Small Molecule Receptor Agonists and Antagonists of CCR3 Provide Insight into Mechanisms of Chemokine Receptor Activation*

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Emma L. Wise, Cécile Duchesnes, Paula C. A. da Fonseca, Rodger A. Allen, Timothy J. Williams, and James E. Pease

From the Leukocyte Biology Section, National Heart and Lung Institute Division, Faculty of Medicine, Sir Alexander Fleming Building, Imperial College London, South Kensington Campus, London SW7 2AZ,

Structural Electron Microscopy Team, Section of Structural Biology, The Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, and UCB-Celltech, Slough SL1 3WE, United Kingdom

Chemokine receptor CCR3 is highly expressed by eosinophils and signals in response to binding of the eotaxin family of chemokines, which are up-regulated in allergic disorders. Consequently, CCR3 blockade is of interest as a possible therapeutic approach for the treatment of allergic disease. We have described previously a bispecific antagonist of CCR1 and CCR3 named UCB35625 that was proposed to interact with the transmembrane residues Tyr-41, Tyr-113, and Glu-287 of CCR1, all of which are conserved in CCR3. Here, we show that cells expressing the CCR3 constructs Y113A and E287Q are insensitive to antagonism by UCB35625 and also exhibit impaired chemoattraxis in response to CCL11/eotaxin, suggesting that these residues are important for antagonist binding and also receptor activation. Furthermore, mutation of the residue Tyr-113 to alanine was found to turn the antagonist UCB35625 into a CCR3 agonist. Screens of small molecule libraries identified a novel specific agonist of CCR3 named CH0076989. This was able to activate eosinophils and transfectants expressing both wild-type CCR3 and a CCR1–CCR3 chimeric receptor lacking the CCR3 amino terminus, indicating that this region of CCR3 is not required for CH0076989 binding. A direct interaction with the transmembrane helices of CCR3 was supported by mutation of the residues Tyr-41, Tyr-113, and Glu-287 that resulted in complete loss of CH0076989 activity, suggesting that the compound mimics activation by CCL11. We conclude that both agonists and antagonists of CCR3 appear to overlap sites within the transmembrane helical bundle, suggesting a fine line between agonism and antagonism of chemokine receptors.

Release of other mediators such as leukotriene C4 and transforming growth factor-β can induce other responses such as bronchoconstriction, mucus hypersecretion (3), and airway remodeling (4).

Chemokines are a family of low molecular weight proteins that are key to the regulation of leukocyte migration, exerting their activity via G protein-coupled receptors expressed on the cell surface (5). While chemokines are associated with homeostatic cell migration and host defense, inadvertent or excessive production of chemokines has been implicated in the inflammatory components of many clinically important diseases, including asthma (6). A chemokine central to the pathology of asthma is the CC chemokine CCL11/eotaxin (7). This chemokine interacts with the receptor CCR3 (8–10), the principal chemokine receptor expressed by the eosinophil (8–10) but also by other cells involved in the allergic response, such as Th2 cells (11) and basophil (12) and mast cells (13). Activation of CCR3 by CCL11 occurs via a two-step model (14) involving tethering of the ligand by the receptor amino terminus and subsequent delivery to the extracellular loops (15, 16). This is postulated to lead to conformational changes in the receptor, resulting in G protein recruitment and activation of intracellular signaling. Data from studies of activation of the related receptor CCR5 suggest that the amino terminus of the chemokine disrupts interactions between the side chains of neighboring transmembrane helices, leading to the induction of the active receptor conformation (17, 18). In contrast, the mechanism of CCR3 activation by CCL11 remains poorly understood despite effort from several groups (19–24).

We have previously described the effects of a bispecific CCR1–CCR3 antagonist, UCB35625 (25), that interacts with the residues Tyr-41, Tyr-113, and Glu-287 of CCR1 (26). A homology model of the structure of the CCR1 transmembrane region, calculated using the structure of the related receptor rhodopsin as a template (27), suggests these residues make up an intrahelical bundle in which the antagonist sits. Here we show that within CCR3 the conserved residues Tyr-113 and Glu-287 are important for the antagonist activity of UCB35625 and also for the agonist activity of CCL11. We also describe a novel small molecule agonist of CCR3, CH0076989, which binds to a site within the receptor overlapping that of the antagonist.

