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A dual antagonist for chemokine CCR3 receptor and histamine H_1 receptor

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Abstract

Eosinophilic chemokines and histamine play distinct but important roles in allergic diseases. Inhibition of both eosinophilic chemokines and histamine, therefore, is an ideal strategy for the treatment of allergic inflammation, such as asthma, allergic rhinitis, and atopic dermatitis. YM-344484 was found to potently inhibit both the CCL11-induced Ca^{2+} influx in human CCR3-expressing cells (K_b=1.8 nM) and histamine-induced Ca^{2+} influx in histamine H_1 receptor-expressing PC3 cells (K_b=47 nM). YM-344484 also inhibited the CCL11-induced chemotaxis of human CCR3-expressing cells (IC_{50}=6.2 nM) and CCL11-induced eosinophil-derived neurotoxin release from human eosinophils (IC_{50}=19 nM). Orally administered YM-344484 inhibited the increase in histamine-induced vascular permeability in mice (82% inhibition at a dose of 10 mg/kg) and the accumulation of eosinophils in a mouse asthma model (74% at a dose of 300 mg/kg). These results indicate that YM-344484, a novel and functional dual antagonist for chemokine CCR3 receptor and histamine H_1 receptor, is an attractive candidate for development as a novel anti-allergic inflammation drug.

Keywords: CCR3; CCL11; Histamine H_1 receptor; Histamine; Asthma; Eosinophil

1. Introduction

When allergy patients are challenged with an allergen to which they have previously been sensitized, they exhibit an immediate phase allergic response, such as bronchoconstriction, a runny nose, and the appearance of wheals and flares on the skin. Immediate phase allergic response is mainly due to chemical mediators, such as histamine, serotonin, and leukotriene, being released from mast cells (Gomez et al., 1986). Histamine H_1 receptor antagonists are widely used for the treatment of wheals, flares, and itch (Meltzer, 1998). Several hours after the immediate phase response, allergic patients often develop a delayed, more sustained local inflammation, known as the late phase allergic response. This is characterized by the local accumulation of activated inflammatory cells, including eosinophils, monocytes, and T-lymphocytes (Gaga et al., 1991). Of these cells, eosinophils are of particular importance in terms of tissue damage (Rothenberg, 1998), and it is chemokine CCR3 receptor and its ligands, such as CCL11, CCL24, CCL26, CCL7, CCL13 and CCL5, which control the selective recruitment of eosinophils from blood into the inflammatory tissue (Jose et al., 1994a,b; Ponath et al., 1996a,b; Daugherty et al., 1996; Forssmann et al., 1997; Shinkai et al., 1999). Preclinical studies using mice suggest that CCR3 and its ligands play a pivotal role in the infiltration of eosinophils during allergic inflammation. Targeted disruption of CCL11 weakened antigen-induced tissue eosinophilia in mouse allergic models, such as those for experimental allergic airway disease and onchocercal keratitis, with 70% and 50% inhibition, respectively (Rothenberg et al., 1997). The recruitment of eosinophils into the bronchoalveolar lavage fluid is impaired in CCR3-deficient mice (89% inhibition), and these mice fail to develop

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airway hyperresponsiveness following antigen inhalation (Ma et al., 2002). Several research groups, including this one, have suggested that the blockade of CCR3 ligands and CCR3 interaction has potential as an effective and attractive therapy for allergic diseases using potent and selective small-molecule CCR3 antagonists (Sabroe et al., 2000; White et al., 2000; Bryan et al., 2002; De Lucca et al., 2005; Suzuki et al., 2006; Morokata et al., 2006).

Since the biosynthesis and/or release of multiple mediators is involved in the pathophysiology of allergic inflammatory diseases, an agent with multi-inhibitory ability, such as a broad spectrum receptor antagonist, is likely to be more effective than single inhibitors for the treatment of inflammatory conditions. In this study, it was discovered that YM-344484 selectively inhibited the ligand-induced Ca^{2+} influx mediated by both CCR3 and histamine H_1 receptor. Orally administered YM-344484 significantly inhibited both increases in histamine-induced vascular permeability in mice and the accumulation of eosinophils in a mouse asthma model at doses of 10 mg/kg and 300 mg/kg, respectively. These results suggest that the novel dual antagonist for CCR3 and histamine H_1 receptor, YM-344484, has good potential for the treatment of allergic inflammatory conditions, such as asthma, allergic rhinitis, and atopic dermatitis.

2. Materials and methods

2.1. Reagents

YM-344484 (Fig. 1) was chemically synthesized in our laboratories. This compound was dissolved in 100% dimethyl sulfoxide before use. All chemokines were purchased from Pepro Tech (Rocky Hill, NJ, USA), cell culture reagents from Gibco (Rockville, MD, USA), and general laboratory reagents from Sigma (St. Louis, MO, USA).

