

Identification and Characterization of a Potent, Selective Nonpeptide Agonist of the CC Chemokine Receptor CCR8

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ABSTRACT

In this study, we report the first example of a nonpeptide chemokine receptor agonist, 2-{2-[4-(3-phenoxybenzyl)piperazin-1-yl]ethoxy}ethanol (ZK 756326), for the CC chemokine receptor CCR8. ZK 756326 inhibited the binding of the CCR8 ligand I-309 (CCL1), with an IC_{50} value of 1.8 μ M. Furthermore, ZK 756326 was a full agonist of CCR8, dose-responsively eliciting an increase in intracellular calcium and cross-desensitizing the response of the receptor to CCL1. In addition, ZK 756326 stimulated extracellular acidification in cells expressing human CCR8. The ability of ZK 756326 to induce a response was receptor-specific and mediated through G_{α_i} , because it could be blocked by treatment with pertussis toxin. The CCR8 agonist activated cells expressing murine CCR8, eliciting their

chemotaxis and inducing phosphorylation of extracellular signal-regulated kinase ERK1/2. Like CCL1, ZK 756326 inhibited human immunodeficiency virus (HIV) fusion of cells expressing CD4 and CCR8. Finally, unlike mCCL1, ZK 756326 bound to and activated a form of mCCR8 that was mutated to eliminate O-linked sulfation at tyrosines 14 and 15. Therefore, ZK 756326 is most probably not binding in the same manner as CCL1 but can activate the switch mechanism involved in transducing signaling events. In summary, we have identified a nonpeptide agonist of CCR8. This compound may be useful in evaluating the physiological role of CCR8 in HIV infection, as well as in the general study of CCR8 biology without the constraints inherent to the use of protein agonists such as its natural ligand.

Chemokine receptors belong to one of the most pharmacologically exploited protein families, the G-protein-coupled receptors (GPCRs) (Flower, 1999). Drugs that target these receptors make up greater than 45% of all known marketed medicines (Drews, 1996). Considerable evidence exists that points to a major role of chemokines in the pathophysiology of a number of autoinflammatory diseases, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, dermatitis, organ transplant rejection, etc. (Gerard and Rollins, 2001). Thus, chemokine receptor antagonists could prove to be useful therapeutics in treating these and other inflammatory

diseases. Based on these considerations, there has been a veritable explosion of activity to discover potent, selective, clinically useful chemokine receptor antagonists (Dhanak et al., 2001a,b; Dorn et al., 2001; Finke et al., 2001a,b,c; Forbes et al., 2000; Horuk et al., 2001; Ichiyama et al., 2003; Widowson et al., 2004), and this has generated more than 250 patent applications and nine human clinical trials (Horuk, 2003).

We have studied the CC chemokine receptor CCR8 for several reasons. In an earlier study, we showed that CCR8, when coexpressed with CD4, can function as an HIV coreceptor for diverse viral strains. The ligand for CCR8, CCL1 (I-309), was a potent inhibitor of HIV-mediated cell-cell fusion and virus infection (Horuk et al., 1998). In addition to its potential interest as an HIV coreceptor, CCR8-deficient mice have been shown to have an impaired Th2 immune response,

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; HIV, human immunodeficiency virus; ZK 756326, 2-{2-[4-(3-phenoxybenzyl)piperazin-1-yl]ethoxy}ethanol; PBS, phosphate-buffered saline; HEK, human embryonic kidney; BSA, bovine serum albumin; ERK, extracellular signal-related kinase; Env, HIV-1 envelope protein; 5-HT, 5-hydroxytryptamine; AMD-3100, 1,1'-(1,4-phenylenebis(methylene))bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride.

suggesting a potential role for this receptor in allergic disease and asthma (Chensue et al., 2001). However, reports from several other laboratories have been unable to confirm these findings, and there is a suspicion that this might reflect strain-dependent differences in the CCR8-deficient animals (Chung et al., 2003; Goya et al., 2003). Nevertheless, based on the potential interest in CCR8 as a therapeutically relevant target, we embarked on a high-capacity receptor binding screen to discover nonpeptide antagonists of CCR8.

Analysis of compounds that were identified in a screen testing inhibition of CCL1 binding showed that one of the nonpeptides was a CCR8 receptor agonist able to stimulate a number of biological responses on both human and mouse CCR8. Here, we present a detailed characterization of 2-[2-[4-(3-phenoxybenzyl)piperazin-1-yl]ethoxy]ethanol (ZK 756326) activity on human and mouse CCR8. This is, to our knowledge, the first description of a nonpeptide agonist of a chemokine receptor, and it might be especially valuable in helping to decipher the ligand/receptor contact points required for chemokine receptor signaling.

Materials and Methods

Materials. Unlabeled chemokines were all human and from R&D Systems (Minneapolis, MN) unless otherwise indicated. 125 I-labeled CCL1 was obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA).

CCR8-Expressing Cells. The human malignant glioma U87 MG cells expressing CCR8 were obtained as described previously (Horuk et al., 1998). Stable cells were selected in minimal essential medium with nonessential amino acids (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum and 300 μ g/ml G418. For binding assays, the cells were harvested and washed once with phosphate-buffered saline (PBS). Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium) containing 10% (v/v) fetal bovine serum and supplemented with glutamine, penicillin, and streptomycin. The mouse thymic lymphoma BW5147.3 (TIB-47) cell line was obtained from the American Type Culture Collection and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and antibiotics (Sigma).

