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Title Page

A novel, selective, and orally available antagonist for CC chemokine receptor 3

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A Novel CCR3 Antagonist

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Abbreviations: CCL, CC chemokine ligand; CCR, CC chemokine receptor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; FBS, fetal bovine serum; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDN, eosinophil-derived neurotoxin; GAFS, gated autofluorescence forward scatter

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Abstract

CC chemokine ligand 11 (CCL11/Eotaxin) and other CC chemokine receptor three (CCR3) ligands (CCL24/Eotaxin-2, CCL26/Eotaxin-3, CCL13/MCP-4, etc) play important roles in the chemotaxis and activation of eosinophils and other CCR3-expressing cells (basophils, mast cells, and $CD4^+$ Th2 cells) in allergic inflammation incidents, including asthma and rhinitis. A newly synthesized compound, YM-355179, inhibited the binding of CCL11 and CCL5/RANTES to CCR3-expressing B300-19 cells with IC_{50} values of 7.6 and 24 nM, respectively. In contrast, YM-355179 did not affect the binding of CCL5 to CCR1 or CCR5. In functional assays, YM-355179 inhibited CCL11-induced, intracellular Ca^{2+} influx, chemotaxis, and eosinophil degranulation with IC_{50} values of 8.0, 24, and 29 nM, respectively. YM-355179 did not, however, affect any CC chemokine receptor (CCR1, CCR2, CCR4, or CCR5) -mediated Ca^{2+} influx signals. Furthermore, oral administration of YM-355179 (1 mg/kg) inhibited CCL11-induced shape change of whole blood eosinophils in cynomolgus monkeys. Intravenous injection of YM-355179 (1 mg/kg) also inhibited eosinophil infiltration into airways of cynomolgus monkeys after segmental bronchoprovocation with CCL11. These results indicate that YM-355179 is a novel, selective, and orally available CCR3 antagonist with therapeutic potential for treating eosinophil-related allergic inflammatory diseases.

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Introduction

Eosinophils play a crucial role in allergic diseases such as bronchial asthma and allergic rhinitis (Durham et al., 1985; Terada et al., 1994). CCR3, a 7-transmenbrane-spanning G protein-coupled receptor, is a major chemokine receptor expressed on allergic inflammatory cells, including not only eosinophils, but also basophils, mast cells, and Th2-type $CD4^+$ cells (Combadiere et al., 1995; Post et al., 1995; Sabroe et al., 1998). This receptor has been identified in humans, monkeys, mice, rats, and guinea pigs (Zhang et al., 2002; Garcia-Zepeda et al., 1996; Forssmann et al., 1997; Sallusto et al., 1997). Human CCR3 binds multiple chemokine ligands, such as CCL11/Eotaxin, CCL24/Eotaxin-2, CCL26/Eotaxin-3, and CCL13/MCP-4, with high affinity, whereas CCR3 binds CCL5/RANTES and CCL7/MCP-3 with lower affinity (Doucet et al., 1998; Stellato et al., 1997; Forssmann et al., 1997). CCR3 ligands are produced by multiple cell types, including lung and mucosal fibroblast cells, bronchial epithelial cells, vascular endothelial cells, smooth muscle cells, macrophages, mast cells, and eosinophils (Shinkai et al., 1997; Lilly et al., 1997; Menzies-Gow et al., 2004; Hirst et al., 2002; Lamkhioued et al., 1997). The binding of CCR3 ligands to CCR3 on the surface of eosinophils causes a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that activates several signal pathways, including actin polymerization (Elsner et al., 1996). With activation of the intracellular motile apparatus, eosinophils undergo shape change followed by acquisition of a polarized morphology, subsequent migration to sites of allergic inflammation, and granule release to combat against exogenous pathogens and allergens (Sambrano et al., 1999; Elsner et al., 1995). A characteristic feature of allergic asthma and rhinitis is leukocyte infiltration into the bronchial and intranasal walls, which is predominantly where eosinophils and other CCR3-expressing cells accumulate and are activated (Sehmi et al., 2003; Terada et al., 2001), therefore CCR3 is an attractive target for treatment of allergic

asthma and rhinitis.

Although several non-peptide compounds that inhibit binding of CCL11 to CCR3 have been reported (Sabroe et al., 2000; White et al., 2000; Saeki et al., 2001; Wan et al., 2002, De Lucca GV et al., 2005), their *in vivo* effectiveness has not yet been demonstrated in any nonhuman primates. YM-355179 is a novel, selective, and orally available low molecular weight CCR3 antagonist that has been chemically synthesized in our laboratories. In this study the effects of YM-355179 on CCL11-induced chemotaxis, eosinophil degranulation, *ex vivo* eosinophil shape change, and *in vivo* eosinophil infiltration into the airways of cynomolgus monkeys were investigated.

In vivo effectiveness of YM-355179 indicates the therapeutic potential of CCR3 antagonists for treatment of conditions caused by eosinophilic allergic inflammation, including asthma and allergic rhinitis.

Materials and Methods

Reagents

All chemokines (recombinant human CCL11, CCL24, CCL26, CCL13, CCL5, CCL7, CCL2/MCP-1, CCL17/TARC, and murine CCL11) were purchased from Pepro Tech (Rocky Hill, NJ). [^{125}I]-CCL11 (74 TBq/mmol) and [^{125}I]-CCL5 (74 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). YM-355179 (Figure 1) was synthesized by Yamanouchi Pharmaceutical (Tsukuba, Japan).

CC chemokine receptor-expressing cells

Previously engineered human CCR1 or CCR3-expressing B300-19 cells (murine pre-B cell lymphoma) were used (Sato et al., 1999). Other CC chemokine receptors were cloned from either a human peripheral blood mononuclear cell (PBMC) cDNA library, human genomic library, or rhesus monkey PBMC library by polymerase chain reaction (PCR). Primers were designed based on sequences from the following Gene Bank™ submissions: CCR2B (U03905), CCR4 (X8540), CCR5 (X91492), and monkey CCR3 (AF405537). Expression vector, pEF-BOS-Neo, carrying each of the full-length receptor cDNA species above, was transfected into B300-19 cells using electroporation, then G-418-resistant stable transformants were isolated (Sato et al., 1999). The transformed B300-19 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 μM 2-mercaptethanol, 1 mg/mL geneticin, 100 U/mL penicillin, and 100 μg/mL streptomycin. cDNA encoding for murine CCR3 (AY049018) and rat CCR3 (AF003954) was obtained from murine and rat splenocyte mRNA using RT-PCR, then subcloned into pCR3.1 and pcDNA3.1 vectors, respectively (Invitrogen, Carlsbad, CA). These constructed vectors were then cotransfected with chimeric G protein Gqi5 (Molecular Devices, Sunnyvale, CA) into Chinese hamster

ovary (CHO) and human embryonic kidney 293 (HEK293) cells, respectively, using Lipofectamine. Transfected-CHO cells were maintained in α Minimal Essential Medium supplemented with 10% FBS, 0.25 mg/mL geneticin, 50 U/mL penicillin, and 50 μg/mL streptomycin. Transfected-HEK-293 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin for 24 h before being used for the Ca^{2+} influx assay.