2.2. Cells and cell culture

All chemokine receptor-expressing cells were generated as described previously (Sato et al., 1999; Suzuki et al., 2006). Briefly, cDNA for human CCR1, 2, 3, 4, 5, and CXCR3 were generated using reverse transcription-polymerase chain reaction (RT-PCR) from human spleen mRNA (Becton Dickinson, Franklin Lakes, NJ, USA). The amplified cDNA fragments were subcloned into pEF-BOS (Mizushima and Nagata, 1990) and stably expressed in murine pre-B cell lymphoma B300-19 cells (Alt et al., 1984). Each clonal line was first screened for ligand-induced Ca^{2+} mobilization in order to identify the cell line that responded best. The lines were then maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml β-mercaptoethanol, and 1 mg/ml G418. The cultures were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. cDNA for murine CCR3 was obtained from murine spleen mRNA (Becton Dickinson) using PCR. Amplified cDNA fragments were subcloned into pCR3.1 and then stably expressed in CHO cells. Transfectants expressing murine CCR3 were obtained and maintained in the same manner as human CCR3-expressing cells.

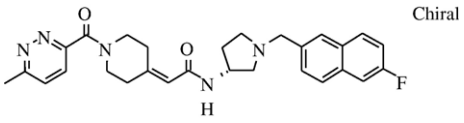

Fig. 1. Chemical structure of YM-344484, *N*-{(3*R*)-1-[(6-fluoro-2-naphthyl) methyl]pyrrolidin-3-yl}-2-{1-[(6-methylpyridazin-3-yl)carbonyl]piperidin-4-ylidene}acetamide.

PC3 (human prostate cancer cells), which express histamine H_1 receptor (Lee et al., 2001), were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Rat C6 BU-1 glioma cells, which express serotonin 5-HT_{2A} receptor (Miyoshi et al., 2001), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After culturing for 3–4 days, C6 cells were incubated with Dulbecco's modified Eagle's medium in the absence of fetal bovine serum for 1 day, as previously described (Miyoshi et al., 2001).

2.3. Calcium mobilization experiments

Human CCR3-expressing B300-19 cells were loaded with 5 μM fura-2 acetoxymethyl ester in RPMI 1640 media containing 1% fetal bovine serum for 30 min at 37 °C in the dark. After being washed twice, the cells were diluted to a concentration of 2×10^6 cells/ml with 20 mM HEPES buffer containing 0.1% bovine serum albumin, 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 to cuvettes. YM-344484 solution (1 μl; 50–50,000 nM in dimethyl sulfoxide) or vehicle (1 μl) was added to a cuvette 1 min prior to the addition of 10 μl CCL11 (final concentration 3 nM) with constant agitation. Changes in fluorescence were monitored at 25 °C using an intracellular ion analyzer CAF-110 (Nihon Bunkoh, Tokyo, Japan) at excitation wavelengths of 340 nm and 380 nm, and an emission wavelength of 510 nm. To assess the inhibitory effect of YM-344484 on other CCR3 ligands, CCL24 (3 nM), CCL26 (10 nM), CCL13 (1 nM), and CCL5 (10 nM) were used. The dissociation constant (K_b) of the antagonist was estimated using an alternative to the Cheng-Prusoff equation (Lazareno and Birdsall, 1993).

To evaluate the ligand and receptor selectivities, human CCR1-expressing cells were loaded with fura-2 and stimulated with CCL5 (6 nM) or CCL7 (5 nM). Human CCR2-, CCR4-, CCR5-, and CXCR3-expressing cells loaded with fura-2 were stimulated with CCL2 (6 nM), CCL22 (1 nM), CCL5 (6 nM), and CXCL10 (3 nM), respectively. To assess cross-reactivity between species, murine CCR3-expressing cells loaded with fura-2 were stimulated with murine CCL11 (10 nM).

To evaluate the effect of YM-344484 on histamine H_1 receptor and serotonin 5-HT_{2A} receptor, PC3 cells and C6 cells were loaded with fura-2, then stimulated with histamine (3 μM) and serotonin (0.3 μM), respectively.

2.4. Radioligand binding assays

Radioligand binding assays were performed to investigate the selectivity of YM-344484 for several G-protein-coupled seven-transmembrane receptors, namely histamine H_1, H_2, serotonin 5-HT_{1A}, bradykinin B_1, B_2, leukotriene BLT_1, $CysLT_1$, muscarinic M_1, M_2, M_3, and tachykinin NK_1. The membrane specimens and radioligands used are listed in Table 1. The effect of 10 μM YM-344484 on these binding assay systems was examined.

2.5. Chemotaxis assays

Measurement of chemotaxis using human CCR3-expressing B300-19 cells was performed using a modified Boyden chamber procedure, as described previously (Boyden, 1962). Briefly, lower chambers were filled with 33 μl of each chemokine, CCL11 (3 nM), CCL24 (10 nM), CCL26 (100 nM), CCL13 (10 nM) or CCL5 (100 nM), and separated from the upper chamber by a polycarbonate filter with 5 μm pores (Neuro Probe, Gaithersburg, MD, USA). The upper chamber was then filled with 200 μl of cell suspension (5×10^5 cells) in chemotaxis assay buffer (RPMI 1640 supplemented with 1 mg/ml bovine serum albumin) containing various concentrations of YM-344484 or 0.2% dimethyl sulfoxide. Cell migration was allowed to proceed for 3 h at 37 °C in a 5% CO_2 incubator, after which the chamber was disassembled. The number of cells that migrated to the lower chamber was quantified by measuring the ATP content using an ATPlite ATP detection system (Perkin Elmer, Wallesley, MA, USA).