Chemokine Binding Studies. U87 MG cells expressing human CCR8 (60,000 cells/well) were incubated with 0.2 mg/well wheat germ agglutinin-coated SPA beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 30 min at room temperature in binding buffer [PBS/Ca/Mg, 0.1% (w/v) bovine serum albumin (BSA), and 30 μ g/ml bacitracin]. Compounds (concentrations varied as indicated in text) or 100 nM unlabeled CCL1 (nonspecific binding) were added to the cell/bead mixture. Finally, 100 pM 125 I-CCL1 (specific activity, 2200 Ci/mmol; approximately 20,000–25,000 cpm/well) was added to the mixture and incubated at room temperature for 2 h. Bound 125 I-CCL1 was measured by scintillation counting (Wallac TriLux; PerkinElmer Life and Analytical Sciences).

Cytosolic Ca^{2+} Measurements on U87 Cell Lines. U87 MG cells expressing CCR8 were plated on poly-D-lysine-coated black 96-well plates (BD Biosciences, Franklin Lakes, NJ) at 10,000 cells/well and were cultured overnight. Cells were then loaded with Calcium 3 (Molecular Devices, Sunnyvale, CA), a Ca^{2+} -sensitive non-wash fluorescence dye, for 60 min at 37°C in Hanks' balanced salts solution (Invitrogen) containing 20 mM HEPES, 3.2 mM CaCl_2 , 1% (v/v) fetal bovine serum, and 2.5 mM probenecid. Changes in intracellular free- Ca^{2+} concentration were measured with Fluorometric Imaging Plate Reader (FLIPR 3) (Molecular Devices) immediately

after the addition of agonist at room temperature. Cross-desensitization experiments were performed by a first addition of the agonist (CCL1 at 30 nM or ZK 756326 at 3 μ M), immediately followed by a second addition of 100 nM CCL1.

Measurement of Extracellular Acidification with the Microphysiometer. Extracellular acidification was measured in a microphysiometer as described previously (Liang et al., 2000a). Human or mouse U87-CCR8 cells were seeded in polycarbonate cell capsules (Molecular Devices) at 1×10^5 cells/capsule and incubated for 48 h at 5% CO_2 , 37°C. Capsules were washed with a modified RPMI 1640 medium (containing 1 mM sodium phosphate, supplemented with 1 mg/ml BSA) and loaded into the chambers of the microphysiometer (Molecular Devices). The chambers were perfused with the low buffering media at a rate of 100 μ l/min. After stabilization, the cells were perfused with the indicated concentrations of chemokine and/or compound for 6 min, during which the rate of acidification was measured. Data shown are representative of at least two separate experiments.

Transient Transfection of mCCR8 Variants and Calcium Mobilization Studies. Mammalian expression plasmids containing the Myc-tagged coding sequences of wild-type or YFFY mutant mouse CCR8 cDNAs have been described previously (Gutierrez et al., 2004). Plasmids were prepared using the Jetstar Maxi Kit 50 (Genomed, Bad Oeynhausen, Germany) and transiently transfected into HEK 293 cells with Lipofectamine Plus (Invitrogen) according to manufacturers' instructions. Forty-eight hours after transfection, HEK 293 cells were harvested, washed, and incubated (30 min) with 3 μ M Indo-1 AM (Invitrogen). Cells were washed twice, resuspended at 3×10^6 cell/ml, and stored on ice until use. All incubations and washes were in Hanks' balanced salt solution with 0.5% (w/v) BSA, 10 mM HEPES, and 0.8 mM CaCl_2 . Aliquots of the cell suspension were heated for 5 min at 37°C, excited with light at 335 nm, and stimulated with purified mCCL1 (TCA-3) (BD Biosciences Pharmingen; San Diego, CA) or ZK 756326. Intracellular Ca^{2+} levels were assessed as Indo-1 fluorescence at 390 nm and recorded as a time trace in an F2500 fluorometer (Hitachi, Tokyo, Japan).

Chemotaxis. Migration assays with BW5147.3 cells were performed in Transwell inserts (Corning Life Sciences, Acton, MA) with a 5 μ m-pore diameter. Cells were resuspended in RPMI with 1% (w/v) BSA and 25 mM HEPES, pH 7.4 (3×10^5 cells/well), and 100- μ l aliquots were loaded into upper inserts. Samples of mCCL1 and ZK 756326 prepared in 600 μ l of the same medium were placed in the lower wells. After incubation (2 h at 37°C), inserts were removed, and the migrated cells were counted in an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). Duplicate wells were used for each point. A migration index was established as the ratio of the number of cells that had migrated in response to the chemokine or to ZK 756326 divided by the number of cells that had migrated in response to buffer alone.

Western Blot Analysis of Extracellular Signal-Related Kinase (ERK) Activation. Exponentially growing BW5147.3 cells were serum-starved for 16 h (overnight) in depletion medium [Dulbecco's modified Eagle's medium, 1% (w/v) BSA, 1 mM sodium pyruvate, and 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and antibiotics]. Cells were centrifuged, washed with the same medium, and resuspended. Cells (4×10^6 /tube) were preincubated (10 min at 37°C) and stimulated with mCCL1 or ZK 756326 in a final volume of 0.2 ml. At different time points after agonist exposure, cells were immediately washed in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris-HCl, pH 8, 137 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10% glycerol, 1% (v/v) Nonidet P-40, 0.5% deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and protease inhibitors]. Cell lysates were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose. Western blotting was performed using an antiphosphospecific ERK-mitogen-activated protein kinase monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's instructions. Blots were then stripped and probed with an anti-total

ERK polyclonal antibody, used at 1:1000 (Cell Signaling Technology, Beverly, MA) for normalization. Antibody staining was evaluated by enhanced chemiluminescence detection (GE Healthcare).

