

Selective Inhibition of Eosinophil Influx into the Lung by Small Molecule CC Chemokine Receptor 3 Antagonists in Mouse Models of Allergic Inflammation

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ABSTRACT

CC chemokine receptor (CCR) 3 is a chemokine receptor implicated in recruiting cells, particularly eosinophils (E Φ), to the lung in episodes of allergic asthma. To investigate the efficacy of selective, small molecule antagonists of CCR3, we developed a murine model of E Φ recruitment to the lung. Murine eotaxin was delivered intranasally to mice that had previously received i.p. injections of ovalbumin (OVA), and the effects were monitored by bronchoalveolar lavage. A selective eosinophilic influx was produced in animals receiving eotaxin but not saline. Furthermore, the number of E Φ was concentration- and time-dependent. Although anti-CCR3 antibody reduced the number of E Φ , the effect of eotaxin in OVA-sensitized mice was not a direct chemotactic stimulus because mast cell deficiency (in WBB6F₁-*Kit^W/Kit^{W-V}* mice) significantly reduced the response. Two representative small molecule CCR3 antagonists from our

program were characterized as being active at mouse CCR3. They were administered p.o. to wild-type mice and found to reduce eotaxin-elicited E Φ selectively in a dose-dependent manner. Pump infusion of one of the inhibitors to achieve steady-state levels showed that efficacy was not achieved at plasma concentrations equivalent to the *in vitro* chemotaxis IC₉₀ but only at much higher concentrations. To extend the results from our recruitment model, we tested one of the inhibitors in an allergenic model of airway inflammation, generated by adoptive transfer of OVA-sensitive murine T helper 2 cells and aerosolized OVA challenge of recipient mice, and found that it inhibited E Φ recruitment. We conclude that small molecule CCR3 antagonists reduce pulmonary eosinophilic inflammation elicited by chemokine or allergenic challenge.

The accumulation and activation of eosinophils (E Φ) in tissue is a hallmark of allergic inflammation. In asthma, E Φ have been implicated in the characteristic pathophysiology of the disease such as mucus hypersecretion, epithelial shedding, and airway remodeling (Lacy and Moqbel, 2002). However, their role in the airway dysfunction that is the hallmark of asthma is more controversial (Bochner, 2004). A good deal of interest has focused on the mechanism(s) responsible for the movement of E Φ from the circulation into the lung. CC

chemokine receptor (CCR) 3 is the principal chemokine receptor on the surface of E Φ (Daugherty et al., 1996; Ponath et al., 1996a) and is bound by ligands such as eotaxin (Ponath et al., 1996b)/CC chemokine ligand (CCL) 11 (Zlotnik and Yoshie, 2000), eotaxin 2 (Forssmann et al., 1997)/CCL24, and eotaxin 3 (Shinkai et al., 1999)/CCL26, all selective for this receptor. Other chemokines such as regulated on activation normal T cell expressed and secreted (RANTES)/CCL5, monocyte chemotactic protein 3/CCL7, and monocyte chemotactic protein 4/CCL13 are CCR3 ligands but also bind other chemokine receptors (Zlotnik and Yoshie, 2000). Biologically active concentrations of these ligands are found in the bronchoalveolar lavage (BAL) fluid of asthmatics after allergen challenge (Brown et al., 1998; Berkman et al., 2001; Lilly et

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ABBREVIATIONS: E Φ , eosinophil(s); CCR, CC chemokine receptor; CCL, CC chemokine ligand; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; BAL, bronchoalveolar lavage(s); Th₂, T helper 2; mAb, monoclonal antibody(ies); OVA, ovalbumin; BSA, bovine serum albumin; *W/W⁺*, WBB6F₁-*Kit^W/Kit^{W-V}*; +/+, WBB6F₁-+/+; i.n., intranasal; AUC, area under the curve; AUMC, area under the moments curve; Cl, clearance; V_{ss}, volume of distribution at steady state; N Φ , neutrophils(s); M Φ , macrophage(s); IL, interleukin; IFN, interferon.

al., 2001), and levels of eotaxin correlate with asthma severity and airway dysfunction (Nakamura et al., 1999, 2001).

In animal models of asthma, neutralization of CCR3 ligands has resulted in inhibition of the E Φ influx following allergen challenge in allergen-sensitized mice. For example, administration of an antieotaxin antibody to sensitized mice reduced BAL E Φ numbers following antigen challenge (Campbell et al., 1998; Gonzalo et al., 1998). Genetic depletion of eotaxin also reduced postchallenge BAL eosinophilia compared with wild-type mice (Rothenberg et al., 1997; Mattes et al., 2002; Schuh et al., 2002). Genetic depletion of CCR3 itself reduced the numbers of E Φ in BAL and lung tissue after allergen challenge (Humbles et al., 2002; Ma et al., 2002).