Table 1
Activity of YM-344484 in radioligand binding assays

Receptor	Source	Radioligand	% Inhibition at 10 μM
Histamine H_1	Human recombinant	[^{3}H] Pyrilamine	95
Histamine H_2	Human recombinant	[^{3}H] Aminopotentidine	10
Serotonin 5-HT_{1A}	Human recombinant	[^{3}H] 8-OH-DPAT	36
Bradykinin B_1	Human IMR-90 cells	[^{3}H] (Des-Arg^{10})-kallidin	8
Bradykinin B_2	Human recombinant	[^{3}H] Bradykinin	8
Leukotriene BLT_1	Human U937 cells	[^{3}H] LTB_4	13
Leukotriene $CysLT_1$	Human recombinant	[^{3}H] LTD_4	2
Muscarinic M_1	Human recombinant	[^{3}H] N-Methylscopolamine	14
Muscarinic M_2	Human recombinant	[^{3}H] N-Methylscopolamine	32
Muscarinic M_3	Human recombinant	[^{3}H] N-Methylscopolamine	27
Tachykinin NK_1	Human recombinant	[^{3}H] SR-140333	2

2.6. 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 the peripheral blood of normal volunteers. The wells of 96-well flat bottom tissue culture plates (Costar, Cambridge, MA, USA) were blocked with 50 μl of 2.5% bovine serum albumin diluted in PBS for 2 h at 37 °C. After washing the wells, 200 μl of a suspension of eosinophils in RPMI 1640 medium containing 0.5% bovine serum albumin (5×10^5 cells), with or without 0.6 nM human CCL11, was added to the wells, and 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-344484. After incubation, the supernatants were collected and frozen at −20 °C until use. The amount of neurotoxin in the supernatants was measured using an enzyme-linked immunosorbent assay kit purchased from MBL International (Woburn, MA, USA).

2.7. Histamine-induced acceleration of plasma leakage in mouse skin

Histamine-induced skin reactions were performed according to a modified method described previously (Inagaki et al., 1999). Briefly, six-week-old female Balb/c mice ($n=6$–7 per group; Charles River, Hamamatsu, Japan) with shaved backs were given an oral administration of the vehicle (0.5% CMC solution, 10 ml/kg), YM-344484 (3, 10, and 30 mg/kg), or ketotifen (0.3 mg/kg). One h later, the mice received saline (200 μl per animal) containing 1.25 mg Evans blue intravenously, and an intradermal injection of histamine solution (2 μg/10 μl) was given as the same time. The normal control mice received an intradermal saline injection instead of histamine, and the naive mice received neither. After 30 min, the mice were sacrificed by decapitation and the skin was removed. The skin was incubated overnight in 2 ml formamide at 37 °C with shaking. The amount of dye, an index of increased plasma leakage, was measured by light absorbance at 620 nm using a spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). This was calculated from a standard curve of dye concentrations in the range of 0.078–10 μg/ml, and was expressed as μg per site.

2.8. Ovalbumin-induced asthmatic model in mice

Female Balb/c mice ($n=10$ per group; Charles River) were sensitized by an intraperitoneal injection of 20 μg ovalbumin and 2 mg aluminum hydroxide gel dissolved in 200 μl PBS on the first day. Animals received a booster injection of this ovalbumin-aluminum hydroxide gel mixture 14 days later. Twenty-eight days after the first sensitization, mice were exposed to aerosolized ovalbumin solution (2% in saline) for 20 min. One day after ovalbumin challenge, the mice were again exposed for 20 min. Normal control mice were sensitized with the ovalbumin–aluminum hydroxide gel mixture twice and inhaled saline (vehicle) instead of aerosolized ovalbumin. Two

days after the first ovalbumin challenge, the mice were sacrificed by intraperitoneal injection of sodium pentobarbital. Their tracheae were cannulated, and bronchoalveolar lavage was performed using four separate 1 ml aliquots of saline containing 1 U/ml heparin. The total number of cells from bronchoalveolar lavage fluid was counted using an automated cell counter (Celltac-α, Nihon Kohden, Tokyo, Japan). A differential cell count was performed using a cytospin preparation stained with May-Giemsa. A total of 500 cells was counted to calculate the eosinophil, neutrophil, and mononuclear cell percentage of the total white blood cell count using standard morphological criteria. Either the vehicle (0.5% CMC solution, 10 ml/kg) or YM-344484 (100, 300, and 600 mg/kg) was orally administered 1 h before the first ovalbumin challenge, and then every 8 h afterward. Bronchoalveolar lavage was performed 8 h after the sixth administration.

2.9. Statistical analysis

Results are expressed as the mean±standard error of the mean (S.E.M.). Either the Student's *t*-test or Dunnett's multiple range test was employed for data evaluation. Values of $P<0.001$, $P<0.01$, and $P<0.05$ were considered to be statistically significant.