Binding assay

Binding assays were performed in 96 well polystyrene microplates (Optiplate^{TM}-96, PerkinElmer, Wellesley, MA) at 25°C. Cells (3×10^6 cells) were incubated for 60 min in 200 μL of binding buffer [50 mM HEPES, pH 7.5, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin (BSA)] containing 100 pM [^{125}I]-CCL11 or 50 pM [^{125}I]-CCL5 and 1 mg/mL wheatgerm agglutinin SPA beads (Amersham Pharmacia Biotech, Uppsala, Sweden), in the presence or absence of various concentrations of YM-355179, under constant agitation. Radioactivity was counted using a microplate scintillation counter (TopCount^{TM}, PerkinElmer, Wellesley, MA). Specific binding of CCL11 or CCL5 to each receptor was defined as the difference between total binding and non-specific binding obtained in the presence of a 200-fold excess of unlabeled CCL11 or CCL5.

Measurement of $[Ca^{2+}]_i$

Cells were loaded with 5 μM Fura-2 acetoxymethyl ester in RPMI 1640 medium containing 1% BSA for 30 min at 37°C. After being washed twice, cells were resuspended at a concentration of 2×10^6 cells/mL in 20 mM HEPES buffer containing 0.1% BSA, 130 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 5.5 mM glucose. The cell suspension (490 μL) was then transferred into glass cuvettes. One microliter of YM-355179 dimethyl

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sulfoxide (DMSO) solution was added to the cuvette 1 min prior to addition of various concentrations of chemokine (10 μL). Changes in fluorescence were monitored at 25°C using an intracellular ion analyzer (CAF-110, JASCO, Tokyo, Japan) at 340 and 380 nm (excitation wavelengths) and at 510 nm (emission wavelength). Calculation of $[Ca^{2+}]_i$ was performed using K_d for Ca^{2+} binding at 224 nm.

Chemotaxis assay

Quantitative chemotaxis assays were performed using 96-well Boyden chambers (Neuro Probe, Gaithersburg, MD), polyvinylpyrrolidone-free polycarbonate filters with 5 μm pores (Neuro Probe, Gaithersburg, MD) for 3 h at 37°C in a humidified 5% CO_2 incubator. Cells suspended at 5×10^6 cells/mL in RPMI 1640 medium containing 0.1% BSA, were preincubated for 1 min with various concentrations of either YM-355179 or vehicle (DMSO), then added to the upper wells (200 μL) without removing compound from the medium. Various concentrations of chemokines were added to the lower wells. Cell numbers migrating to the lower wells were quantified using the ATP-lite™ (PerkinElmer, Wellesley, MA) bioluminescence assay.

Human eosinophil degranulation

CCL11-induced-eosinophil degranulation assays were performed as previously described, with minor modifications (Kaneko et al., 1995). Human eosinophils were isolated from peripheral blood of normal volunteers. Wells in 96-well flat bottom tissue culture plates (Costar, Cambridge, MA) were blocked with 50 μL of 2.5% BSA diluted in PBS for 2 h at 37°C. After washing, a suspension of 200 μL of eosinophils in RPMI 1640 medium containing 0.5% BSA (5×10^5 cells) with or without 0.6 nM human CCL11 was added to wells, then incubated for 4 h in a humidified incubator at 37°C and 5% CO_2 in the presence or

absence of various concentrations of YM-355179. After incubation, supernatants were collected and frozen at -20°C until assay for eosinophil-derived neurotoxin (EDN). The amount of released EDN was measured using an enzyme-linked immunosorbent assay kit purchased from MBL International (Woburn, MA).

Animals

Male cynomolgus monkeys (*Macaca fascicularis*), were imported by Hamuri Co., Ltd. (Sanwa, Japan), and housed individually in stainless steel cages. They were provided with feed twice a day and water *ad libitum*. Animals used for *ex vivo* whole blood assays were 3 to 4 years old and weighed from 4 to 7 kg. Animals used for *in vivo* CCL11-induced eosinophil infiltration were 6 to 8 years old and weighed from 6 to 9 kg.

Cynomolgus monkey whole blood-gated autofluorescence forward scatter (GAFS) assay

The procedure for the GAFS assays was modified from that created by Sabroe et al. (Sabroe et al., 1999). Briefly, cynomolgus monkeys were fasted overnight and either YM-355179 or placebo (distilled water) was administered orally with a gastric tube. At 2, 6, and 12 h after administration, blood samples (80 μL) were collected in polypropylene tubes containing 10 μL of 177 mM citric acid solution, mixed with various concentrations of CCL11 (10 μL), then incubated at 37°C for 10 min. CCL11-stimulated blood samples were mixed with 2 mL of ice-cold fixative solution containing 2.5% Cell FIX™ (BD Bioscience, San Jose, CA), then 2 mL of ice-cold lysis buffer (168 mM NH_4Cl, 10 mM $KHCO_3$, pH7.35) was added to the mixture. After being left on ice for 10 min to achieve uniform red blood cell lysis, remaining white blood cells were washed twice with phosphate-buffered saline (PBS) containing 5% BSA, then resuspended in PBS containing 10 mM HEPES, 0.1% 10 mM glucose, and 0.1% BSA. Samples were processed using a FACS Calibur™ flow cytometer

(BD Bioscience, San Jose, CA), and data from the 20000 cells contained in each sample were collected. Eosinophils were gated out based on high autofluorescence and mean forward scatter was calculated using CELLQuest™ software (BD Bioscience, San Jose, CA).