HIV Cell-Cell Fusion Assay. The assay was performed as described previously (Doranz et al., 1996). Target cells (quail QT6) were cotransfected with plasmids expressing CD4, the indicated chemokine receptor, and a luciferase expression plasmid under the control of the T7 promoter (T7-luc; Promega). Effector cells (QT6) were infected with a recombinant vaccinia expressing the T7 polymerase (vTF1.1) and transfected with an Env expression plasmid. The effector cells were added to the targets approximately 18 h after transfection and were allowed to interact for a period of 7.5 h at 37°C. Productive cell-cell fusion was assessed by assaying for T7 polymerase driven production of luciferase.

Results

In addition to its ability to act as an HIV coreceptor, CCR8 seems to play a role in triggering Th2 responses and, as such, could participate in asthma and allergic processes. Based on these potential activities of CCR8, we set up a high throughput screen to identify nonpeptide CCR8 antagonists. These assays used a SPA binding protocol, measuring the ability of the compounds to compete with ^{125}I -CCL1 for binding to CCR8 stably expressed on a U87 cell line. One of the compounds identified, ZK 756326 (Fig. 1), showed more than 50% inhibition of binding of CCL1 when tested at 5 μM . The compound was resynthesized and retested in the competition binding assay. Both unlabeled CCL1 and ZK 756326 competed with the radiolabeled ^{125}I -CCL1 in a concentration-dependent manner (IC_{50} values of 3.4 nM and 1.8 μM , respectively) (Fig. 2).

Binding competition assays were performed on a series of other G-protein-coupled receptors to determine whether the interaction of ZK 756326 was specific for CCR8 (Table 1). In these assays, ZK 756326 was tested at 50 μM for inhibition of radiolabeled ligand binding. At this concentration, ZK 756326 showed >28 fold specificity for CCR8 compared with 26 other GPCRs, all with IC_{50} values of >50 μM . There was less selectivity when ZK 756326 was tested against the serotonergic receptors 5-HT_{1A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{5A}, 5-HT₆, and the adrenergic receptor α_{2A} , in which IC_{50} values of 5.4, 4.4, 34.8, 16, 5.9, and <20 μM (at 20 μM 65% inhibition), respectively, were observed. The compound is unlikely to be an agonist on these biogenic amine receptors, because when tested at concentrations up to 10 μM on a representative receptor, 5-HT_{1A}, it showed no agonist activity in a GTP γ S binding assay.

Because ZK 756326 could block binding of CCL1 with CCR8, we tested whether it could also inhibit the functional activation of CCR8 by CCL1. To determine this, we measured agonist-induced Ca^{2+} mobilization in U87 cells expressing

CCR8. The cells responded in a concentration-dependent manner to CCL1 (data not shown). However, to our surprise, we discovered that ZK 756326 was itself an agonist of CCR8, inducing a concentration-dependent calcium response (Fig. 3). The peak response of the CCR8-expressing cells to ZK 756326 was reached in the submicromolar to micromolar range, suggesting a reduced potency compared with CCL1. However, sample traces indicate that the receptor is stimulated in a similar manner (Fig. 4). As predicted from the binding competition studies, the compound had no inherent agonist activity on CCR4 (Fig. 3) or on other chemokine receptors including CXCR3, CXCR4, and CCR5 (data not shown). Thus, the agonist like properties of the compound seemed to be CCR8-specific.

After ligand stimulation, chemokine receptors undergo desensitization whereby subsequent ligand challenges generate a reduced response. Using the calcium release assay, ligands can be tested for cross-desensitization, an indication that the ligands act on the same receptor population. The CCR8 ligand CCL1 (30 nM) was able to desensitize the receptor to subsequent challenge with CCL1 (100 nM) (Fig. 4A). ZK 756326 at a concentration of 3 μM was able to inhibit the subsequent signaling elicited by 100 nM CCL1 in a similar manner, suggesting that it was acting specifically on CCR8.

Signaling of CCL1 through CCR8 has previously been shown to be pertussis toxin sensitive, an indication of signaling through the G-protein G_{α_i} (Goya et al., 1998). The U87-CCR8 cell line was incubated with pertussis toxin and challenged with CCL1 (as indicated in the Fig. 4B legend). Pertussis toxin treatment reduced signaling of both CCL1 and ZK 756326, completely eliminating the CCL1-induced calcium response and reducing the ZK 756326-induced calcium response by >75% (Fig. 4B). In additional experiments, pretreatment with pertussis toxin eliminated all signaling by ZK 756326. These data further indicate that the agonist-

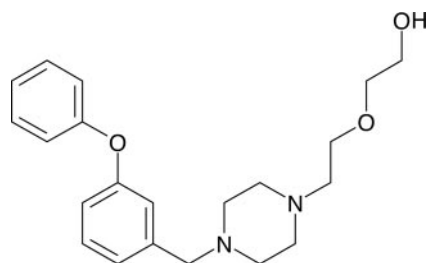


Fig. 1. Structure of ZK 756326.