2.10. Animal ethics

All experiments were performed in accordance with the regulations of the company's Animal Ethical Committee.

3. Results

3.1. A CCR3 and histamine H_1 receptor dual antagonist

YM-344484 (Fig. 1) was discovered through focused synthesis of potent CCR3 antagonists. Human CCR3-expressing cells showed transient concentration-dependent (0.03–100 nM) increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to CCR3 ligands, such as CCL11, CCL24, CCL26, CCL13, and CCL5, as previously described (Morokata et al., 2006). Fig. 2A shows the curves for YM-344484 inhibition in the presence of a fixed concentration of ligands. YM-344484 potently and concentration-dependently inhibited the CCL11 (3 nM), CCL24 (3 nM), CCL26 (10 nM), CCL13 (1 nM), and CCL5 (10 nM)-induced elevation of $[Ca^{2+}]_i$ with K_b values of 1.8, 2.5, 2.5, 1.7, and 0.69 nM, respectively. The murine CCR3 amino acid sequence is only 68% homologous to human CCR3 (Post et al., 1995; Daugherty et al., 1996; Zhang et al., 2002). Therefore YM-344484 showed obvious species selectivity, with a K_b value of 88 nM for the murine CCL11-induced elevation of $[Ca^{2+}]_i$ in murine CCR3-expressing cells (Fig. 2A), which was about 50-fold weaker than the K_b value for human CCR3.

It has been reported that certain CCR3 antagonists also interact with CCR1 (Sabroe et al., 2000) due to the 62% amino acid sequence homology between the two (Ponath et al., 1996a). Human CCR1-expressing cells showed a transient increase in $[Ca^{2+}]_i$ in response to CCR1 ligands, such as CCL5 and CCL7. However, YM-344484 showed neither agonistic nor antagonistic activity toward CCR1 at concentrations up to 10 μM (Fig. 2B). Furthermore, YM-344484 failed to affect CCR2-, CCR4-, CCR5-, and CXCR3-mediated $[Ca^{2+}]_i$ elevation (CCL2-induced, CCL22-induced, CCL5-induced, and CXCL10-induced, respectively, data not shown). These results suggest that YM-344484 is at least 1000 times more selective for CCR3 over the other chemokine receptors tested.

Chemokine receptors belong to a family of G-protein-coupled seven-transmembrane receptors. Radioligand binding assays were conducted in order to investigate the receptor selectivity of YM-344484 over other G-protein-coupled seven-transmembrane receptors associated with inflammation or bronchoconstriction. Although YM-344484 had subtle effects on the ligand binding of histamine H_2 receptor, serotonin 5-HT_{1A} receptor, bradykinin B_1, B_2 receptor, leukotriene BLT_1, $CysLT_1$ receptor, muscarinic M_1, M_2, M_3 receptor, and tachykinin NK_1 receptor at 10 μM, it was found to strongly

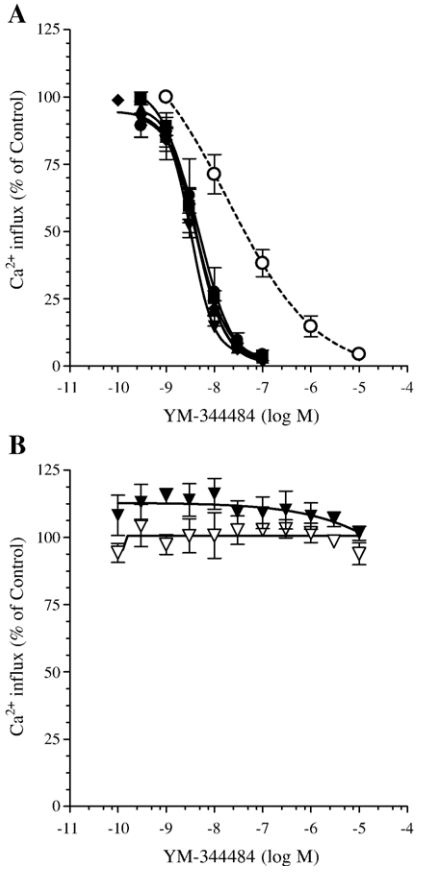

Fig. 2. CCR3 antagonistic activity of YM-344484 *in vitro*. A: Inhibition of increase in $[Ca^{2+}]_i$ stimulated by various ligands in CCR3-B300-19 cells. Data are the mean±S.E.M. of multiple experiments ($n=3$–4). Chemokines and cells are designated using the following symbols: human CCL11 (●), CCL24 (▲), CCL26 (■), CCL13 (◆), CCL5 (▼) in human CCR3-B300-19 cells, and murine CCL11 (○) in murine CCR3-B300-19 cells. B: Effect on the increase in $[Ca^{2+}]_i$ stimulated by ligands in CCR1-B300-19 cells. Data are the mean±S.E.M. of multiple experiments ($n=4$). Chemokines and cells are designated using the following symbols: human CCL5 (▼), and CCL7 (▽) in human CCR1-B300-19 cells.