CCL11-induced eosinophil infiltration into the airways of cynomolgus monkeys

This study was conducted in a placebo-controlled, crossover fashion with two weeks interval for washout. Each animal was anesthetized with an intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (1 mg/kg), intubated with a cuffed endotracheal tube, then placed in the supine position. A pediatric fiberoptic bronchoscope (BF 3C40, Olympus, Tokyo, Japan) was inserted through the cuffed endotracheal tube, guided past the carina, and wedged into a bronchus. CCL11 solution (1 mL; 30 pmole/mL, containing 2% Evan's Blue) was infused through the lavage channel of the bronchoscope into the bronchus. Each animal received two bolus intravenous injections of YM-355179 or placebo (saline) 5 min before and 24 h after CCL11 provocation in order not to interfere with absorption of YM-355179 from the gastrointestinal tract, as peristaltic motion was affected by anesthesia. At 48 h after provocation, animals were anesthetized and intubated again with a cuffed endotracheal tube, then the bronchoscope was inserted into the same bronchus stained with Evan's Blue. Bronchoalveolar lavage (BAL) was performed through the lavage channel using two 10 mL aliquots of saline. Recovery rate for each BAL fluid sample was approximately the same, about 60%. The BAL fluid was centrifuged at 4 °C, then the sediment was resuspended in 1 mL of saline containing 1 U/mL heparin. Total cell number was counted using an automatic cell counter (Celltac-α, Nihon Kohden, Tokyo, Japan). Differential cell counts were performed on slides prepared using a centrifuge (Cytospin 3™, Shandon, Pittsburgh, PA), then stained with Wright-Giemsa stain (Diff-Quik™ Kit, International Reagents Corporation, Kobe, Japan). A total of 300 cells were counted using standard morphological techniques to

calculate the ratio of eosinophil, neutrophil, monocyte, macrophage, lymphocyte, basophil, and mast cells to total leukocytes in the BAL fluid.

Data Analysis

Data were expressed as mean ± SEM. Statistical significance of differences between the mean of groups was determined using Paired *t*-test or Two-way Dunnett-test. Probabilities of < 0.05 were considered significant.

Ethical considerations

All experiments were performed in accordance with the regulations of the Yamanouchi Pharmaceutical Animal Ethics Committee.

Results

YM-355179 binds to CCR3, but not other CC chemokine receptors

We examined the competitive displacement of [^{125} I]-CCL11 by various CCR3 ligands when binding to CCR3-expressing B300-19 cells. CCL11, CCL13, and CCL24 are reported to be full agonists, which can stimulate the exchange of GTP for GDP on the active site of the G_α protein during CCR3 signal induction, while CCL5, CCL7, and CCL26 are only partial agonists (Wan et al., 2002). As shown in Figure 2A, CCL11, CCL24, CCL26, and CCL13, competitively displaced the binding of [^{125} I]- CCL11 to CCR3-expressing B300-19 cells with IC_{50} values of 1.0, 0.45, 3.2, and 0.052 nM, respectively. CCL5 and CCL7 showed less potent displacement activity (approximately 50% inhibition), even at 100 nM, during the binding of [^{125} I]-CCL11 to CCR3. The order of displacement potency (CCL13 > CCL24 > CCL11 > CCL26 >> CCL5 = CCL7) using CCR3-expressing B300-19 cells was approximately of the same order as CCR3 ligand affinities for CCR3 on human eosinophils, which was demonstrated during the evaluation of CCR3 agonists (Doucet et al., 1998; Stellato et al., 1997; Forssmann et al., 1997). YM-355179 (Figure 1) is a novel CCR3 antagonist, chemically synthesized in our laboratories. To demonstrate the effectiveness of YM-355179 in the binding assay, CCL11 and CCL5 were chosen as full and partial agonists, respectively. YM-355179 demonstrated competitive displacement activity during the binding of both [^{125} I]-CCL11 and [^{125} I]-CCL5 to CCR3 with potent IC_{50} values of 7.6 and 24 nM, respectively (Figure 2B). In contrast, YM-355179 did not inhibit the binding of [^{125} I]-CCL5 to CCR1 or CCR5, even at a concentration of 10 μM. These results indicate that YM-355179 selectively antagonizes CCR3.

YM-355179 acts as a CCR3 antagonist in functional assays

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Recombinant human CCL11, CCL24, CCL26, and CCL13 are potent CCR3 agonists (Doucet et al., 1998; Stellato et al., 1997; Forssmann et al., 1997). CCL11, CCL24, CCL26, CCL13, and CCL5 showed $[Ca^{2+}]_i$ elevation in a concentration-dependent manner (Figure 3A). EC_{50} values were 2.4, 4.1, 10, 0.74, and 72 nM, respectively. CCL11 and CCL13 had more potent $[Ca^{2+}]_i$ elevation than the other CCR3 ligands, CCL24, CCL26, and CCL5. YM-355179 potently inhibited CCL11-, CCL24-, CCL26-, CCL13-, and CCL5-induced $[Ca^{2+}]_i$ elevation with IC_{50} values of 8.0, 5.6, 7.0, 6.1, and 4.5 nM, respectively (Figure 3B). However, YM-355179 did not affect CCR1-, CCR2-, CCR4-, or CCR5-mediated signals (CCL5-, CCL2-, CCL17-, or CCL5-induced, respectively). Next the effect of YM-355179 on chemotaxis induced by each CCR3 ligand was examined. All CCR3 ligands tested induced the migration of CCR3-expressing B300-19 cells in a bell-shaped manner, a common feature of all chemokines (Figure 4A). CCL11 and CCL13 were more potent and effective chemoattractants than the other CCR3 ligands, CCL24, CCL26, and CCL5. YM-355179 inhibited CCL11-, CCL24-, CCL26-, CCL13-, and CCL5-induced chemotaxis with IC_{50} values of 24, 13, 9.5, 5.9, and 11 nM, respectively (Figure 4B). No effect was observed on CCR1-, CCR2-, CCR4-, or CCR5-mediated chemotaxis at any YM-355179 concentration, ranging from 0.1 to 10000 nM (data not shown). Effect of YM-355179 on EDN release was also examined. YM-355179 dose-dependently inhibited EDN release from CCL11-induced human peripheral eosinophils with an IC_{50} value of 29 nM (Figure 5). YM-355179 inhibited EDN release at approximately the same concentration as in the Ca^{2+} influx and chemotaxis assays.

Effect of YM-355179 on monkey, rat, and murine CCR3

It is important to investigate species specificity of YM-355179 to design *in vivo* preclinical studies. As shown in Table 1, YM-355179 antagonizes not only human CCR3, but also

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monkey CCR3. IC_{50} value of human CCL11-induced $[Ca^{2+}]_i$ elevation in monkey CCR3-expressing B300-19 cells was 12 nM. In contrast, IC_{50} values of murine CCL11-induced $[Ca^{2+}]_i$ elevation in murine CCR3-transfected CHO and rat CCR3-transfected HEK293 cells were 460 nM and greater than 10 μM, respectively. CCR3 antagonistic activity of YM-355179 was less potent in rodents than in primates.

