry 41 (2002) 10418–10425.

[21] V. Booth, I. Clark-Lewis, B.D. Sykes, NMR structure of CXCR3 binding chemokine CXCL11 (ITAC), Protein Sci. 13 (2004) 2022–2028.

[22] I. Clark-Lewis, C. Schumacher, M. Baggiolini, B. Moser, Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH_2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities, J. Biol. Chem. 266 (1991) 23128–23134.
[23] K.E. Cole, C.A. Strick, T.J. Paradis, K.T. Ogborne, M. Loetscher, R.P. Gladue, W. Lin, J.G. Boyd, B. Moser, D.E. Wood, B.G. Sagahan, K. Neote, Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3, J. Exp. Med. 187 (1998) 2009–2021.
[24] P. Loetscher, I. Clark-Lewis, Agonistic and antagonistic activities of chemokines, J. Leukoc. Biol. 69 (2000) 881–884.