inhibit the binding of [^{3}H] pyrilamine to histamine H_1 receptor (95% inhibition at 10 μM; Table 1). It is known that PC3 cells express histamine H_1 receptor (Lee et al., 2001). In this study, histamine dihydrochloride (30–100,000 nM) caused a transient elevation of $[Ca^{2+}]_i$ in PC3 cells. This $[Ca^{2+}]_i$ elevation was inhibited when cells were preincubated briefly with histamine H_1 receptor antagonists, such as pyrilamine and ketotifen (K_b values of 2.7 and 6.4 nM, respectively, Fig. 3A), which suggests that the histamine-induced elevation of $[Ca^{2+}]_i$ in PC3 cells was mediated by histamine H_1 receptor. YM-344484 was found to inhibit histamine H_1 receptor-mediated $[Ca^{2+}]_i$ elevation in a concentration-dependent fashion, with K_b values of 47 nM (Fig. 3A). Antagonistic activity against histamine H_1 receptor was 17 and 7 times less potent than that against pyrilamine and ketotifen, respectively. C6 cells are known to express serotonin 5-HT_{2A} receptor (Miyoshi et al., 2001). Serotonin (1–100,000 nM) causes a transient $[Ca^{2+}]_i$ elevation in C6 cells. Although a 5-HT_{2A} receptor antagonist (ketanserin) inhibited this elevation in $[Ca^{2+}]_i$ [K_b: 1.1 nM (Fig. 3B)], YM-344484 did not potently inhibit 5-HT_{2A} receptor-mediated $[Ca^{2+}]_i$ signals (25% inhibition at 10 μM, Fig. 3B). These *in vitro* assays suggest that YM-344484 is a functional and selective dual antagonist for chemokine CCR3 and histamine H_1 receptor.

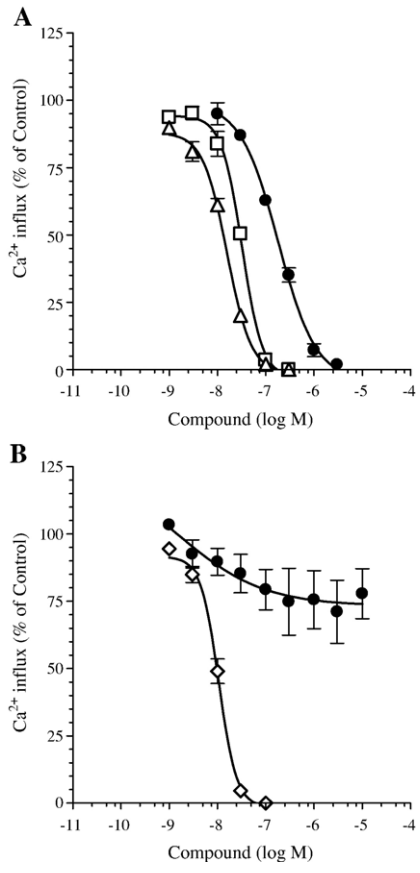

Fig. 3. Histamine H_1 receptor antagonistic activity of YM-344484 *in vitro*. A: Inhibition of increase in $[Ca^{2+}]_i$ stimulated by histamine in PC3 cells. Data are the mean±S.E.M. of multiple experiments (n=3–4). Compounds are designated using the following symbols: YM-344484 (●), pyrilamine (△), and ketotifen (□). B: Effect on increase in $[Ca^{2+}]_i$ stimulated by serotonin in C6 cells. Data are the mean±S.E.M. of multiple experiments (n=3–4). Compounds are designated using the following symbols: YM-344484 (●), and ketanserin (◇).

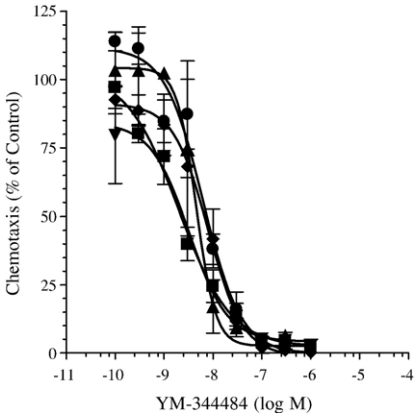

Fig. 4. Inhibition of ligand-induced chemotaxis of CCR3-B300-19 cells. Data are the mean±S.E.M. of multiple experiments (n=3–4). Chemokines are designated using the following symbols: CCL11 (●), CCL24 (▲), CCL26 (■), CCL13 (♦), and CCL5 (▼).

3.2. Inhibition of CCR3-mediated chemotaxis

The effect of YM-344484 on the inhibition of chemotaxis mediated by CCR3 was examined. Human CCR3-expressing cells readily migrated toward all five CCR3 ligands (CCL11, CCL24, CCL26, CCL13, and CCL5, Morokata et al., 2006). CCL11 (3 nM), CCL24 (10 nM), CCL26 (100 nM), CCL13 (10 nM), and CCL5 (100 nM)-induced chemotaxis were concentration dependently inhibited by YM-344484 with potent IC_{50} values of 6.2, 4.5, 2.0, 8.0, and 3.2 nM, respectively (Fig. 4).