CCR3 is also expressed by other cells implicated in the allergic response. These include basophils (Uguccioni et al., 1997), mast cells (Romagnani et al., 1999), and a subpopulation of T helper 2 (Th₂) lymphocytes (Sallusto et al., 1997). Accordingly, CCR3 antagonism has the potential to affect the infiltration of all these cells into the lungs of asthmatics and therefore is a compelling therapeutic target. To date, the *in vivo* pharmacologic activity of CCR3-selective small molecule antagonists has not been reported. The present report shows that orally active small molecule antagonists of CCR3 are capable of significantly reducing airway eosinophilia in mouse models of allergic inflammation.

Materials and Methods

Reagents. Recombinant human and murine chemokines and anti-murine CCR3 monoclonal antibody (mAb) were obtained from R&D Systems (Minneapolis, MN). ¹²⁵I-labeled human eotaxin was from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Ovalbumin (OVA), aluminum hydroxide, low-endotoxin bovine serum albumin (BSA), and Wright's Giemsa stain were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). RPMI 1640 medium and HEPES buffer were from Invitrogen (Carlsbad, CA).

Animals. Female BALB/c (18–20 g) mice were obtained from Charles River Laboratories (Raleigh, NC). Female WBB6F₁-*Kit^{W^v}*/*Kit^{W^v}* (W/W^v) (mast cell-deficient mice) and WBB6F₁-+/+ (+/+) (congenic control mice) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice expressing the transgene for the DO11.10 T cell receptor α/β were also obtained from Jackson. Animals were housed at the Bristol Myers Squibb animal care facility under a 12-h light/dark cycle and were allowed food and water *ad libitum*. All of the animal protocols were reviewed and approved by the Bristol Myers Squibb Animal Care and Use Committee.

Sensitization of Mice to OVA. Mice received an i.p. injection of 10 μ g of OVA and 1 mg of aluminum hydroxide (in a volume of 0.1 ml) on days 1 and 8. On days 15 through 17, mice were subjected to either a single intranasal (i.n.) instillation of murine eotaxin or aerosolized OVA challenge.

Administration of Intranasal Eotaxin. OVA-sensitized BALB/c mice were administered either vehicle (phosphate-buffered saline + 0.1% low endotoxin BSA) or different concentrations (3–30 μ g) of murine recombinant eotaxin in 50- μ l volume under Metofane (Mallinckrodt Veterinary Inc., Mundelein, IL) anesthesia. Small molecule antagonist or vehicle was administered by p.o. gavage 30 min before eotaxin challenge. In the standardized protocol, BAL was performed at 6 h with 1 ml of phosphate-buffered saline (Ca²⁺/Mg²⁺-free) containing 10 mM EDTA (lavage buffer). BAL samples were centrifuged (320g, 10 min) and resuspended in 200 μ l of lavage buffer containing 0.1% BSA (resuspension buffer). An aliquot of the cell suspension was used for total counts, performed using a Neubauer hemacytometer, and for cytospin preparations used to count differ-

entials after staining in Wright's Giemsa. In some experiments requiring predictable steady-state plasma levels, compound was delivered via osmotic minipumps (Alzet, Cupertino, CA) at a rate of 1 μ l/h for 3 days beginning 2 days before eotaxin challenge.

Pharmacokinetics of Compounds 1 and 2. The pharmacokinetics of compounds 1 and 2 were investigated in male BALB/c mice following a single i.v. dose of 1 mg/kg or a p.o. dose of 10 mg/kg. Blood samples were collected at 3 (i.v. only) and 30 min and 1, 3, 6, and 8 h postdose either via the orbital sinus or via a terminal cardiac puncture. Blood was allowed to coagulate on ice and was centrifuged at 4°C to obtain serum. The serum concentrations of compounds 1 and 2 were determined by using a selective, specific, and sensitive liquid chromatography/tandem mass spectrometry assay, with appropriate calibration curves and quality control samples. The concentration-time data ($n = 6$ mice, composite profile) for the two compounds was used to calculate the pharmacokinetic parameters using a noncompartmental method in WINNONLIN. In brief, the systemic exposure of compounds after i.v. or p.o. dosing was determined by calculating the area under the concentration (C) versus time (t) plot (AUC), using the linear trapezoidal approximation. The area under the moments curve (AUMC) was calculated similarly, where AUMC is the area under the curve of a plot of the product of concentration and time (C*t) versus time (t). Terminal phase rate constant (k_{el}) was determined as the inverse of the slope of the linear regression of log C versus t plot, and the terminal phase half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. The peak concentration after p.o. dosing (C_{max}) was recorded directly from experimental observations. The equations used to calculate additional pharmacokinetic parameters are described as follows: clearance (Cl) = dose/AUC; volume of distribution at steady state (V_{ss}) = dose $\times$ AUMC/AUC; and Bioavailability (F) = dose_{i.v.} $\times$ AUC_{p.o.}/dose_{p.o.} $\times$ AUC_{i.v.}.