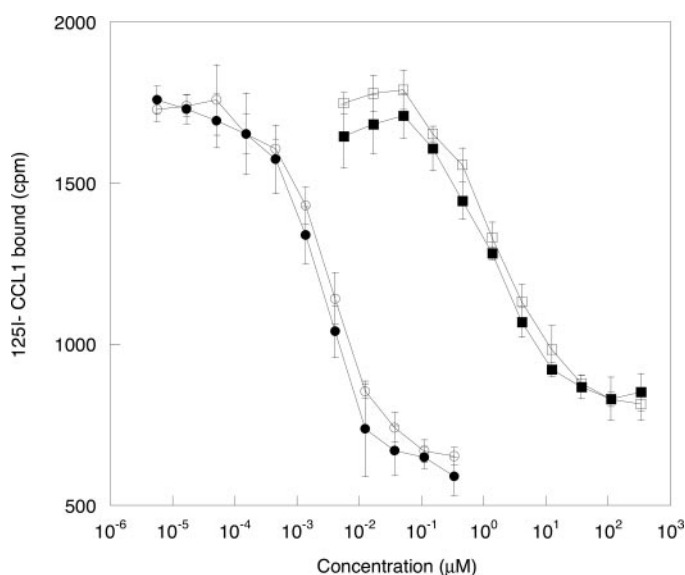


Fig. 2. ^{125}I -CCL1 binding to human CCR8 is displaced by CCL1 and ZK 756326. U87 cells expressing human CCR8 were incubated with ^{125}I -CCL1 and increasing concentrations of unlabeled ligand (circles) or ZK 756326 (squares). The data shown are ^{125}I -CCL1 binding \pm S.D. The binding results from ZK 756326 are two representative assays, each with four replicates. Calculated IC_{50} values are 3.4 ± 0.3 nM for CCL1 and 1.8 ± 0.2 μM for ZK 756326.

Often when nonpeptide agonists and antagonists are discovered by screening for activity on human GPCRs, subsequent analyses show low activity on the receptor homologs from other species (Fong et al., 1992; Link et al., 1992; Cotte et al., 1998; Liang et al., 2000b). To test whether ZK 756326 had agonist activity on murine CCR8, a U87 cell line expressing murine CCR8 was generated (U87-mCCR8). When tested in the microphysiometer, the murine CCR8 ligand mCCL1 was able to stimulate mCCR8 in a potent, concentration-responsive manner. As with the human CCR8 receptor, mCCR8 was activated by mCCL1 with an EC₅₀ value of 12

ZK 756326 was tested in receptor binding assays on a number of GPCRs. All receptors were expressed in Chinese hamster ovary cells except for the following: adrenergic α_2 in insect Sf9 cells, adrenergic β_1 in Rex 16 cells, adrenergic β_2 in NBRI cells, bradykinin in HST 29 cells, cannabinoid and serotonin 5-HT₂ in HEK 293 cells, cholecystokinin in NIH-3T3 cells, muscarinic M1 and M2 in Sf9 insect cells, neuropeptide Y in KAN-TS neuroblastoma cells, serotonin 5-HT₆ in He La cells, and leukotriene, serotonin 5-HT_{2B}, 5-HT_{2C}, and 5-HT_{5A}, CCR1, CCR2B, CCR4, CCR5, and CX3CR1 in CHO-K1 cells. Binding assays were carried out in whole cells at 25°C or 37°C and at compound concentrations of 20 and 50 μ M, or with dose curves ranging from 10 nM to 1 mM.

Receptor	IC ₅₀
	μM
Adrenergic α_{2A}	<20 ^a
Adrenergic β_1	>50
Adrenergic β_2	>50
Bradykinin B ₁	>50
Cannabinoid B ₁	>50
Cholecystokinin CCK ₁	>50
Dopamine D ₁	>50
Dopamine D _{2S}	>50
Endothelin A	>50
Leukotriene, cysteinyl CysLT ₁	>50
Leukotriene BLT ₁	>50
Muscarinic M ₁	>50
Muscarinic M ₂	>50
Neuropeptide Y	>50
Opiate δ	>50
Serotonin 5-HT _{1A}	5.4
Serotonin 5-HT _{2B}	4.4
Serotonin 5-HT _{2C}	34.8
Serotonin 5-HT ₃	>50
Serotonin 5-HT _{5A}	16
Serotonin 5-HT ₆	5.9
Tachykinin NK ₁	>50
CCR1	>50
CCR2B	>50
CCR4	>50
CCR5	>50
CXCR2	>50
CX3CR1	>1000

Chemokines were originally defined and classified as potent leukocyte chemoattractants mediating their effects