3.3. Inhibition of eosinophil-derived neurotoxin release from human eosinophils

YM-344484 was evaluated for its ability to inhibit human eosinophil function. CCL11 is a strong initiator for the release of the eosinophil granules that are partially responsible for the lung damage that results from asthma (Gutierrez-Ramos et al.,

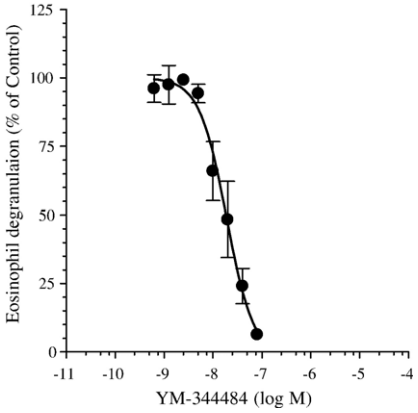

Fig. 5. Inhibition of CCL11-induced eosinophil-derived neurotoxin release from human eosinophils. Human peripheral eosinophils were stimulated with CCL11 (0.6 nM) in the presence of YM-344484. Data are the mean of four experiments using eosinophils obtained from two separate volunteers.

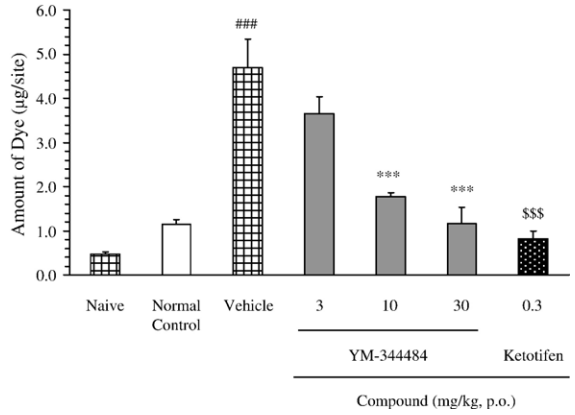

Fig. 6. Prevention of histamine-induced acceleration of plasma leakage in mouse skin. Vehicle (0.5% CMC solution, 10 ml/kg), YM-344484 (3, 10, and 30 mg/kg), or ketotifen (0.3 mg/kg) were orally administered 1 h before intradermal injection of histamine (2 μg). Each value represents the mean + S.E.M. for 6 or 7 mice. ###$P<0.001$ compared to the normal control group (Student's *t*-test); ***$P<0.001$ compared to vehicle control (Dunnett's multiple range test); $$$$P<0.001$ compared to vehicle control (Student's *t*-test).

1999; Rankin et al., 2000). The amount of eosinophil-derived neurotoxin from purified human eosinophils was measured as a marker of CCL11-induced degranulation. YM-344484 inhibited CCL11 (0.6 nM)-induced eosinophil-derived neurotoxin release from human eosinophils with an IC_{50} value of 19 nM (Fig. 5).

3.4. Prevention of histamine-induced skin reaction in mice

Histamine is important in the acceleration of vascular permeability. YM-344484 was evaluated for its ability to behave as a histamine H_1 receptor antagonist *in vivo* using histamine-induced cutaneous reactions in mice. Although intradermal injection of saline (10 μl) had no effect on vascular permeability, intradermal injection of histamine (2 μg) induced a skin reaction, which was caused by an increase in plasma leakage. Histamine-induced vascular permeability was totally inhibited when mice were given the anti-histamine drug ketotifen (0.3 mg/kg). Lower doses of ketotifen (0.01 and 0.1 mg/kg) had no effect (data not shown). YM-344484 (3, 10, and 30 mg/kg) dose-dependently suppressed the increase in vascular permeability when orally administered 1 h before histamine injection. This suppression was statistically significant at doses of 10 mg/kg or greater, and was complete at a dose of 30 mg/kg (Fig. 6). These results suggest that YM-344484 is a strong histamine H_1 receptor antagonist *in vivo*.

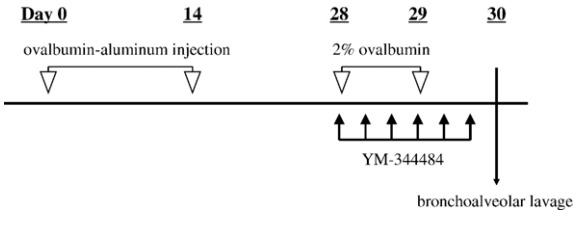

Fig. 7. Protocols for the ovalbumin-induced asthma model and treatment with YM-344484. On days 0 and 14, mice were sensitized by means of intraperitoneal injections of ovalbumin–aluminum hydroxide gel mixture. The airway challenge was performed on days 28 and 29. Vehicle (0.5% CMC solution) or YM-344484 (100, 300, and 600 mg/kg) were administered every 8 h on the challenge days. Bronchoalveolar lavage was performed 8 h after the last administration.