Pharmacodynamic analysis of YM-355179 using whole blood GAFS assay in cynomolgus monkeys

Ex vivo whole blood GAFS assay was used to estimate the pharmacodynamics of YM-355179 in monkeys. YM-355179 (1 mg/kg) was orally administered to one monkey, then whole blood samples were collected 2, 6, and 12 h after the administration. As shown in Figure 6A, CCL11 (12 and 36 nM)-induced eosinophil shape change in whole blood was observed in the placebo group. In contrast, in blood obtained 2 h after oral administration of YM-355179 (1 mg/kg), CCL11-induced eosinophil shape change was significantly inhibited compared to the placebo group (Figure 6A). When 12 nM CCL11 was used for stimulation, inhibitory effect of YM-355179 was maintained up to 12 h after administration (Figure 6B). When 36 nM CCL11 was used, effect of YM-355179 was observed at 2 and 6 h after administration.

***In vivo* effect of YM-355179 on CCL11-induced eosinophil infiltration into the lung in cynomolgus monkeys**

Effect of YM-355179 on eosinophil infiltration into the airways of cynomolgus monkeys was investigated. After segmental bronchoprovocation with CCL11, at 48 h a significant increase in total leukocyte number in BAL fluid was observed (Figure 7A). Cells observed in BAL fluid included eosinophils, basophils, mast cells, lymphocytes, monocytes, macrophages, and neutrophils. Compared to the untreated group, number of each cell type

significantly increased after CCL11 provocation (Figure 7A-7F). When YM-355179 (1 mg/kg i.v.) was administered twice, 5 min before and 24 h after CCL11 provocation, number of eosinophils was only 17% of the number in the placebo group, and the difference is statistically significant (Figure 7B). YM-355179 inhibited not only the number of eosinophils, but also the number of lymphocytes, basophils, and mast cells, which are all CCR3-expressing cells, with statistical significance (Figure 7C and 7D). As expected, YM-355179 had no inhibitory effect on the number of non-CCR3-expressing cells, such as monocytes, macrophages, and neutrophils, in BAL fluid (Figure 7E and 7F).

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Discussion

CCR3 antagonists are good candidates for development as drugs for treatment of inflammatory diseases mediated by CCR3. In murine asthma models, eosinophil infiltration and airway hyperresponsiveness are inhibited in CCR3 gene knockout mice and also by administration of monoclonal antibody to CCR3 in OVA-challenged mice (Ma et al., 2002; Grimaldi et al., 1999). The prototype of YM-355179, YM-207086, was discovered in our chemical library by high-throughput screening using Ca^{2+} influx assay with CCR3-expressing B300-19 cells. YM-355179 was about 160 times more potent than YM-207086 in the Ca^{2+} influx assay and also inhibited binding of [^{125}I]-CCL11 to CCR3. CCR3 ligands such as CCL11, CCL13, and CCL24 play important biological roles in the activation of eosinophils, including inducing shape change, endothelial migration, chemotaxis and degranulation at the sites of inflammation (Zhang et al., 2002; Bryan et al., 2002). YM-355179 inhibited the binding of [^{125} I]-CCL11, CCL11-induced $[Ca^{2+}]_i$ elevation, chemotaxis, and EDN release from human eosinophils at approximately the same range of concentration (IC_{50}=7.6-29 nM). These results suggest that YM-355179 achieved these inhibitory effects by blocking CCR3. YM-355179 alone did not exhibit agonistic activity similar to CCL11 in chemotaxis or any other functional assays (data not shown).

YM-355179 preferentially inhibited human and monkey CCR3-mediated signals compared to rodent CCR3. Non-peptide, small molecular, chemokine receptor antagonists sometimes show species specificity (Onuffer et al., 2003). Disparity in the potency of CCR3 antagonism between primates and rodents was more than 60 times based on IC_{50} values. This is supported by the fact that monkey CCR3 sequence is reportedly 92-96% identical to human CCR3 at the amino acid level, while murine CCR3 and rat CCR3 show only 67 and

69% homology to human CCR3, respectively (Zhang et al., 2002; Iino et al., 2002). Therefore, cynomolgus monkeys were used for *ex vivo* and *in vivo* evaluation of CCR3 antagonism. Oral administration of YM-355179 (1 mg/kg) inhibited CCL11-induced *ex vivo* eosinophil shape change for an extended period (6-12 h). In pharmacokinetic studies of YM-355179 (1 mg/kg, *po*) in cynomolgus monkeys, mean maximal plasma concentration (C_{max}) was 456 ng/mL (907 nM), AUC $_{(0\text{-}24h)}$ was 3198 ng·h/mL, and bioavailability was 67% (data not shown). At 2 h after administration, when *ex vivo* eosinophil shape change was significantly suppressed, mean plasma concentration was 180.7 ng/mL (359.5nM), approximately 30 times as much as the IC_{50} value (12 nM) for monkey CCR3 antagonism in the Ca^{2+} influx assay. YM-355179 (1 mg/kg, *i.v.*) also inhibited infiltration of eosinophils into the airways of monkeys after segmental bronchoprovocation with CCL11. At 48 h after the first *i.v.* bolus, when BAL was performed, mean plasma concentration was 72.75 ng/mL (144.8 nM). This concentration is also 10 times higher than the IC_{50} value obtained for monkey CCR3 antagonism. *Ex vivo* and *in vivo* CCR3 antagonistic activities of YM-355179 were supported by sufficient plasma concentrations of the compound in cynomolgus monkeys.

IL-5 plays a primary role in maturation and differentiation of eosinophils in bone marrow, and CCL11 also plays some important roles in their production and migration (Clutterbuck et al., 1989; Peled et al., 1998). CCL11 stimulates differentiation of myelopoietic progenitors to eosinophil precursors in order to ensure trafficking of immature $CD34^{+}$ eosinophils from bone marrow into non-hematopoietic tissues, such as the intestine and lung mucosal tissues, after antigen provocation (Sehmi et al., 2003; Peled et al., 1998; Matthews et al., 1998). CCR3 antagonists are therefore expected to suppress generation of resident immature eosinophils in mucosal tissues during allergic inflammation. CCR3 antagonists like YM-355179 can

suppress the function of all CCR3-expressing cells, not only eosinophils, but also basophils, mast cells, and $CD4^+$ Th2 cells. However, anti-IL-5 antibody will suppress only eosinophils. CCR3 antagonist rather than anti-IL-5 antibody is likely to be useful in the treatment of allergic inflammation.

Anti-CCL11 monoclonal antibody (bertilimumab) is currently being tested in clinical trials for the treatment of seasonal allergic rhinitis. It has shown a tendency to reduce the numbers of submucosal eosinophils and basophils, thus improve post allergy nasal obstruction (Ding et al., 2004). Compared to bertilimumab, CCR3 antagonist, YM-355179, demonstrates a more promising effectiveness in the control of allergic diseases as it can inhibit not only CCL11, but also other CCR3 ligands, such as CCL24, CCL26, CCL13, and CCL5.