Human E Φ Isolation. Peripheral venous blood drawn from healthy volunteers was separated over Percoll (density 1.087), and the granulocyte/red blood cell fraction was collected. Red blood cells were removed by hypotonic lysis, leaving a granulocyte fraction consisting principally of neutrophils (N Φ) and E Φ . The granulocytes were incubated with anti-CD16 magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany), and the E Φ were collected by negative separation in an AutoMACS (Miltenyi).

Generation of Mouse E Φ for *in Vitro* Assays. BALB/c mice were sensitized to OVA (as described above) and on day 15 were given an i.n. challenge of 10 μ g of OVA. Forty-eight hours later, BAL was performed using lavage buffer, pooled, and kept on ice. All the remaining procedures were performed at 4°C. The pooled lavages were centrifuged (as described above), and the resulting cell pellet was resuspended in 1 ml of resuspension buffer. The red blood cells were lysed by hypotonic lysis (0.2% NaCl followed by 1.6% NaCl containing 10 mM glucose). Remaining intact cells were centrifuged again and resuspended in buffer for total and differential cell counts. Typically, the procedure yielded a cell population comprising E Φ ($51 \pm 2.9\%$), macrophages (M Φ) ($45.5 \pm 2.2\%$), and N Φ ($4.8 \pm 2.1\%$) (data derived from 4–5 mouse separate preparations).

CCR3 Binding Assay. Human. Chinese hamster ovary cells transfected with human CCR3 were suspended in binding buffer consisting of RPMI 1640 medium buffered with HEPES (20 mM) and 0.1% BSA. Chinese hamster ovary/CCR3 at 3×10^5 cells/well were transferred to 96-well filter plates. Cells were incubated with ¹²⁵I-recombinant human eotaxin in the presence or absence of varying concentrations of compound for 30 min at room temperature before filter separation of bound versus free eotaxin. Nonspecific binding was determined in the presence of 100 nM unlabeled eotaxin.

Mouse. Binding assays were performed using BAL E Φ elicited from OVA-sensitized and aerosol-challenged mice. A total of 1×10^5 E Φ were added to each well, and the protocol was the same as for human CCR3, including the use of labeled human eotaxin. Nonspecific binding was determined in the presence of 100 nM unlabeled mouse eotaxin. Competition assay showed that mouse eotaxin bound

to BAL cells with a K_d of 0.3 nM, a value similar to that reported for human eotaxin binding to human CCR3 (Daugherty et al., 1996).

In Vitro Chemotaxis Assay. The chemotaxis assays used freshly isolated human or mouse E Φ . In both cases, the chemotaxis protocol was similar, the only difference being that human eotaxin was used as chemoattractant for human E Φ and mouse eotaxin was used for the mouse E Φ . Cells were washed once and resuspended in chemotaxis buffer (HEPES-buffered RPMI 1640 medium, 0.1% BSA). The assay was performed in NeuroProbe MBA96 chemotaxis chambers with upper and lower wells separated by a polycarbonate filter containing pores of 5 μ M diameter. The protocol was similar to that used by Harvath et al. (1980) but was adapted for a 96-well apparatus as suggested by the manufacturers. Chemotaxis buffer containing eotaxin at a final concentration of 10 nM was placed in the bottom wells. The top wells were filled with 1.5×10^5 cells in buffer. The chambers were incubated at 37°C in humidified air containing 5% CO₂ for 45 min. Nonmigrated cells were aspirated from the top wells, and filters were removed, gently washed, and stained with Wright's Giemsa. Migrated cells on the underside of the filter were counted under a microscope. For each data point, the mean cell number was determined from three high-power fields per well in each of three wells.

Adoptive Transfer Th₂ Cell-Dependent Model of Antigen Challenge. Splenocytes were harvested from transgenic DO11.10 mice and cultured in complete RPMI 1640 medium with OVA 323–339 (1 μ g/ml) and mitomycin-treated splenocytes. The cells were differentiated to a Th₂ phenotype in medium containing murine interleukin (IL) 4 (50 ng/ml) and anti-IL-12 antibody (10 μ g/ml). Cells were cultured for three rounds of antigenic stimulation under these conditions. The Th₂ polarization of the cells was confirmed by activating them with anti-CD3 mAb in the presence of IL-2 (10 U/ml) for 48 h and measuring culture supernatants for IL-4 and IL-5 (high levels) and interferon (IFN)- γ (low levels). Polarized cells in saline were injected into naive recipient BALB/c mice at 5×10^6 /mouse via the tail vein. For OVA challenge, the recipient mice were placed in a Plexiglas pie chamber connected to a nebulizer (Pari Respiratory Equipment, Midlothian, VA) driven by compressed air. Mice were exposed to an aerosol of either vehicle (saline containing 0.01% Tween 20) or 1% OVA for 30 min/day for 3 consecutive days. Mice were p.o. treated b.i.d. with either vehicle or small molecule antagonist. BAL was performed 24 h following OVA challenge as described above.