Asthma is characterized by the accumulation within the bronchial wall of leukocytes, of which the eosinophil predominates (1). Once recruited, eosinophils release a variety of mediators implicated in asthma pathology. Some of these, such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase, can directly induce tissue damage (2).

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1 To whom correspondence should be addressed. Tel.: 44-20-7594-3162; Fax: 44-20-7594-3119; E-mail: j.pease@imperial.ac.uk.
EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma-Aldrich and Invitrogen unless stated otherwise. Recombinant human CCL11 was purchased from PeproTech EC Ltd. (London, UK). CH0076989 was provided by UCB-Celltech (Slough, UK). The monoclonal mouse anti-hemagglutinin (HA)2 antibody was purchased from Covance Research Products (Berkley, CA), and its corresponding IgG1 isotype control antibody was from Sigma-Aldrich. The rat monoclonal anti-CCR3 was from R & D Systems and its corresponding IgG2A isotype from BD Biosciences. Fluorescein isothiocyanate-conjugated goat anti-mouse antibody and the rhodamine phycoerythrin-conjugated goat anti-rat IgG were purchased from DAKO Cytomation (Ely, UK) and AbD Serotec (Kidlington, UK), respectively.

Generation of HA-CCR3 Point Mutants—A CCR3 construct tagged at the amino terminus with the HA epitope was used as a template for mutagenesis. Point mutants of human HA-CCR3 were generated by PCR according to the manufacturer’s instructions, using the QuikChange™ site-directed mutagenesis kit (Stratagene). Verification of mutation was performed by DNA sequencing on both strands (MWG Biotech, Ebersberg, Germany).

Cell Culture and Transfection—The murine pre-B lymphoma cell line L1.2 was maintained in suspension in HEPES-modified RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 1× minimal essential medium non-essential amino acids at 37 °C with 5% CO2 at a density of no more than 1×106 cells/ml. The previously described 4DE4 cell line stably transfected with CCR3 (15) was cultured in the aforementioned medium supplemented with 1 mg/ml G-418. L1.2 cells were transiently transfected by electroporation with 1 μg of DNA/1×106 cells as described previously (23) and were incubated overnight in the presence of 10 mM sodium butyrate to increase responsiveness.

Flow Cytometry—Approximately 5×105 cells were harvested, washed once with fluorescence-activated cell sorting (FACS) buffer (0.25% bovine serum albumin and 0.01% NaN3 in HEPES-modified phosphate-buffered saline), and incubated with 50 μg/ml anti-HA/anti-CCR3 antibody or the appropriate isotype control in 100 μl of ice-cold FACS buffer for 20 min. Cells were then washed with FACS buffer and incubated with 50 μg/ml of the corresponding secondary antibody for 20 min in 50 μl of ice-cold FACS buffer after 20 min. Cells were washed twice and resuspended in 400 μl of FACS buffer before being analyzed by flow cytometry as previously described (26).

Chemotaxis Assay—The chemotactic responsiveness of cells was ascertained using ChemoTx™ plates (NeuroProbe, Gaithersburg, MD) as described previously (26). Using an assay buffer of RPMI supplemented with 0.1% bovine serum albumin, duplicate concentrations of agonist in the presence or absence of UCB35625 were applied in a final volume of 31 μl to the lower wells of the chemotaxis chamber. The filter was put in place and 2×105 cells in the same assay buffer were applied to the upper surface. Following a 5-h incubation in a humidified chamber at 37 °C in the presence of 5% CO2, the number of migrating cells traversing a 5-μm pore filter were counted using a hemocytometer. Data are shown as the percentage of migrating cells.

Internalization Assay—Chemokine receptor internalization was assessed by the loss of cell surface receptor expression as determined by flow cytometry using an antibody directed against CCR3 (28). 1×106 cells were resuspended in duplicate in 100 μl of ice-cold culture medium with an additional tube serving as a buffer control. One set of cells was incubated at 37 °C for 20 min prior to the addition of appropriate stimuli while the duplicates remained on ice. Either 100 nM CCL11, 10 μM CH0076989, or 10 μM UCB35625 were added to tubes incubating at 37 °C or their controls on ice. All samples were then incubated for 20 min at either 37 °C or on ice before washing, staining, and receptor expression analysis by flow cytometry. The remaining receptor expression was calculated as the mean fluorescence of antibody-stained chemokine cells minus mean fluorescence of isotype-stained chemokine-treated cells. This was expressed as a percentage of mean fluorescence of buffer-treated cells.