YM-355179 is an attractive novel CCR3 antagonist because of its potency and oral availability. YM-355179 shows good potential as a new therapy for the prevention and treatment of allergic asthma and rhinitis.

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Legends for Figures

Figure 1

Chemical structure of YM-355179,

N-{(3*R*)-1-[(6-fluoro-2-naphthyl)methyl]pyrrolidin-3-yl}-2-{1-[(5-hydroxy-3-methylpyridin-2-yl)carbonyl]piperidin-4-ylidene}acetamide hemifumarate.

Figure 2

Binding of [^{125}I]-CCL11 to CCR3 in the presence of unlabeled CCL11, CCL24, CCL26, CCL13, CCL5, CCL7, and YM-355179. A: Competitive binding of [^{125}I]-CCL11(100 pM) to CCR3-expressing B300-19 cells in the presence of various concentrations (0.001-100 nM) of each chemokine. B: Competitive binding of [^{125}I]-CCL11 (100 pM) to CCR3-expressing B300-19 cells or [^{125}I]-CCL5 (50 pM) to CCR5-expressing B300-19 cells in the presence of various concentrations (0.1-1000 nM) of YM-355179. Results are representative of the mean ± SEM from three independent experiments performed in duplicate.

Figure 3

CCR3 ligand-stimulated [Ca^{2+}]$_i$ elevation in CCR3-expressing B 300-19 cells and inhibition of [Ca^{2+}]$_i$ elevation by YM-355179. Functional calcium assay was performed using CCR1-, CCR2-, CCR3-, CCR4-, or CCR5-expressing B300-19 cells as described in Materials and Methods. A: CCR3 ligand-induced [Ca^{2+}]$_i$ elevation was examined in the presence of various concentration of CCR3 ligands, CCL11, CCL24, CCL26, CCL13, and CCL5 (0.01-100 nM). B: Effect of YM-355179 (0.1 -1000 nM) on CCR3 ligand-induced [Ca^{2+}]$_i$ elevation was investigated in the presence of CCL11 (3 nM), CCL24 (3 nM), CCL26 (10 nM), CCL13 (1 nM), CCL5 (10 nM for CCR3, 3 nM for CCR1 and CCR5), CCL2 (1 nM),

or CCL17 (1 nM). Results are representative of the mean ± SEM from three independent experiments performed in duplicate.

Figure 4

CCR3 ligand-stimulated chemotaxis of CCR3-expressing B300-19 cells and inhibition of chemotaxis by YM-355179. Chemotaxis assays were performed using CCR3-expressing B300-19 cells in Boyden chambers as described in Materials and Methods. A: CCR3 ligand-induced chemotaxis was examined in the presence of various concentrations of CCR3 ligands: CCL11, CCL24, CCL26, CCL13, and CCL5 (0-100 nM). B: Effect of YM-355179 (0.1-1000 nM) on CCR3 ligand-induced chemotaxis was investigated in the presence of CCR3 ligands: CCL11 (3 nM), CCL24 (3 nM), CCL26 (100 nM), CCL13 (10 nM), and CCL5 (100 nM). Results are representative of the mean ± SEM from three independent experiments performed in duplicate.

Figure 5

Inhibition of EDN release from CCL11-stimulated eosinophils by YM-355179. Human peripheral eosinophils were stimulated with 0.6 nM human CCL11 in the presence of various concentrations of YM-355179 (0.63-80 nM). Results are presented as the mean of three independent experiments using eosinophils obtained from two separate volunteers.

Figure 6

***Ex vivo* CCL11-induced eosinophil shape change in cynomolgus monkeys and inhibition of shape change by the oral administration of YM-355179.** Whole blood samples were collected 2 h (A), and 0, 2, 6, and 12 h (B) after oral administration of YM-355179 (1 mg/kg). Blood samples were stimulated with various concentrations of human CCL11 (0-36 nM).

Increases in eosinophil shape change were determined using GAFS assay, as described in Materials and Methods. Results are representative of the mean ± SEM from n=5 monkeys, performed in duplicate. Statistical significance between the placebo group and the YM-355179-treated group was analyzed using Paired *t*-test with**P*<0.05. Statistical significance between the time point control group (0 h) and YM-355179-treated groups at various times after administration was analyzed using Two-way Dunnett-test with #*P*<0.05.

Figure 7

Effect of CCR3 antagonist, YM-355179, on leukocyte infiltration into airways of cynomolgus monkeys after segmental provocation with CCL11. A: Total leukocytes, B: Eosinophils, C: Basophils and mast cells, D: Lymphocytes, E: Monocytes and macrophages, and F: Neutrophils. YM-355179 (1 mg/kg) was intravenously injected 5 min before and 24 h after segmental provocation with CCL11 (30 pmol/site). Results are presented as mean ± SEM (n=7 monkeys per group) and are representative of two separate experiments. Statistical significance between the baseline group (not CCL11-infused) and placebo group (saline-treated), plus between the placebo group and YM-355179-treated group were analyzed using a Paired *t*-test with **P*<0.05 and #*P*<0.05, respectively.

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Table 1. Effect of YM-355179 on CCR3 from various species

	Species			
CCR3	Human	Monkey	Mouse	Rat
CCL11	Human	Human	Mouse	Mouse
IC_{50} (nM)	7.6	12	460	>10000

Human and monkey CCR3-expressing B300-19 cells were stimulated with human CCL11 at 3 and 12-nM, respectively. Murine CCR3-transfected CHO and rat CCR3-transfected HEK293 cells were stimulated with murine CCL11 (12 nM). Ca^{2+} influx methods are described in Materials and Methods. Results are presented as the mean of two independent experiments.