Statistical Analysis. All of the data are presented as the mean $\pm$ S.E.M. of n animals (in vivo) or separate experiments (in vitro). Statistical differences between groups were analyzed by analysis of variance. If a statistical difference was indicated, Tukey's post-test analysis was applied to identify differences between multiple groups. Significance was accepted at $P < 0.05$.

Results

Development and Characterization of a Model of CCR3-Dependent Airway Inflammation. To test small molecule antagonists in vivo, we established a mouse model in which eotaxin administered by i.n. instillation elicits airway eosinophilia as monitored by BAL. Mice received two i.p. injections of OVA over 2 weeks and were then challenged with i.n. (murine) eotaxin, and the number of E Φ in the BAL fluid was quantified over time. Eotaxin administration resulted in a concentration- and time-dependent increase in the number of E Φ in the BAL fluid. Influx was detectable at 3 μ g of eotaxin but was appreciably greater at 10 μ g and above (Fig. 1A). The influx was seen with eotaxin, not with vehicle alone, and was specific for E Φ (Table 1). Time course studies indicated that the number of E Φ was significantly increased at 4 h after challenge and was further increased at 6 h. The

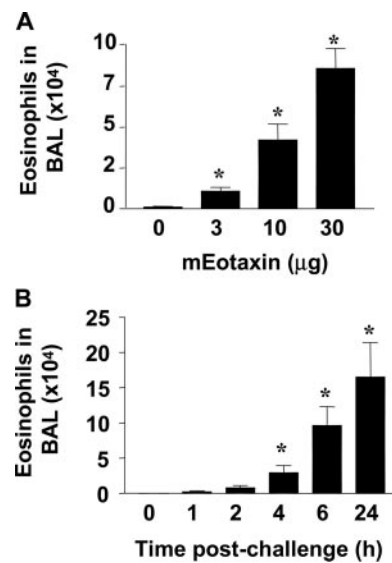


Fig. 1. Effect of eotaxin in mouse lungs in vivo. A, OVA-sensitized BALB/c mice were challenged with vehicle ($n = 11$) or different concentrations of murine eotaxin ($n = 13$ –15 per concentration). BAL was performed at 6 h after challenge, and the number of E Φ was quantified. Data are consolidated from two separate experiments. B, OVA-sensitized BALB/c mice were challenged with 10 μ g of mEotaxin, and BAL was performed at different time points following challenge ($n = 5$ mice per time point), and the number of E Φ was quantified. *, significant compared with vehicle challenge (A) or 0 h (B).

TABLE 1

Number of cells ($\times 10^4$) harvested by BAL following i.n. eotaxin challenge

Treatment	Eosinophils	Neutrophils	Mononuclear Cells
Vehicle	0.05 $\pm$ 0.03	1.2 $\pm$ 0.3	14.4 $\pm$ 3.0
Eotaxin (10 μ g)	4.2 $\pm$ 1.0*	2.0 $\pm$ 0.6	16.0 $\pm$ 4.6

* Significant when compared with the vehicle-challenged group.

number of E Φ underwent no further significant increase at 24 h (Fig. 1B). The number of E Φ was significantly greater in OVA-sensitized mice than in naive (nonsensitized) mice ($42 \pm 10 \times 10^3$ and $7 \pm 2 \times 10^3$ E Φ , respectively). The i.n. administration of other known CCR3 ligands, murine RANTES, or macrophage inflammatory protein-1 α to OVA-sensitized mice did not result in an appreciable E Φ influx: 10 μ g of eotaxin ($n = 8$) and 10 μ g of RANTES ($n = 5$) elicited $18.0 \pm 4.0 \times 10^4$ and $1.0 \pm 0.7 \times 10^4$ E Φ in the BAL, respectively, and 10 μ g of eotaxin ($n = 6$) and 10 μ g of macrophage inflammatory protein-1 α ($n = 6$) elicited $14.2 \pm 6.6 \times 10^4$ and $0.8 \pm 0.6 \times 10^4$ E Φ in the BAL, respectively.

Previous studies have shown a role for endogenous mast cells in mediating eotaxin-mediated E Φ migration into the peritoneal cavity (Das et al., 1997b, 1998; Harris et al., 1997). To investigate the role of mast cells in mediating eotaxin-induced lung eosinophilia, mast cell-deficient W/W^o mice were used. Administration of i.n. eotaxin elicited a BAL eosinophilia in wild-type mice. In contrast, the number of E Φ in the BAL fluid of mast cell-deficient mice was significantly reduced following eotaxin administration to a level not significantly different from the i.n. vehicle control (Fig. 2). Although eotaxin-dependent migration of E Φ into the peritoneal cavity has been shown to be delayed in the absence of mast cells (Harris et al., 1997), lung eosinophilia was still not