Gated Autofluorescence Forward Scatter (GAFS) Assay—GAFS assays were performed as described previously (29). Blood was taken from healthy donors according to a protocol approved by the St. Mary’s Hospital Ethics Committee, and granulocytes (comprising neutrophils and eosinophils) were isolated over discontinuous platelet-depleted plasma/Percoll gradients (30). Granulocytes were preincubated at a concentration of 5×106 cells/ml for 30 min at room temperature in filtered GAFS buffer (phosphate-buffered saline containing 0.9 mM CaCl2, 0.5 mM MgCl2, 10 mM HEPES, 10 mM glucose, and 0.1% bovine serum albumin). The cells were washed and then incubated in GAFS buffer with varying concentrations of CH0076989 for 4 min at 37 °C in a shaking water bath. Cells were then placed on ice and 250 μl of ice-cold fixative added. Finally, the cells were analyzed on a FACScaliber (BD Biosciences) as described previously (29).

Homology Modeling—Generation of a CCR3 model structure and docking of antagonists and agonists were carried out as previously described (26), using our earlier model of CCR1 as a template.

Data and Statistical Analysis—Data are expressed as the mean ± S.E. of at least three separate experiments and were analyzed with a relevant statistical test, where stated, using PRISM v4.03 software (GraphPad, San Diego, CA).

RESULTS

Identification of the Binding Pocket within CCR3 for UCB35625—We have previously described the bispecific CCR1–CCR3 antagonist UCB35625 (Fig. 1A), which antagonizes responses mediated by either CCR1 and CCR3, with the compound an order of magnitude more potent at CCR1 (25). The mode of action of this compound at either receptor is intriguing. While the compound is unable to effectively displace chemokines from either receptor (as deduced by radiolabeled binding studies), in assays of eosinophil shape change the

2 The abbreviations used are: HA, hemagglutinin; GAFS, gated autofluorescence/forward scatter; TM, transmembrane; WT, wild type; FACS, fluorescence-activated cell sorting.
compound induces profiles of insurmountable antagonism (reduced maximal response) from CCR1-expressing cells and profiles of surmountable antagonism (right-shifted response) from CCR3-expressing cells (25). Using L1.2 CCR3 transfec-
tants, chemotactic responses to increasing concentrations of CCL11 were assessed in the presence or absence of 500 nM UCB35625 (Fig. 1B). In keeping with earlier reported GAFS data (25) antagonist was surmountable, with high concentra-
tions of CCL11 able to induce migration albeit with reduced efficacy. This is indicative of a competitive element to the antagonist activity of UCB35625 at CCR3.

A previous study from our group determined the binding site of UCB35625 within CCR1, with mutation of the transmembrane (TM) amino acids Tyr-41 (TM1), Tyr-113 (TM3), and Glu-287 (TM7) leading to resistance to UCB35625 (26).

Because CCR3 and CCR1 exhibit a high level of homology at the amino acid level and all three residues are conserved between both receptors, we postulated that the compound might interact with the same residues within CCR3. HA-tagged Y41A, Y113A, and E287Q mutant CCR3 constructs were generated via site-directed mutagenesis. These were transiently trans-
fected into L1.2 cells along with wild-type (WT) HA-CCR3 and surface receptor expression measured by flow cytometry using an anti-HA antibody (Fig. 2A). All three mutants were expressed at the cell surface, although the Y41A mutant appeared to be expressed at slightly reduced levels compared with WT CCR3. The same transfecteds were subsequently examined in a chemotaxis assay in response to increasing concen-
trations of CCL11 in the presence or absence of 500 nM UCB35625 (Fig. 2, B–E). In contrast to our previous findings with CCR1, mutation of Tyr-41 to alanine in CCR3 did not lead to resistance to blockade by UCB35625, with cells expressing this construct responding to CCL11 with an identical profile to cells expressing the WT receptor (Fig. 2, C and B, respectively), albeit with a reduced maximum. This correlates with the apparent reduced expression of the Y41A construct. However, mutagenesis of both Tyr-113 and Glu-287 yielded CCR3 con-
structs resistant to antagonism of the CCL11 response by UCB35625 (Fig. 2, D and E). Also of interest was the reduced potency of CCL11 for cells expressing the Y113A and E287Q constructs, with little activity observed at the 1 nM concentra-
tion of CCL11 compared with cells expressing WT CCR3. This suggests that Tyr-113 and Glu-287 are involved in both antag-
onist binding and receptor activation by the agonist CCL11. On closer inspection of the response of Y113A transfectants (Fig. 2D), we identified an apparent increase in basal chemotaxis in the absence of CCL11 but in the presence of 500 nM UCB35625, suggesting that the compound might possess agonist activity at this mutated receptor. This was subsequently verified by assess-
ment of the chemotaxis of Y113A and WT CCR3 transfectants to increasing concentrations of the antagonist (Fig. 2F). While UCB35625 was not chemotactic for the WT CCR3 transfect-
tants, Y113A transfectants responded with a typical bell-
shaped profile, although with significantly lower efficacy com-
pared with the natural ligand CCL11. Collectively, these data suggest that Tyr-113 and Glu-287 are involved in both antag-
onist binding and receptor activation by agonist and that muta-
tion of Tyr-113 to alanine converts UCB35625 from an antag-
onist of CCR3 to a partial agonist of the receptor.