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Figure 1

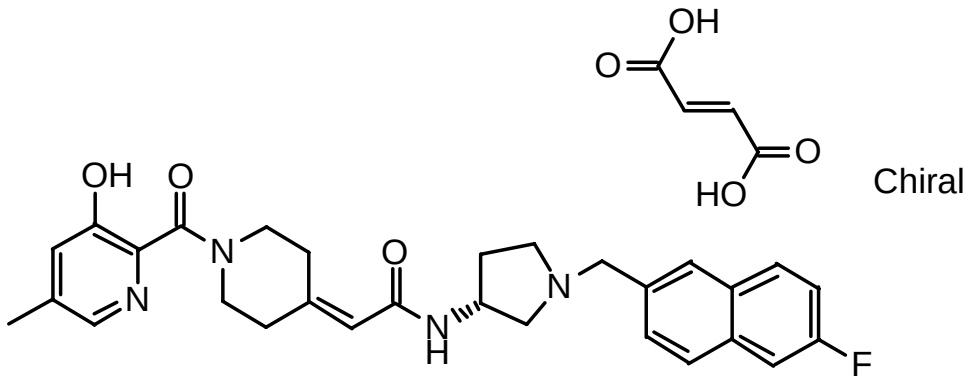

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Figure 2A

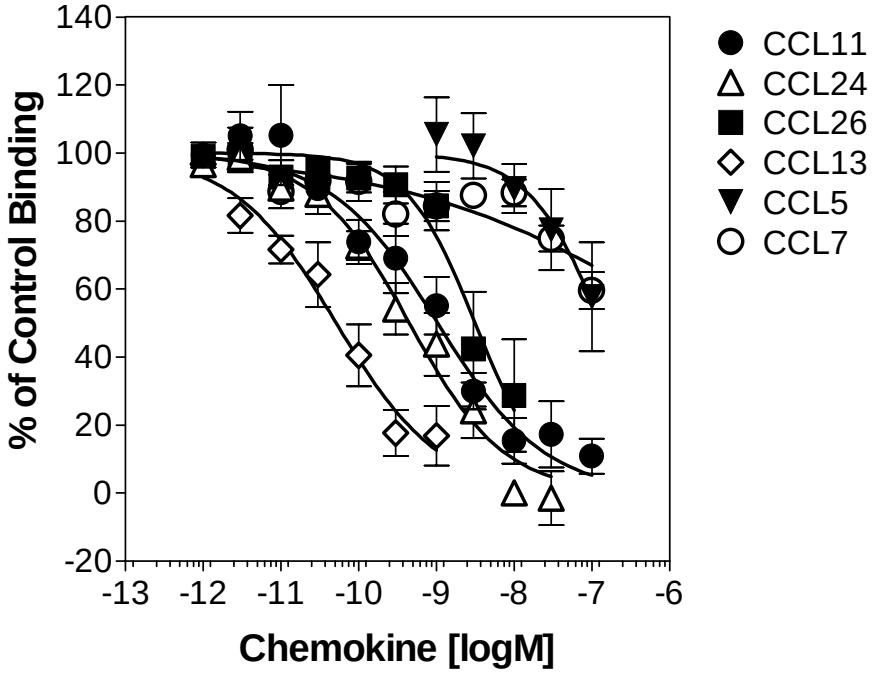

Figure 2B

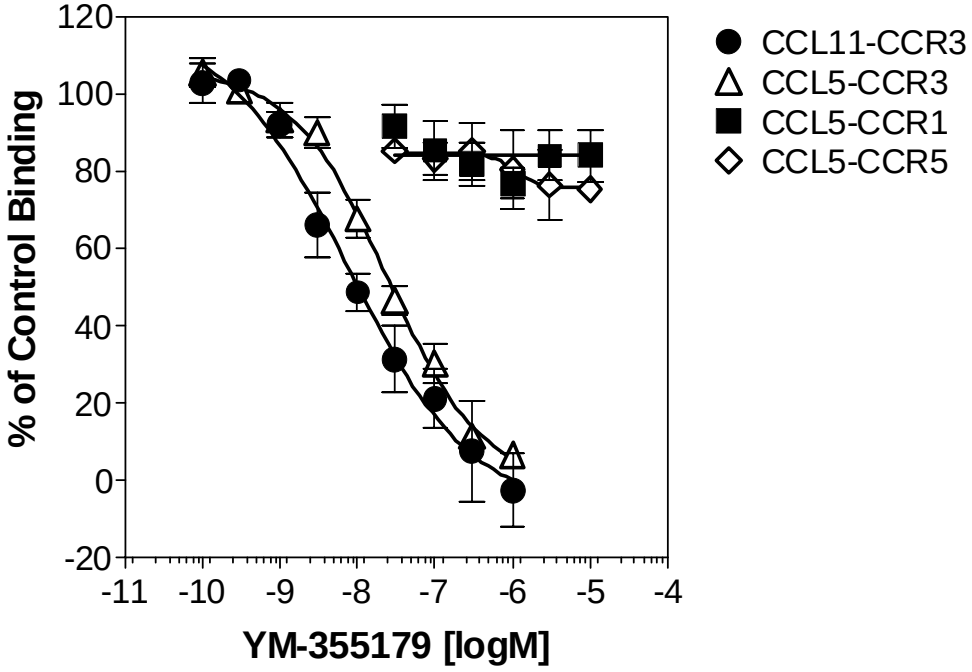

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Figure 3A