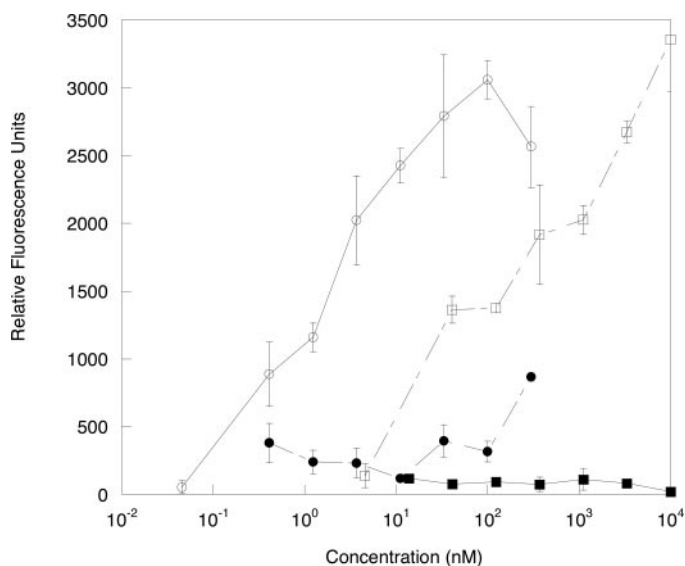


Fig. 3. Signaling at CCR8 by CCL1 and ZK 756326. U87 cells expressing human CCR8 (empty symbols) and cells expressing human CCR4 (full symbols) were loaded with the Calcium 3 dye reagent and stimulated with CCL1 (circles) or ZK 756326 (squares). CCR8 expressing cells respond to both CCL1 and ZK 756326 in a concentration dependent manner, whereas CCR4 expressing cells do not respond to ZK 756326. The data shown are the means of triplicate \pm S.D. These data are representative of two assays.

through GPCRs like CCR8. Thus, it was important to establish whether ZK 756326 was able to mimic this classic function of a chemokine and stimulate leukocyte trafficking. In some instances, ERK1/2 signaling has been reported to be necessary for chemokine-induced cell migration, because inhibitors of this kinase block chemotaxis (Coxon et al., 2003; Floridi et al., 2003). When tested in a transwell migration assay, the murine CCR8 agonist mCCL1 could induce chemotaxis, attracting cells with a peak response at 400 pM (Fig. 7). This concentration response was biphasic, which is typical

of chemokine-induced cell migration. It is noteworthy that ZK 756326 was also able to mimic this specific chemokine function and attracted cells in a chemotactic manner, with an observed peak migration at a concentration of 2.4 μ M (Fig. 7).

We have previously shown that a double tyrosine-to-phenylalanine mutation at amino acid residues 14 and 15 of mouse CCR8 (described previously as the YFFY mutant) severely impaired its ability to mediate calcium transients when stimulated with mCCL1 (Gutierrez et al., 2004). Thus, it was interesting to determine whether the nonpeptide CCR8 agonist ZK 756326 would mimic mCCL1 in this aspect. HEK 293 cells were transiently transfected with plasmids encoding wild-type or YFFY mutant mCCR8; 48 h later they

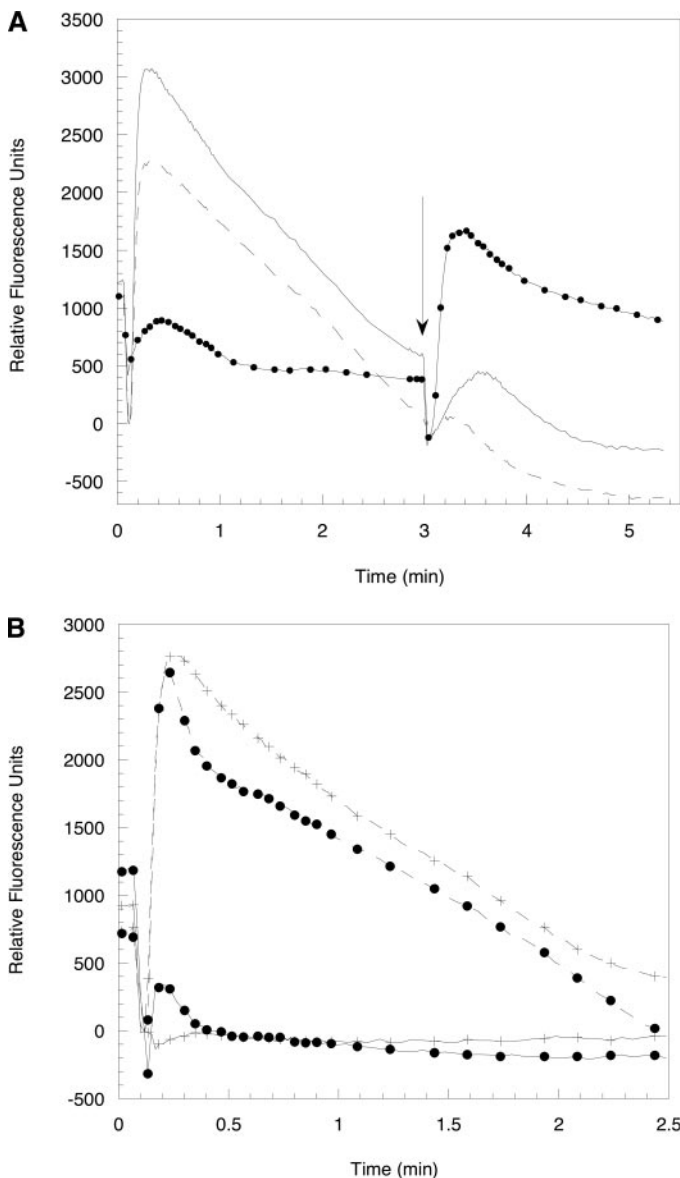


Fig. 4. Signaling of ZK 756326 is mediated through CCR8 and is G-protein-dependent. U87 cells expressing human CCR8 were loaded with the Calcium 3 dye reagent and assayed for an increase in intercellular calcium on the fluorometric imaging plate reader. **A**, desensitization. Cells were stimulated with CCL1 (30 nM) (—), ZK 756326 (3 μ M) (---) or buffer (•••). Three minutes after initial stimulation, cells were rechallenged with CCL1 (100 nM) (arrow). Response traces were set to zero (relative fluorescent units) at 8 s, immediately after the first addition. These traces are representative of three assays. **B**, pertussis toxin treatment. Cells were pretreated with pertussis toxin (200 ng/ml, 1 h, 37°C) (—) or buffer (---) and then challenged with CCL1 (30 nM) (+) or ZK 756326 (3 μ M) (•••). These traces are representative of two repeats. Response traces were set to zero relative fluorescent units at 8 s.