The Small Molecule CH0076989 Is an Agonist of CCR3—A subsequent screen of a small molecule library identified a com-
 Pound, CH0076989 (Fig. 3A), that to our surprise raised the base line of CCL11 responses in the GAFS assay (data not shown). We subsequently investigated whether CH0076989 had agonist activity independent of CCL11. Eosinophils iso-
 lated from four different donors were found to respond in a dose-dependent manner to the compound (Fig. 3B), with opti-
mal activity at 1 μM CH0076989, suggesting that the compound was indeed an agonist at an unidentified receptor. Because eosinophils express CCR3 but can also express CCR1 (29, 31), we hypothesized that one of these receptors might be mediating the response to CH0076989. Stable transfecteds expressing CCR3 or CCR1 were used in chemotaxis assays and the
responses to increasing amounts of CH0076989 determined (Fig. 4A). Although CCR1 transfectants were unresponsive to the compound, CCR3 transfectants migrated in a concentration-dependent fashion to CH0076989 with optimal chemotaxis observed at 1 μM CH0076989 (Fig. 5A). Upon incubation with a concentration of ligand in excess of that required for optimal chemotaxis, chemokine receptors typically undergo endocytosis or internalization. Because the small molecule showed activity at CCR3 in GAFS and chemotaxis assays, we assessed whether or not the compound could modulate receptor internalization as assayed by flow cytometry using CCR3-specific antibodies. Cell surface expression of CCR3 was seen to be reduced following treatment of L1.2 CCR3 transfectants with 100 nM CCL11 for 20 min at 37 °C (Fig. 5B) in agreement with previous findings (28). A 10-μM concentration of CH0076989 was also seen to induce a loss of CCR3 at the cell surface. In contrast to the reduced efficacy of the compound in chemotaxis assays, CH0076989 reduced surface receptor expression levels to those observed with CCL11 (59.4 and 59.6% of original expression, respectively). Surface expression was not significantly reduced following incubation with 10 μM of the previously characterized CCR1–CCR3 bispecific antagonist UCB35625 (25) suggesting that agonist activity at the receptor, and not simply the ability to bind to CCR3, is needed to induce receptor endocytosis.

We have previously shown that in order to activate CCR3 CCL11 has to bind to the amino terminus and extracellular regions of the receptor (15, 24). Indeed, CCR1–CCR3 chimeric constructs in which the amino termini of both receptors are exchanged are unable to efficiently activate G proteins, despite being able to bind ligand (15). Because CH0076989 is several orders of magnitude smaller than CCL11, we hypothesized that it may activate CCR3 by binding to different receptor determinants than the natural ligand CCL11. To test this hypothesis, a chimeric receptor (Chi-1) containing amino acids 1–32 from CCR1 fused to amino acids 33–355 of CCR3 (15) was employed in chemotaxis assays (Fig. 6A). Cells transiently expressing either the chimeric construct Chi-1 or wild-type CCR3 were assessed for their chemotactic responses to increasing concentrations of CH0076989 or a
fixed concentration of 10 nM CCL11 (Fig. 6, B and C). CCR3 transfectants responded robustly to CCL11 and CH0076989 as seen before (Fig. 5B). Cells expressing Chi-1 transfectants were able to respond to CH0076989 in a manner identical to CCR3 transfectants but showed markedly reduced responsiveness to CCL11 as previously described (15), suggesting that although the amino terminus of CCR3 is required for effective CCL11 activity, it is dispensable for functional responses to CH0076989.