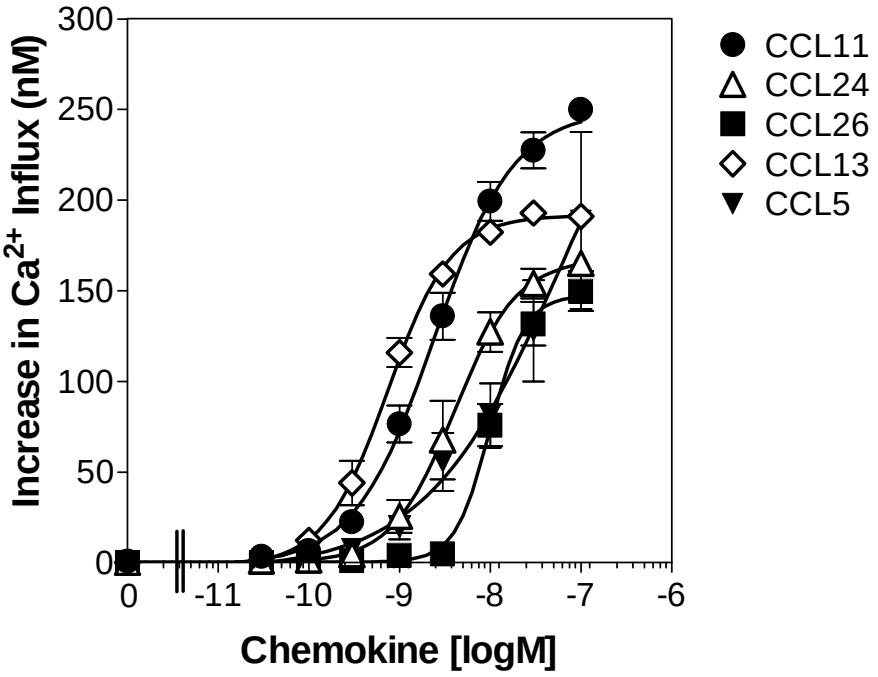

Figure 3B

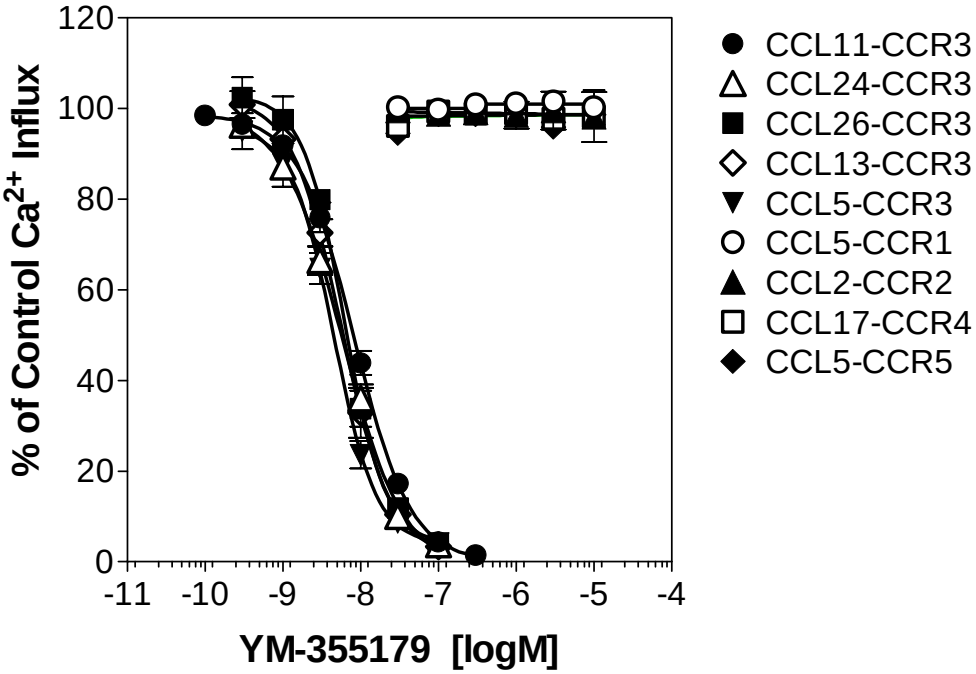

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Figure 4A

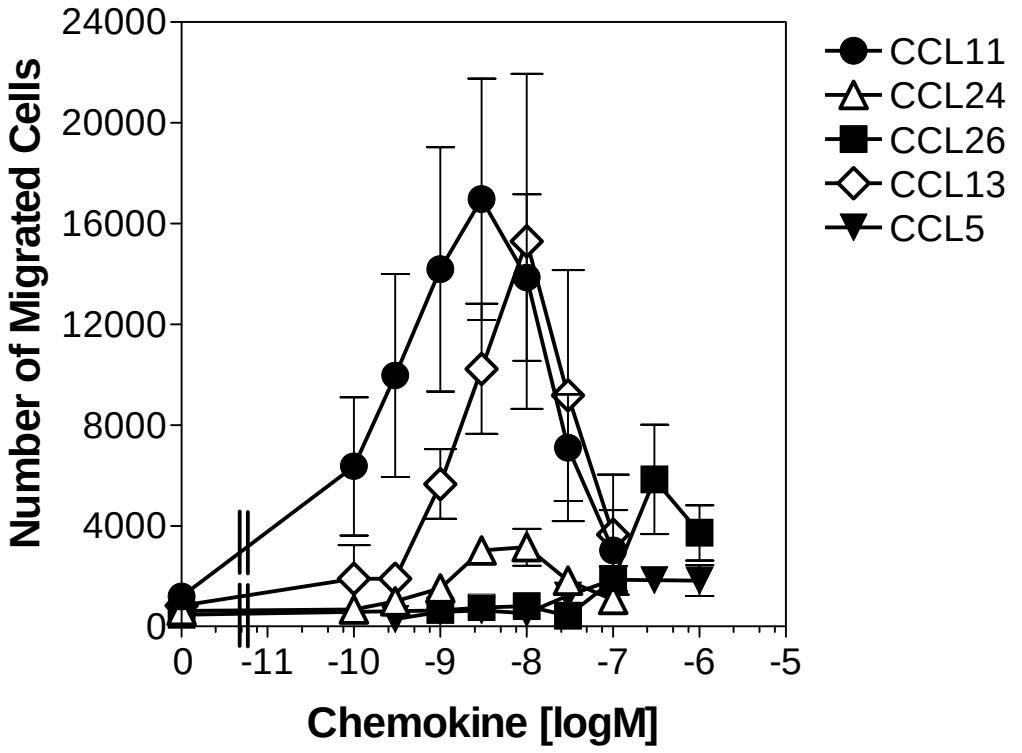

Figure 4B

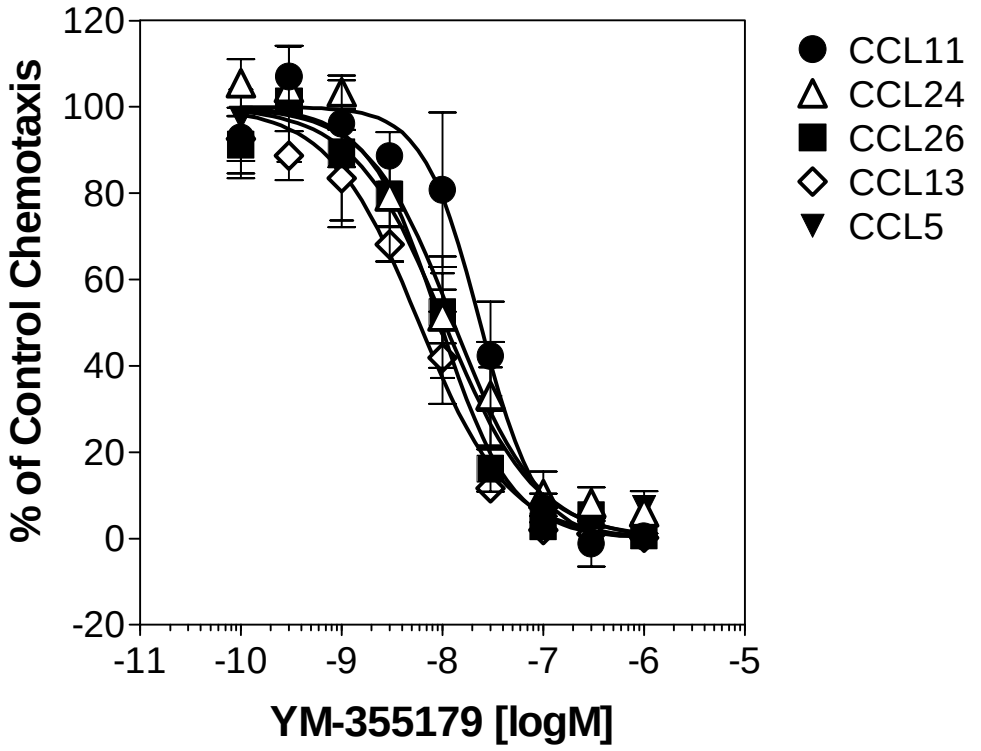