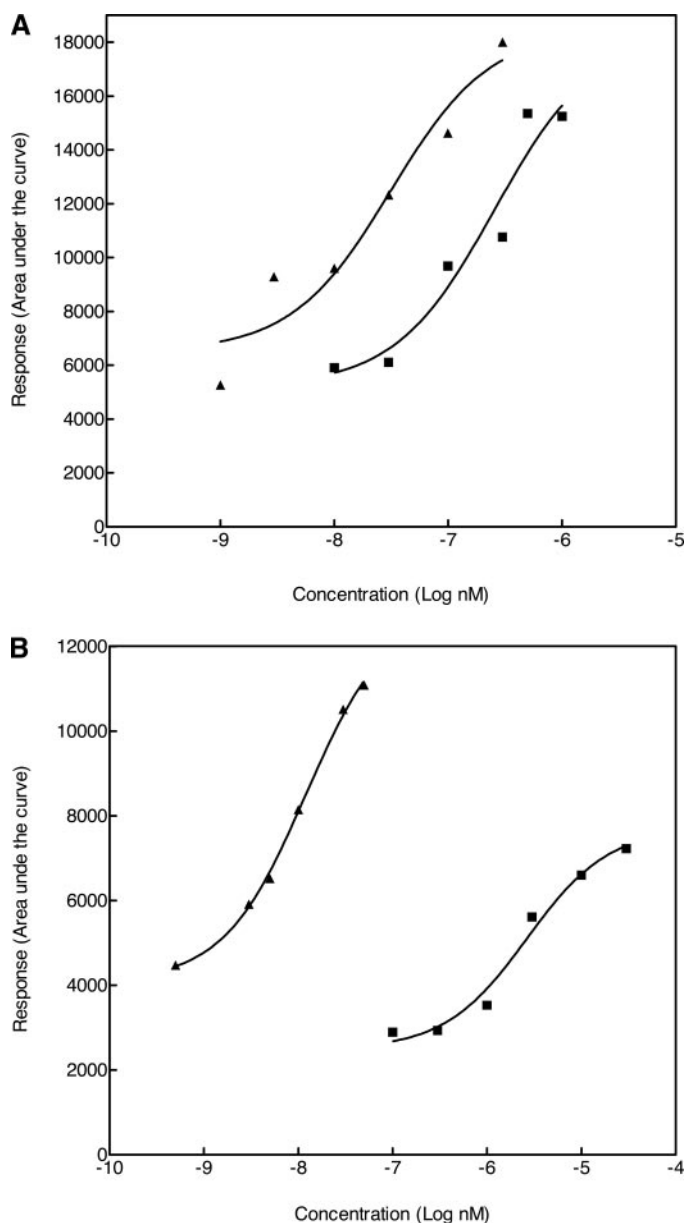


Fig. 5. ZK 756326 and CCL1 increase the extracellular acidification rate of U87-CCR8 cells. **A**, human CCR8 expressing U87 cells were stimulated with CCL1 (triangles) or ZK 756326 (squares); and **B**, murine CCR8 expressing U87 cells were stimulated with mCCL1 (triangles) or ZK 756326 (squares). The increase in acidification rate was monitored using a microphysiometer and the area under the curve is plotted as "Response." Data shown are representative of at least two separate studies.

were tested for receptor expression at the cell surface. The expression levels of wild-type and mutant CCR8 were identical (data not shown), and calcium transients were measured. As expected, cells transfected with wild-type mCCR8 responded readily to 1 nM mCCL1 (Fig. 8A, top trace) and to 18 μ M ZK 756326 (Fig. 8A, bottom trace). As shown previously (Gutierrez et al., 2004), 10 nM mCCL1 did not induce an increase in intracellular calcium in cells transfected with the YFFY mutant (Fig. 8B, top trace). However, they showed a strong response to 18 μ M ZK 756326 (Fig. 8B, bottom trace), similar to that of wild-type mCCR8. Both the wild

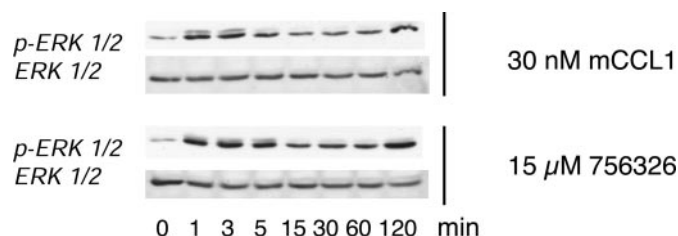


Fig. 6. CCL1 and ZK 756326 (756326) induce phosphorylation of ERK in a murine cell line expressing CCR8, a murine cell line, BW5147.3, expressing murine CCR8 was stimulated for 1 to 120 min with control medium, CCL1 (30 nM), or ZK 756326 (15 μ M). Cell lysates were prepared and used in Western blot analysis with phosphospecific antibodies directed against ERK1/2. Results show the phosphorylated form of ERK1/2 (p-ERK1/2) and total ERK of a representative experiment from three independent analyses performed.