**CH0076989 Interacts with a Binding Pocket within CCR3 That Overlaps with That of UCB35625**—Because CH0076989 is predominantly hydrophobic and does not appear to interact with the amino terminus of CCR3, we hypothesized that, like UCB35625, CH0076989 might bind to CCR3 within a pocket located between the TM helices. We therefore examined the three mutants of CCR3 that we had generated in our analysis of UCB35625 function, namely Y41A, Y113A, and E287Q. Whereas transfectants expressing WT CCR3 were able to migrate in response to CH0076989, to our surprise, chemotaxis to CH0076989 was ablated in cells expressing either of the three point mutants (Fig. 7A). As a control, the same transfectants were simultaneously assessed for their ability to respond to a fixed concentration of 10 nM CCL11 (Fig. 7B). Responses of the same transfectants to CCL11 remained intact, as seen previously (Fig. 2, B–D), suggesting that the lack of efficacy of CH0076989 at the three mutant constructs was specifically related to the inability of the small molecule to activate the receptor and not simply due to an impaired ability of the transfectants to undergo chemotaxis. Because the CCR3 residues Tyr-113 and Glu-287 also line the binding pocket for the antagonist UCB35625, we hypothesized that the sites for both antagonist and agonist binding might overlap. If this is the case then UCB35625 should be an antagonist of CH0076989-induced responses. We therefore assessed chemotactic responses of L1.2 CCR3 transfectants toward a fixed 1 μM concentration of CH0076989 in the presence of increasing amounts of UCB35625 (Fig. 7C). In agreement with our hypothesis, UCB35625 was observed to readily inhibit the chemotactic
activity of CH0076989, with equimolar concentrations of the antagonist greatly reducing cell migration. Thus, despite having diametrically opposed activities at CCR3, the compounds UCB35625 and CH0076989 appear to utilize an overlapping binding site within the transmembrane intrahelical bundle of CCR3.

**DISCUSSION**

We describe here a small molecule named CH0076989 that is an agonist of the chemokine receptor CCR3 and appears to activate the receptor by binding to a site also used by a well characterized receptor antagonist, UCB35625 (25). Chemokines are thought to activate their receptors via a two-step model involving an initial tethering of the chemokine by the amino terminus of the chemokine receptor followed by interactions of the chemokine with the receptor extracellular loops. In the case of the related receptor CCR5, this has been postulated to facilitate the interaction of the chemokine amino terminus with side chains of residues located within the transmembrane helices, notably residues within helices 2 and 3 (17, 18). An earlier study from our group using chimeric CCR1–CCR3 constructs supports the notion that both CCR1 and CCR3 follow the two-step model of receptor activation, as exchange of their respective amino termini resulted in constructs with a considerably reduced affinity for ligand and ability to induce receptor activation (15). This model is also supported by NMR data describing interactions in solution between an amino-terminal peptide of CCR3 and CCL11 (19). Our present results indicate that the molecule CH0076989 bypasses such restraints and utilizes the transmembrane resi-
dues Tyr-41, Tyr-113, and Glu-287 of CCR3 to interact with the receptor, leading to activation. Fig. 8A shows a schematic representation of CCR3 based on our previous model of the highly homologous CCR1 (26), with Tyr-41 of TM1, Tyr-113 of TM3, and Glu-287 of TM7 highlighted. A possible docking of CH0076989 to these residues is shown in Fig. 8B. Here the acidic side chain of Glu-287 is postulated to form a salt bridge with the positively charged quaternary nitrogen of the agonist, while the hydroxyl groups of Tyr-41 and Tyr-113 hydrogen bond to oxygen moieties within the ring structure (Fig. 8C).