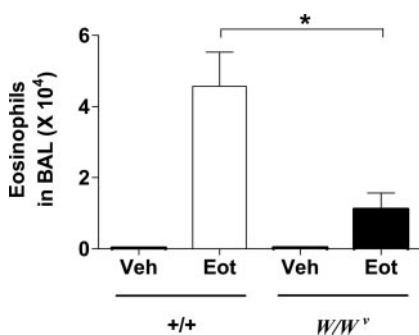


Fig. 2. Effect of i.n. eotaxin in mast cell-deficient mice. OVA-sensitized +/+ mice (congenic control mice) or W/W^v (mast cell-deficient mice) were challenged with 10 μ g of mEotaxin. Six hours later, BAL was performed, and the number of E Φ was quantified. Data are the consolidated data from two separate experiments with $n = 6$ to 10 per experiment. *, significant compared with E Φ numbers in +/+ mice.

evident at 24 h in W/W^v mice, whereas it was abundant in wild-type mice.

To establish a role for CCR3 in the BAL eosinophilia following i.n. eotaxin, we tested the effect of an mAb directed against murine CCR3. Although the eosinophilia in eotaxin-challenged, untreated animals and in the IgG isotype-treated controls was modest, anti-CCR3 mAb significantly reduced the number of E Φ by 67% (Fig. 3). Because anti-CCR3 antibodies have been reported to deplete circulating E Φ in the mouse (Grimaldi et al., 1999), we determined whether this effect played a role in our study. Accordingly, blood E Φ numbers were evaluated 3 h following i.v. injection of anti-CCR3 mAb or isotype (IgG_{2A}) control. Anti-CCR3 mAb reduced circulating E Φ numbers but not significantly: $7.25 \pm 0.51 \times 10^3$ and $4.07 \pm 1.66 \times 10^3$ E Φ / μ l in IgG_{2A}-treated and anti-CCR3-treated mice, respectively ($n = 4$ /group, $P = 0.07$, unpaired t test).

Characterization of Two CCR3 Antagonists. The chemical structures of the two CCR3 antagonists used in this study are shown in Fig. 4. These molecules are representative analogs of two distinct chemical series developed in our laboratories: compound **1** is a heterocyclic derivative of the cyclohexyl-linked series of inhibitors (Ko et al., 2002; Delucca et al., 2005), and compound **2** is a propyl-linked derivative that contains an alternate piperidine headgroup (Watson et al., 2002; Varnes et al., 2004). Both compounds were found to be potent antagonists of human CCR3 binding and function (Table 2). When tested against mouse E Φ , binding IC₅₀ were somewhat higher than for human CCR3, but were similar for the two compounds (Table 2). However, chemotaxis IC₅₀ were

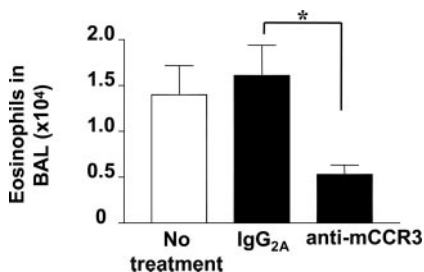


Fig. 3. Inhibition of E Φ influx following i.n. eotaxin by anti-murine CCR3 mAb. OVA-sensitized BALB/c mice (5–8 per treatment group) were either untreated or pretreated at 30 min before i.n. mEotaxin with 200 μ g of IgG_{2A} or anti-CCR3 mAb i.v. Six hours after i.n. mEotaxin administration (10 μ g), BAL was performed, and the number of E Φ was quantified. *, significant compared with IgG_{2A}-treated mice.

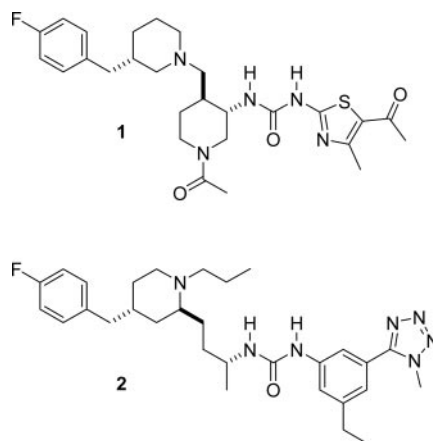


Fig. 4. The chemical structures of the two CCR3 antagonists, compounds **1** and **2**.

TABLE 2

IC₅₀ values (in nanomolars) against human and mouse CCR3

Human binding IC₅₀ was determined on Chinese hamster ovary cells transfected with human CCR3 using ¹²⁵I-hEotaxin (0.15 nM); human chemotaxis IC₅₀ was determined on freshly purified human eosinophils using hEotaxin (10 nM) as chemoattractant. Mouse binding and chemotaxis IC₅₀ were determined on eosinophils obtained by BAL of OVA-sensitized mice 24 h after OVA challenge. Binding was carried out using ¹²⁵I-hEotaxin at 0.15 nM. Chemotaxis used mEotaxin (10 nM) as chemoattractant; means $\pm$ S.E.M.