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Figure 5

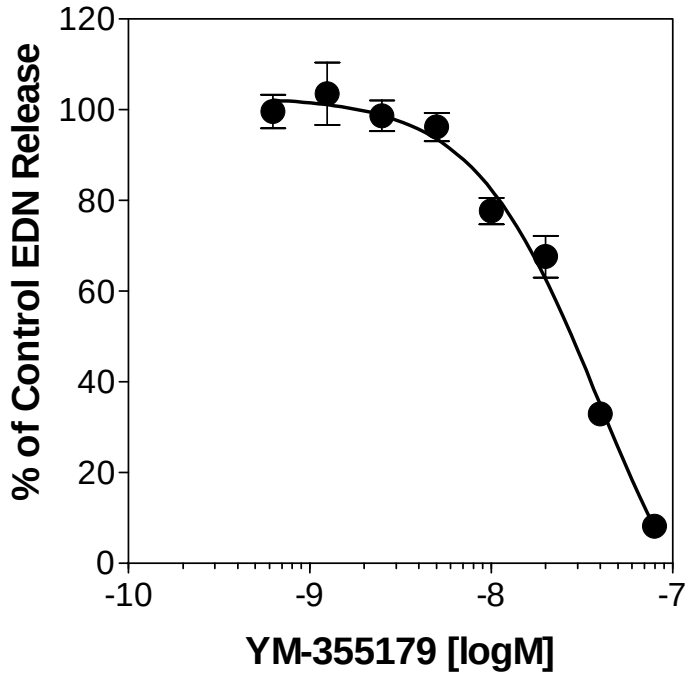

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Figure 6A

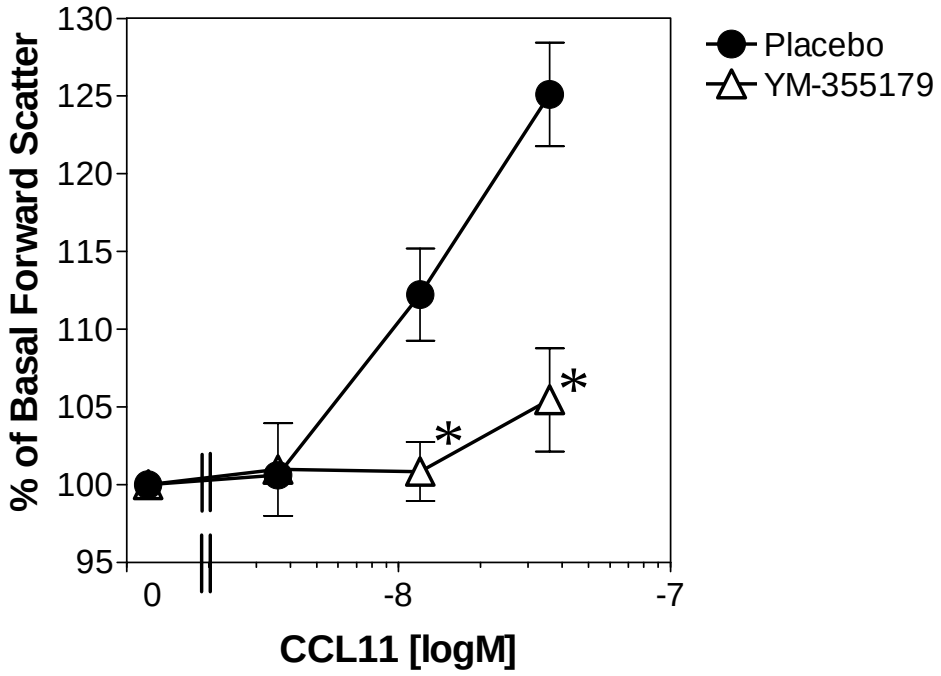

Figure 6B

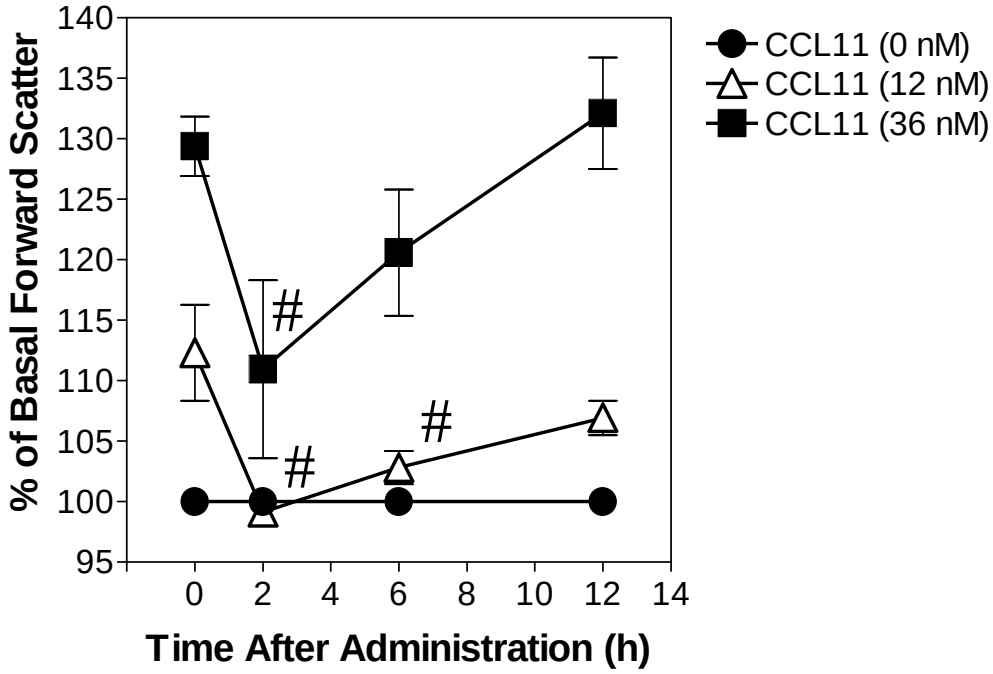

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Figure 7