Table 2
Effect of YM-344484 on antigen-induced lung inflammation in mice

Ovalbumin challenge	Treatment	mg/kg, po	Number of cells ($\times 10^5$ cells)		
			Eosinophils	Neutrophils	Mononuclear cells
−	−	−	0.01±0.00	0.03±0.01	3.52±0.16
+	Vehicle	−	1.41±0.22 [a]	0.60±0.11 [a]	3.58±0.18
+	YM-344484	100	1.74±0.34	0.40±0.14	3.64±0.20
+	YM-344484	300	0.38±0.13 [b]	0.32±0.09	3.25±0.16
+	YM-344484	600	0.01±0.00 [b]	0.42±0.12	4.37±0.41

Animals ($n=10$ per group) were dosed orally with vehicle (0.5% CMC solution, 10 ml/kg) or YM-344484 (100, 300, and 600 mg/kg) 1 h before the first antigen challenge, then every 8 h thereafter.
[a] $P<0.001$ compared with the unchallenged group (Student's *t*-test).
[b] $P<0.05$ compared with the vehicle control (Dunnett's multiple range test).

3.5. Protection against lung inflammation in an ovalbumin-induced asthmatic model

The effect of YM-344484 on lung inflammation was examined in an antigen-induced asthmatic model in mice (Fig. 7). The dose and dose regimen of YM-344484 was determined based on its pharmacokinetic data, plasma protein binding, and the K_b value for murine CCR3. YM-344484 was highly protein bound (97.2%) in murine plasma. At 1 and 8 h after administration of YM-344484 (300 mg/kg, po), the plasma concentrations of the protein-unbound form were 528 and 78 nM, respectively (data not shown). When 300 mg/kg was dosed every 8 h, the plasma concentration of the unbound form of YM-344484 was considered to be continually higher than or equal to the K_b value for murine CCR3 (88 nM, Fig. 2A). Therefore, YM-344484 was administered orally at doses ranging from 100 to 600 mg/kg. Given the data above, it is likely that YM-344484 would strongly antagonize histamine H_1 receptor at these dose levels (Fig. 6). Abnormal behavior was not seen at any of the tested dose levels. When challenged with aerosolized ovalbumin, a significant increase in the numbers of eosinophils and neutrophils in the bronchoalveolar lavage fluid in sensitized mice was observed by means of differential cell count analysis. Although a lower dose of YM-344484 (100 mg/kg, t.i.d.) did not change the number of eosinophils, YM-344484 significantly attenuated eosinophil infiltration at 300 and 600 mg/kg, t.i.d. (74% and 100% inhibition, respectively, Table 2). In contrast, YM-344484 had no inhibitory effect on the number of neutrophils or mononuclear cells in the bronchoalveolar lavage fluid.

4. Discussion

Eosinophilic chemokines and histamine play distinct but important roles in allergic diseases. Inhibition of both

eosinophilic chemokines and histamine, therefore, is an ideal strategy for the treatment of allergic inflammation, such as asthma, allergic rhinitis, and atopic dermatitis. YM-344484 was found to potently inhibit both the CCL11-induced Ca^{2+} influx in human CCR3-expressing cells (K_b=1.8 nM) and the histamine-induced Ca^{2+} influx in histamine H_1 receptor-expressing PC3 cells (K_b=47 nM). However, YM-344484 showed no antagonism against CCR1-, CCR2-, CCR4-, CCR5-, CXCR3-or 5-HT_{2A} -mediated $[Ca^{2+}]_i$ elevation. YM-344484 also had subtle effects on the ligand binding of histamine H_2 receptor, serotonin 5-HT_{1A} receptor, bradykinin B_1, B_2 receptor, leukotriene BLT_1, $CysLT_1$ receptor, muscarinic M_1, M_2, M_3 receptor, and tachykinin NK_1 receptor at a concentration of 10 μM. These results demonstrate that YM-344484 is a novel and selective dual antagonist for CCR3 and histamine H_1 receptor.

It has been shown that murine CCL11 mRNA levels parallel the kinetics of eosinophil accumulation in the lungs during experimentally-induced allergic inflammation (Gonzalo et al., 1996). Detailed immunohistochemistry testing performed on lung tissue from both asthmatic patients and animal models of asthma indicate that there are many potential cellular sources of CCL11, including the airway epithelium, alveolar macrophages, bronchial smooth muscle, and chondrocytes (Ying et al., 1997).

CCR3 and its ligands are important not only for eosinophil chemotaxis and migration (Jose et al., 1994a,b; Ponath et al., 1996a,b; Daugherty et al., 1996; Forssmann et al., 1997; Shinkai et al., 1999), but also for the activation of their cytotoxic response, which is mediated by the release of proteins stored in their granules (Gutierrez-Ramos et al., 1999; Rankin et al., 2000). These proteins include eosinophil-derived neurotoxin, major basic protein, eosinophil cationic protein, and eosinophil peroxidase. The physiological role of these cytotoxic products is thought to be the destruction of parasitic helminthes, but they are also likely to be partially responsible for the airway epithelium damage and late-phase allergic hypersensitivity response seen with asthma (Gutierrez-Ramos et al., 1999; Rankin et al., 2000). YM-344484 inhibited eosinophil-derived neurotoxin release from human eosinophils (IC_{50}=19 nM) at approximately the same concentration that was effective in the $[Ca^{2+}]_i$ influx (K_b=1.8 nM) and chemotaxis assays (IC_{50}=6.2 nM). This suggests that this compound inhibits human eosinophil cytotoxic function as well as eosinophil migration.