Compound	Human		Mouse	
	Binding	Chemotaxis	Binding	Chemotaxis
1	1.9 $\pm$ 0.35	0.06 $\pm$ 0.02	3.6 $\pm$ 1.93	7.0 $\pm$ 3.18
2	0.9 $\pm$ 0.20	0.02 $\pm$ 0.00	3.0 $\pm$ 0.84	0.2 $\pm$ 0.02

divergent. Like other compounds in the cyclohexyl series (Delucca et al., 2005), compound **1** was somewhat less potent in the chemotaxis assay than in binding; in contrast, compound **2** was 15-fold more potent in mouse chemotaxis, just as it was more potent in human chemotaxis than in human binding. When dosed p.o. in mice, compounds **1** and **2** delivered moderate serum exposures as judged by AUC values of $\sim$ 1800 nM/h (Table 3). Taken together, the murine binding potency, chemotaxis potency, and oral bioavailability suggested that compounds **1** and **2** were suitable for study in murine models of E Φ influx.

In Vivo Efficacy of CCR3 Antagonists in the Intranasal Eotaxin Model. In studies of compounds **1** and **2**, compound or vehicle alone was administered p.o. to mice 30 min before challenge with eotaxin. Six hours following eotaxin administration, mice were euthanized, and the number of E Φ in the BAL fluid was measured. Compound **1** dose-dependently reduced the number of E Φ in the BAL fluid (Fig. 5A). Although the numbers of N Φ were reduced at 1, 3, and 10

TABLE 3

Pharmacokinetic parameters of compounds **1** and **2** in mice

Route	Parameter	Compound 1 ^a	Compound 2 ^b
i.v.	Cl (l/h/kg)	2.5	1.0
	V _{ss} (l/kg)	2.5	0.4
p.o.	AUC 0–8 h (nM/h)	1870	1839
	C _{max} (nM)	1030	233
	t _{1/2} (h)	1.2	1.3
	Oral bioavailability (%)	25	4

^a Compound **1** was dosed p.o. at 10 mg/kg and i.v. at 2 mg/kg using 10% dimethylacetamide/90% dextrose (5%) as a vehicle.

^b Compound **2** was dosed p.o. at 11 mg/kg and i.v. at 1 mg/kg using 10% dimethylacetamide/90% aqueous HCl (0.01 N) as a vehicle.

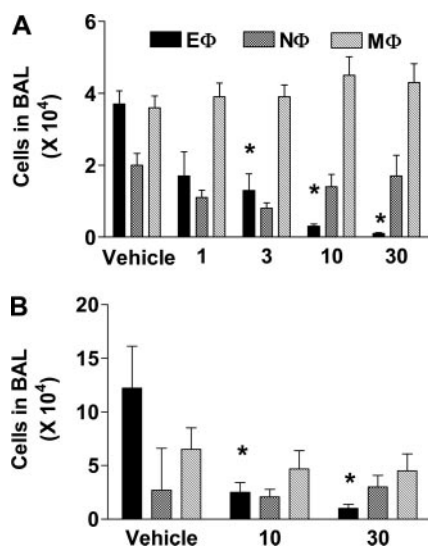


Fig. 5. Inhibition of cellular influx by compound 1 (A) and 2 (B) following i.n. eotaxin. OVA-sensitized BALB/c mice (5–9 per group) were pretreated at 30 min before i.n. mEotaxin with graded p.o. doses of compound. Mice were challenged i.n. with 10 μ g of mEotaxin. BAL was collected 6 h following challenge, and the number and type of cells were determined. *, significant compared with vehicle. Comparison of NΦ numbers with vehicle, $p > 0.05$.

mg/kg, these changes did not reach significance. A dose-response curve was constructed (not shown), and the effective doses that inhibited 50% (ED_{50}) and 90% (ED_{90}) of the eosinophilia were determined as 1.5 and 10.5 mg/kg, respectively. Delivery p.o. of compound 2 also inhibited BAL eosinophilia in a dose-dependent manner (Fig. 5B).

We wished to probe the pharmacodynamic relationship between plasma levels of antagonist and efficacy and determine how this reflected the in vitro potency of the molecule.

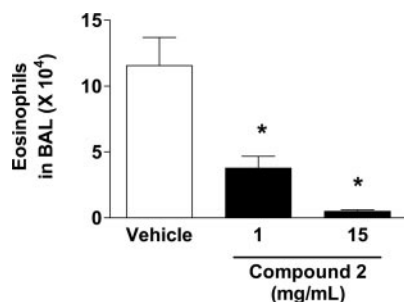


Fig. 6. Inhibition of EΦ influx in the i.n. eotaxin model by compound 2 contained in s.c. minipumps at concentrations of either 1 or 15 mg/ml. Three-day pumps were implanted s.c. in OVA-sensitized BALB/c mice. Two days later, the mice received 10 μ g of i.n. mEotaxin. BAL was collected 6 h following challenge, and the number of EΦ was determined. This is the first of two experiments shown in Table 4. *, significant compared with vehicle.