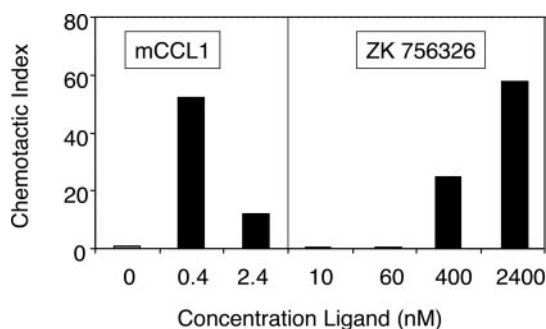


Fig. 7. CCL1 and ZK 756326 induce chemotaxis in a murine cell line expressing CCR8. A murine cell line, BW5147.3, expressing murine CCR8 was tested for migration in response to mCCL1 and ZK 756326, as described under *Materials and Methods*. The mCCL1 ligand range was 400 to 2400 pM, whereas ZK 756326 was tested from 10 to 2400 nM. Results are reported as a chemotactic index, or the ratio of cells migrated in response to ligand divided by cells migrating to buffer alone. The data are the mean of duplicate samples.

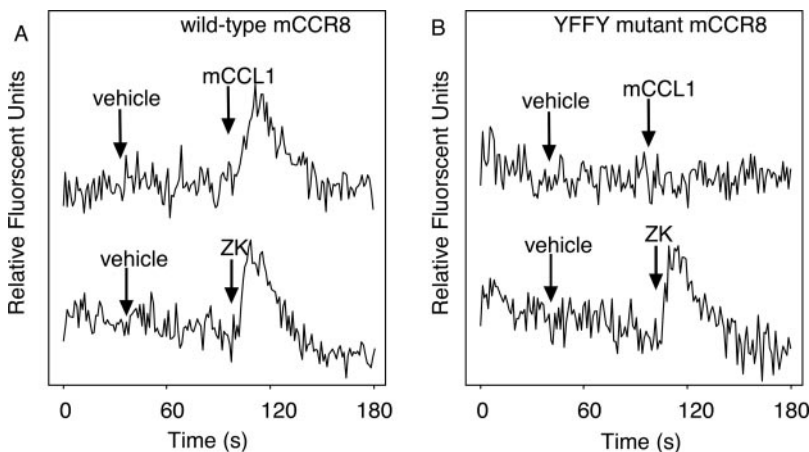


Fig. 8. Calcium flux assay with mutant mCCR8. HEK 293 cells transfected with the indicated versions of mCCR8 were subjected to calcium flux assays. Vehicle and agonists were sequentially added at the times indicated by arrows. mCCL1 was added at 1 nM in A and 10 nM in B. ZK 756326 was added at 18 μ M.

type and the YFFY mutant of mCCR8 were sensitive and responded similarly to lower concentrations of ZK 756326; as expected, HEK 293 cells transfected with void vector did not respond to mCCL1 or ZK 756326 (data not shown).

In addition to binding and signaling by CCL1, CCR8 can serve as a coreceptor for HIV-1, and CCL1 can inhibit this process (Horuk et al., 1998). To determine whether ZK 756326 could mimic the HIV-1 inhibition of CCL1, we tested it in an Env-mediated cell-cell fusion assay (Fig. 9). Various Env-expressing effector cells were allowed to elicit membrane fusion with target cells expressing CD4 and CCR8. In this assay, effector cells also express T7 RNA polymerase, whereas target cells bearing CD4 and a coreceptor contain a luciferase plasmid under control of the T7 promoter. Fusion between target and effector cells results in cytoplasmic mixing and the subsequent production of luciferase, whose enzymatic activity can be easily quantified. We found that a number of HIV-1 Envs, both CCR5-tropic (ADA, YU2, JRFL, SF162) and dual-tropic (580, 148, 89.6, UGO24), were able to produce membrane fusion via CCR8. Cell-cell fusion mediated by all of these Envs was inhibited specifically by ZK 756326 (10 μ M) to well below the IC_{50} level, with most Envs now showing fusion at between 4 and 25% (Fig. 9). When cells expressing CD4 and CCR5 were used as targets, ZK 756326 did not inhibit fusion, confirming its specificity for CCR8 (data not shown).

Discussion

These studies demonstrate that a nonpeptide, ZK 756326, can act as an agonist on a chemokine receptor, CCR8, although the natural agonist CCL1 is a protein of much larger molecular mass. What are the possible mechanisms by which this occurs? It is well known that receptor signaling in GPCRs comprises two separate events: ligand binding and signal transduction. Ligand binding of GPCRs induces a conformational change that triggers an activation domain in the receptor that induces receptor signaling. The signal is transduced by G-proteins that are coupled to the intracellular side of the GPCR. The ligand binding domains of GPCRs are probably quite diverse, given the broad range of ligands that can induce activation (e.g., light, metals, nucleotides, biogenic amines, lipids, and proteins). By contrast, it is thought that the residues involved in coupling receptor binding to receptor activation comprise very similar domains in all GPCRs. Based on these considerations, it is possible that ZK

756326 and CCL1 bind to different but perhaps overlapping binding domains in CCR8, both of which can induce identical conformational changes that are able to trigger a common receptor activation domain.