In the immediate phase allergic response, histamine is released from mast cells and basophils. The interaction of histamine with histamine H_1 receptor mediates a variety of effects, such as vasodilatation, bronchial smooth muscle contraction, mucus secretion, and pruritus (Gomez et al., 1986). In the pathophysiology of asthma, increased vascular permeability leads to not only edema of the airway wall, which contributes to increases in airway hyperresponsiveness, but also leakage of plasma proteins into the airways, causing increased mucus viscosity (Simons, 2002). Since orally administered YM-344484 inhibited microvascular leakage induced by histamine to a significant degree (Fig. 6), it may be beneficial for the treatment of allergic asthma.

In the late-phase allergic response in rat asthma model, Liu et al. (2005) demonstrated that the number of mucosal mast cells in the tracheal epithelial layer and histamine levels in the bronchoalveolar fluid increased. Histamine induces the expression of mRNA encoding CCL11, CCL2, CCL5, and CCL7 through the histamine H_1 receptor in explant cultures of human nasal mucosa (Fujikura et al., 2001). Endothelial cells express functional histamine H_1 receptor, and activation of this receptor induces the expression of adhesion molecules, such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and P-selectin (Lo and Fan, 1987; Kubes and Kanwar, 1994; Yamaki et al., 1998), which are involved in eosinophil adhesion. Recently, it was reported that histamine acts as a T cell chemotactic factor via the histamine H_1 receptor, and that histamine H_1 receptor-deficient mice, which have significantly fewer eosinophils and lymphocytes, fail to develop lung inflammation following ovalbumin challenge (Bryce et al., 2006). Like histamine H_1 receptor-deficient mice, wild-type animals receiving histamine H_1 receptor antagonists, such as fexofenadine and desloratadine, exhibited a marked attenuation of eosinophil infiltration in the bronchoalveolar lavage fluid (Gelfand et al., 2002; Bryce et al., 2003; Blumchen et al., 2004). These results suggest that histamine also regulates the chemotaxis and adhesion of eosinophils and neutrophils, and that CCR3/CCR3 ligands and histamine H_1 receptor/histamine both play important roles in the pathogenesis of asthma, including eosinophilic inflammation.

YM-344484, a functional dual antagonist for CCR3 and histamine H_1 receptor, selectively attenuated eosinophil infiltration, but not neutrophil or monocyte infiltration, into bronchoalveolar lavage fluid in an ovalbumin-induced asthmatic model in mice (every 8 h dosing of 300 mg/kg or greater). This dosing regimen was expected to maintain the plasma concentration of the protein-unbound form at a level higher than the K_b value for murine CCR3 (88 nM). It was also expected to be a strong histamine H_1 receptor antagonist *in vivo* (Fig. 6). These results would suggest that its pharmacological action against both CCR3 and histamine H_1 receptor resulted in the significant reduction in eosinophil infiltration. However, since the peak concentration of the unbound form of YM-344484 is somewhat high (528 nM), the possibility that YM-344484 affected other pathways cannot be ruled out. Considering that the *in vitro* antagonistic activity of YM-344484 against human CCR3 is about 50 times stronger than that against murine CCR3, it can be assumed that a lower dosage (6–12 mg/kg) would be effective in humans.

Most older histamine H_1 receptor antagonists have the potential for adverse effects on the central nervous system due to poor receptor specificity. They bind to muscarinic receptors, which results in marked anticholinergic effects. Newer antihistamine agents have improved the side-effect profiles, but some, such as astemizole and terfenadine, result in severe cardiovascular liabilities, including torsades de pointes, a potentially fatal cardiac abnormality characterized by ventricular tachycardia (Simons et al., 1988; Woosley et al., 1993). This is believed to be due to blockade of the I_{kr} channel, which inhibits the rapid delayed rectifier K^+ current and prolonged

action potential (Woosley et al., 1993). This study demonstrated YM-344484 to be a potent and selective histamine H_1 receptor antagonist with low affinity for the muscarinic receptors, M_1, M_2, and M_3. Furthermore, 1 μM YM-344484 only inhibited the human ether-a-go-go-related gene (hERG) channel current (8.4% compared to the control, data not shown). This concentration was about 560 and 20 times higher than the CCR3 and histamine H_1 receptor K_b values, respectively, which suggests low potential for ventricular arrhythmias.

YM-344484 acted as a novel and functional dual antagonist for CCR3 and histamine H_1 receptor. Orally administered YM-344484 significantly inhibited the histamine-induced vascular permeability increase in mice and the accumulation of eosinophils in a mouse asthma model at doses of 10 mg/kg and 300 mg/kg, respectively. These results suggest that dual antagonists for CCR3 and histamine H_1 receptor have good potential as treatments for allergic inflammation conditions, such as asthma, allergic rhinitis, and atopic dermatitis.

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