TABLE 4

Efficacy of compound 2 following steady-state infusion

Shown are data from two experiments in which compound was delivered from minipumps implanted s.c. For the lower plasma concentration, one pump/mouse contained compound at 1 mg/ml; for the higher concentration, two pumps/mouse were used, each containing 7.5 mg/ml. Mice were challenged i.n. with 10 μ g of mEotaxin.

% Reduction in BAL Eosinophils	Experiment 1		Experiment 2	
	67	96	50	85
Steady-state plasma concentration (nM)	24 ± 1.2	216 ± 20	57 ± 12	208 ± 42
Free ^a plasma concentration (nM)	1.2 ± 0.06	10.8 ± 1	2.9 ± 0.6	10.4 ± 2.1
Multiple of chemotaxis IC_{50} (0.2 nM)	6	54	14	52
Multiple of binding IC_{50} (3 nM)	0.4	3.6	1	3.5

* Calculated based on mouse protein binding = 95%.

As indicated above, the murine potencies of compound 2 in the binding and chemotaxis assays were divergent, being 15-fold more potent in the latter. Therefore, we selected to administer compound 2 by infusion so as to achieve steady-state levels that were multiples of the in vitro potency values and remained at steady state during the 6-h interval post-i.n. eotaxin challenge. The results from two separate experiments show that ~90% inhibition of eosinophilic influx into the airway is achieved at plasma levels that, when adjusted for protein binding, represent 52- to 54-fold multiples of the chemotaxis IC_{50} but a lower multiple (3–4-fold) of the binding IC_{50} (Fig. 6 and Table 4).

Effect of CCR3 Antagonism on Antigen-Induced EΦ Migration into Mouse Lungs. The i.n. eotaxin model is useful for determining pharmacodynamic relationships because it is exclusively CCR3-dependent. However, it does not reflect the more complex conditions operating in allergen challenge where the concentration and localization of potentially multiple CCR3 ligands may be quite different from the simple eotaxin model. Because the allergen response in human airways is prompted by sensitization and challenge via the same aerosol route, we employed an adoptive transfer model in which mice were injected with OVA-sensitive T cell receptor-transgenic Th₂ lymphocytes and were then challenged with aerosolized OVA for 30 min on each of up to 3 consecutive days (Lloyd et al., 2000). Twenty-four hours after the first OVA challenge, eosinophilia was already evident in the BAL, albeit at modest levels ($1.5 \pm 0.6 \times 10^4$). The response was not seen in mice that did not receive T cells or received Th₂ cells but were not challenged with OVA. Compound 1, administered p.o. at 100 mg/kg b.i.d., selectively reduced this by 82% (to $0.3 \pm 0.1 \times 10^4$). After 3 days of OVA challenge, the level of airway inflammation was much more pronounced, but EΦ still comprised the major cell type (EΦ, 26.3 ± 4.0 ; NΦ, 3.5 ± 1.0 ; MΦ, 6.8 ± 1.8 ; all $\times 10^4$). BAL levels of the Th₂ cytokines, IL-4 and IL-5, were 89.4 ± 10.5 and 89.5 ± 16.6 pg/ml, respectively, whereas levels in unchallenged animals were undetectable. The level of the Th₁ cytokine, IFN- γ , was undetectable. Compound 1 reduced the eosinophilia by 78% (Fig. 7) but had no effect on the other cell types. It did not affect the BAL levels of cytokines IL-4, IL-5, and IFN- γ .

Discussion

Eotaxin requires a readily available pool of circulating EΦ from which to recruit cells into tissues (Mould et al., 1997; Grimaldi et al., 1999). In mice, blood eosinophilia has been produced by sensitizing mice to an antigen that will induce a bone marrow and blood eosinophilia (Das et al., 1997a,b). In

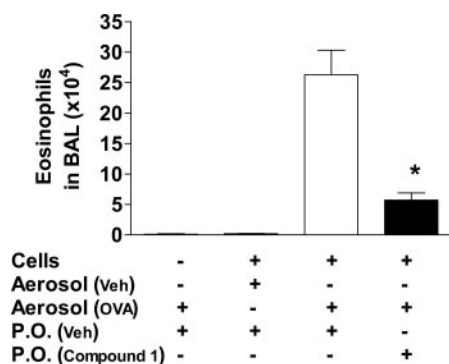


Fig. 7. Inhibition of E Φ influx by compound 1 dosed p.o. in mice that had undergone adoptive transfer of OVA-sensitive Th₂ lymphocytes and subsequent OVA challenge. Mice (7–8 per group) were injected with 5×10^6 differentiated Th₂ cells by tail vein injection and then aerosol-challenged with OVA for 30 min on 3 consecutive days. They were dosed p.o. with compound 1 (100 mg/kg) or with vehicle in a b.i.d. regimen 30 min before OVA challenge and 12 h later. BAL was performed 24 h following the third OVA challenge. The number of E Φ in the BAL was determined. This is the second of two experiments giving similar results. *, significant compared with vehicle.