In previous work, we showed that mCCL1 does not stimulate calcium flux on YFFY-expressing cells because it does not bind to the mutant receptor (Gutierrez et al., 2004). The fact that ZK 756326 stimulates this mutant as well as wild-type mCCR8 suggests that the manner in which ZK 756326 and mCCL1 bind the receptor is different and that ZK 756326 binding to mCCR8 is independent of tyrosines 14 and 15. Because both tyrosines 14 and 15 in mCCR8 are *O*-sulfated (Gutierrez et al., 2004), it has been proposed that the negative charges contributed by these sulfate groups play an important role in the binding of mCCL1, which is a cationic polypeptide with a pI of 9.5 (Gutierrez et al., 2004). At neutral pH, ZK 756326 is predicted to have a slightly positive net charge (+1), which would not drive the type of charge interactions predicted to occur between mCCL1 and the receptor sulfate groups. In addition, because the molecular mass of ZK 756326 (356 Da) is considerably smaller than that of mCCL1 (7.8 kDa), ZK 756326 would not be expected to interact with mCCR8 using the same residues. It has been reported that other small-molecule ligands for GPCRs interact at the heptahelical bundle near the extracellular side of transmembrane domains (Onuffer and Horuk, 2002; Paterlini, 2002). The fact that ZK 756326 is insensitive to mutations at the N-terminal domain of mCCR8 further suggests that it does not interact with this region and supports the idea that it could interact exclusively with the putative transmembrane site. Further work will be needed to precisely define this site.

The coreceptors used by HIV to infect cells are attractive targets for antiviral therapy. Small molecule inhibitors of both CCR5 and CXCR4 have been developed and some are in human clinical trials. Whether inhibitors of other viral coreceptors will prove to be useful for antiviral therapy remains to be determined. So far, there is no compelling evidence that coreceptors other than CCR5 or CXCR4 are important in mediating HIV-1 infection of relevant primary cell types,

although there is some evidence that CCR8 may be important for thymocyte depletion and infection by HIV-1. However, if small-molecule inhibitors potently block HIV-1 infection mediated by CCR5 or CXCR4, HIV-1 may evolve to use other coreceptors to infect cells. An example of unexpected coreceptor use in the face of strong selective pressure is provided by SIV_{rcm} infection of red-capped mangabeys. Many red-capped mangabeys are CCR5-negative because of a naturally occurring polymorphism in the CCR5 open-reading frame. Viruses isolated from these infected animals use CCR2 as their major coreceptor to infect cells. Thus, development of small-molecule inhibitors that target alternative HIV coreceptors could play a role in antiviral therapy in some circumstances.

It is interesting to note that many of the compounds identified in the binding screen displayed specific CCR8 agonist activity in the calcium assay. These results emphasize the need for consideration of primary functional assays when screening for agonistic activity. There may be situations in which a chemokine receptor agonist could be favorable; i.e., CCR5 activation by an agonist will lead to potential inhibition by HIV infection and therefore justify the use of a functional primary assay. At this time, we cannot say whether this is a characteristic of the receptor or simply a bias that exists in the nonpeptide library used to screen this receptor. It has been reported that AMD-3100, a drug developed as a CXCR4 antagonist, did show at least partial agonist activity on CXCR4 (Zhang et al., 2002), although there is controversy surrounding this activity. In the calcium response assays, ZK 756326 is acting as a full agonist, given that the peak response generated by the compound is similar to that seen with CCL1. In other ways, the ZK 756326-mediated signaling of CCR8 is similar to that of the natural ligand: inhibition by pertussis toxin and the induction of ERK1/2 phosphorylation. To our knowledge, this is the first example of a small molecule acting as an agonist at a chemokine receptor.

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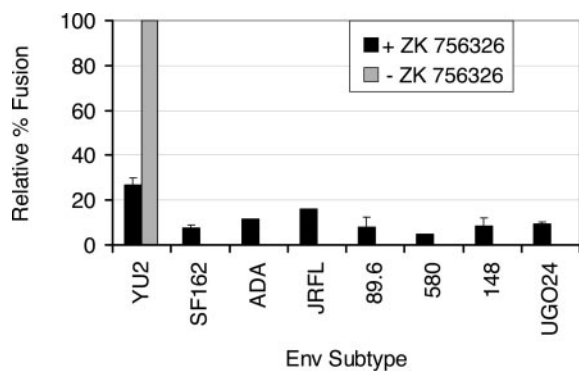


Fig. 9. ZK 756326 inhibition of CCR8-mediated HIV fusion. Relative inhibition was assessed by a cell-cell fusion luciferase reporter assay. QT6 effector cells expressed the indicated Envs and T7 RNA polymerase. QT6 target cells expressing CD4 and coreceptor and containing a luciferase reporter gene under the control of the T7 promoter were used. Fusion levels are shown for the indicated Envs on CD4/CCR8+ cells with ZK 756326 (10 μ M). Data are expressed as a percentage of wild-type fusion on CD4/CCR8+ cells without ZK 756326. The fusion levels without ZK 756326 are shown only for the YU2 Env, although they were run for all Envs. Results represent the means \pm S.D. of at least three independent experiments with at least two independent Env DNA preparations.

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