In keeping with the importance of Tyr-113 and Glu-287 in the activation of CCR3, Y113A and E287Q transfectants exhibited reduced potency in chemotactic responses to CCL11 when compared with those of wild type-expressing cells. Relating this data to the two-step model for CCR3 activation (15), we therefore postulate that CH0076989 mimics the interactions of the CCL11 amino terminus with Tyr-113 and Glu287 and induces receptor activation. Such a model would also be in keeping with other class A G protein-coupled receptors such as rhodopsin and the β2 adrenergic receptor, where movement of both TM3 and TM7 has been reported to occur during the activation process (32, 33). The solution structure of CCL11 has been previously solved by NMR (34), and backbone dynamics calculations have shown the amino-terminal region of CCL11 to be highly flexible upstream of the constraining CC disulfide bridge with little interaction with the core of the chemokine (35, 36). Using this NMR structure (PDB accession number 1EOT), we subsequently docked the amino terminus of CCL11 within our model of CCR3 (Fig. 8, D and E). In this scenario, the hydroxyl group of Tyr-113 is postulated to hydrogen bond with the hydroxyl group of the S4 residue of CCL11 and Glu-287 hydrogen bond with the backbone oxygen of the Pro-2 residue of CCL11. Although these interactions are at present speculative, such a model fits previous experimental data generated by other groups. First, that removal of the first two amino acids of CCL11 by CD26/DPPIV severely reduces its biological activity (37), and, second, mutation of Ser-4 of CCL11 to alanine results in a loss of both binding affinity and biological activity (20).

Tyr-113 and Glu-287 of CCR3 are also contact points for the small molecule antagonist UCB35625, which has activity at both CCR3 and its close relative CCR1 (26). We have previously shown that Tyr-113 and Glu-287, together with Tyr-41, are critical for the activity of UCB35625 at CCR1 (26). Although it is not surprising that the antagonist interacts with a similar pocket in both receptors, it is curious that the same residues should be implicated in receptor activation by both the natural ligand CCL11 and the small molecule CH0076989. This may help explain the surmountable (right-shifted) profiles of antagonism obtained with UCB35625 following activation of CCR3 by CCL11 both here and in a previous study (25).

Homology modeling of CCR3 produced a helical bundle of very similar structure to that of CCR1, which is unsurprising as the receptors share considerable identity in these regions and the homology model of CCR3 was obtained using the previous presented model of CCR1 as template (25, 38). Although allowing the docking of agonists and antagonists to CCR3, such a model does not help to explain the different activities we observed at each receptor. For example, Tyr-113 and Glu-287

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**FIGURE 7.** The CCR3 binding pocket of the small molecule agonist CH0076789 overlaps that of the antagonist UCB35625. Panel A shows the chemotactic responses to CH0076789 of L1.2 cells transiently expressing CCR3 and the mutants Y41A, Y113A, and E287Q. Panel B shows the chemotactic responses of the same transfectants to a fixed concentration of 10 nM CCL11. Panel C shows dose-dependent inhibition of the chemotactic response of CCR3 transfectants to 1 μM CH0076789 by increasing concentrations of UCB35625. Basal migration to buffer alone was subtracted. Data are represented as the mean ± S.E. from three separate experiments.
are conserved in both CCR1 and CCR3, yet CH0076989 only had activity at CCR3. High resolution structural information will be required to definitively answer these points. In its absence, recent developments in the ab initio modeling of chemokine receptors show much promise (39) and may help the refinement of our current models, highlighting structural differences between such closely related proteins.

Our finding that antagonists and agonists of CCR3 have overlapping binding sites and that a single point mutation in the receptor can turn an antagonist into an agonist suggests that there is a fine line between agonist and antagonist activity at CCR3. Supportive of this, other CCR3 antagonist programs have also reported the identification of small molecule agonists of the receptor following optimization of the structure-activity relationships of lead compounds (40, 41). This may also be the case for other chemokine receptors, with recent reports of small molecule agonists of CCR5, 8, and CXCR3 (42–44). Because genetic variations such as single nucleotide polymorphisms are relatively abundant within the genes encoding G protein-coupled receptors (45), it is also plausible that certain individuals within a population harbor mutations that unexpectedly turn receptor antagonists into agonists or render the antagonists ineffective. It may therefore be beneficial to screen individuals prior to their entry into clinical trials of such compounds.

In summary, we describe here the characterization of a small molecule CCR3 agonist, CH0076989, that we postulate activates the receptor by mimicking the amino terminus of the natural ligand CCL11. The elucidation of the binding site of CH0076989 within CCR3 along with that of the prototypic antagonist UCB35625 gives insight into the mechanisms of chemokine receptor activation and may aid the development of more potent,
selective, CCR3 antagonists. These have potential as therapeutics for the future treatment of allergic diseases such as asthma.

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