the present study, i.n. administration of eotaxin to OVA-sensitized mice elicited a reproducible eosinophilia in the BAL fluid. The poor response to MIP-1 α and RANTES is consistent with previous reports, which showed that, in contrast to eotaxin, these two murine ligands failed to stimulate in vitro migration of purified mouse E Φ (Borchers et al., 2002). The eosinophilic response to eotaxin was markedly reduced in mast cell-deficient mice. Although this has been observed in the lung following aerosolized OVA challenge (Kung et al., 1994), our finding is the first to suggest a role for mast cells in eotaxin-elicited pulmonary eosinophilia. Mast cells also appear to fill a similar role in the peritoneum following eotaxin challenge (Harris et al., 1997; Das et al., 1998). Tryptase/chymase-double positive human mast cells express CCR3 (Ochi et al., 1999; Romagnani et al., 1999), which is functionally active in chemotaxis. Potentially, then, eotaxin may prompt the resident pulmonary mast cells to release mediators critical to eosinophil recruitment. Kung et al. (1994) suggested that the mast cells served as a source of IL-5 in the allergen challenge model, but this would seem unlikely to be a major source compared with the large numbers of infiltrating Th₂ cells. Recruitment of E Φ from the blood into the tissue is facilitated by chemoattractant(s), but the initial margination of the cells is likely to require the up-regulation of adhesion molecules on vascular endothelium. Eotaxin itself has not been shown to stimulate their expression directly so that the mast cell involvement may be to mediate this effect following eotaxin-CCR3 engagement on the mast cells.

Anti-murine CCR3 mAb reduced eotaxin-induced BAL eosinophilia in vivo but had no effect on circulating E Φ numbers. Administration p.o. of either compound 1 or 2 provided dose-dependent inhibition of eotaxin-elicited BAL eosinophilia.

The two compounds showed a discrepancy between their binding and chemotaxis potencies with subnanomolar potency in the latter. This has been typical of some chemical series of our CCR3-active molecules (Delucca et al., 2005) but has not been reflected in their potencies against mouse CCR3. Compound 2 was unusual in exhibiting subnanomolar

chemotaxis IC₅₀ against mouse CCR3, a 15-fold increase in potency over the binding number. Accordingly, this compound was selected to help determine which in vitro potency better predicted in vivo efficacy. Somewhat to our surprise, the pump infusion studies show that ~90% efficacy was achieved only at high multiples of the chemotaxis IC₅₀, whereas 3- to 4-fold multiples of the binding IC₅₀ were adequate. These results suggest that the assumption that inhibiting chemotaxis is sufficient to prevent E Φ influx in vivo is incorrect. The sequestration of E Φ at the vascular endothelium is a prerequisite of their transmigration, and the action of eotaxin in stimulating this process, possibly via the mediation of the mast cell, is not a chemotactic function but may require a potency closer to that in the binding assay. Although the mouse cells have not been examined for functions other than chemotaxis, compounds tested on human E Φ show potencies in eotaxin-stimulated calcium flux similar to those of binding.

The i.n. eotaxin model is useful for screening the pharmacodynamic activity of CCR3 antagonists because its endpoint, airway eosinophilia, is exclusively CCR3-dependent. The adoptive transfer/aerosol challenge model offers an opportunity to test molecules in the context of a prolonged allergenic airway inflammation mediated exclusively through Th₂ cells. Because the model does not employ sensitization before antigen challenge, there is no immediately available circulating pool of mobilized E Φ . Nevertheless, after only one aerosol challenge, E Φ were recruited to the lung, presumably drawn from the bone marrow. This recruitment was largely CCR3-dependent because compound 1, at a p.o. dose higher than was fully efficacious in the i.n. model, selectively reduced the BAL eosinophilia by 82%. After three consecutive aerosol challenges, however, the number of E Φ was greatly increased. In this situation, the compound still achieved 78% selective inhibition. The remaining E Φ are likely to enter the lung through non-CCR3 mechanisms. This has been confirmed in CCR3 knockout mice, in which the eosinophilia resulting from allergen challenge is reduced relative to wild-type mice by a proportionately similar degree (Humbles et al., 2002; Ma et al., 2002).

In conclusion, these studies show that small molecule CCR3 antagonists have potential to reduce eosinophilic influx into allergic airways. The clinical benefits of such an outcome are controversial because reduction of airway eosinophilia in the clinical trials of anti-IL-5 antibodies failed to relieve active bronchoconstriction, the principal manifestation of asthma (Bochner, 2004). However, subepithelial fibrosis was diminished, and this may have a positive impact on restrictive airway remodeling (Kay et al., 2004). CCR3 antagonism may offer advantages over anti-IL-5 because, in addition to E Φ , it is likely to affect additional cell types that express the receptor and are important in the asthmatic response, including mast cells, basophils, and lymphocytes.

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