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Identification of full, partial and inverse CC chemokine receptor 3 agonists using [^{35}S]GTPγS binding

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Abstract

Study of the CC chemokine receptor 3 (CCR3) has been limited to using radiolabeled agonist chemokines. A small molecule CCR3 antagonist, 2-[(6-amino-2-benzothiazolyl)thio]-*N*-[1-[(3,4-dichlorylphenyl)methyl]-4-piperidinyl]acetamide, Banyu (I), was tritiated and used for pharmacological studies. Banyu (I) has a K_d of 5.0 ± 0.4 and 4.3 ± 1.8 nM on human CCR3 transfectants and eosinophils, and noncompetitively inhibits [^{125}I]eotaxin binding and eotaxin-induced [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding. The proportion of [^{125}I]eotaxin: [^{3}H]Banyu (I) binding sites in eosinophils or transfectants was 35% or 13%, although both binding sites were overexpressed in transfectants. CCR3 spontaneously couples to G-proteins in CCR3 transfectants, demonstrated by changes in basal and eotaxin-induced [^{35}S]GTPγS binding under reduced NaCl and GDP concentrations. Consequently, Banyu (I) was identified as an inverse agonist. In contrast, CCL18 and I-TAC (interferon-inducible T cell α-chemoattractant) were neutral antagonists, inhibiting eotaxin-induced [^{35}S]GTPγS binding, with minimal effect on basal coupling of CCR3 to G proteins. Eotaxin, eotaxin-2 and monocyte chemoattractant protein (MCP)-4 are full agonists inducing [^{35}S]GTPγS binding; eotaxin-3, MCP-3, RANTES (regulated on activation normal T cell expressed and secreted), vMIP-I (Kaposi's sarcoma-associated herpesvirus macrophage inflammatory protein-) and vMIP-II are partial agonists, indicating that this is a sensitive method to quantitate agonist efficacy.

Keywords: Receptor binding; Ca^{2+}, intracellular; CCL18, I-TAC; vMIP; Small molecule antagonist; Constitutive activity

1. Introduction

CC chemokine receptor 3 (CCR3) is a β-chemokine receptor primarily expressed on eosinophils (Daugherty et al., 1996; Ponath et al., 1996b), basophils (Uguccioni et al., 1997) and a subpopulation of Th2 cells (Gerber et al., 1997; Sallusto et al., 1997). It is also expressed by nonhematopoietic cells, such as bronchial epithelial cells, where it is known to be a functional receptor in vitro (Stellato et al., 2001). Based upon this expression pattern, it is probable that CCR3 plays a role in allergic inflammation. This is supported by studies in mice in which blockade of eotaxin, an important CCR3 ligand (Ponath et al., 1996a), by administration of neutralizing antibody (Gonzalo et al., 1996) resulted in reduced pulmonary eosinophilic inflammation in response to inhaled allergen challenge. A similar reduction was observed in mice deficient in eotaxin (Rothenberg et al., 1997). Using an antigen sensitization and challenge regimen to induce lung inflammation, CCR3 knock-out mice displayed reduced eosinophilic lung inflammation following allergic inhalation challenge. This was accompanied by enhanced (Humbles et al., 2002) or reduced bronchoconstrictor responses to methacholine (Ma et al., 2002) depending on the route of sensitization.

In addition to eotaxin, other CCR3 ligands exist, such as eotaxin-2 (Forssmann et al., 1997), eotaxin-3 (Uguccioni et al., 1996), MCP-3 (monocyte chemoattractant protein-3) (Ponath et al., 1996b), MCP-4 (Uguccioni et al., 1996), and RANTES (regulated on activation normal T cell expressed and secreted) (Ponath et al., 1996b) as well as the virally encoded chemokines, vMIP-I (Kaposi's sarcoma-associated herpesvirus macrophage inflammatory protein-) (Boshoff et al., 1997) and vMIP-II (Fernandez et al., 2000). Interestingly, several other chemokines have been described as naturally occurring antagonists of CCR3. These include

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the CXCR3 ligands, MIG (monokine induced by γ-interferon), IP-10 (interferon-inducible protein 10) and I-TAC (interferon-inducible T cell α-chemoattractant) (Loetscher et al., 2001) as well as CCL18 (Nibbs et al., 2000), whose cognate receptor is currently unidentified. Given the physiological effects of blockade of eotaxin or CCR3 in mouse models of pulmonary inflammation, as well as the existence of multiple endogenously occurring ligands and inhibitory molecules, the development of CCR3 receptor antagonists represents a possible therapeutic approach to block the pulmonary eosinophilic inflammation that is characteristic of asthma (Broide et al., 1992; Egan et al., 1996). Towards this end, it is important to understand the pharmacology of interaction of CCR3 with its ligands.

Pharmacological study of CCR3 has been limited because radioligands have been restricted to agonists, such as [^{125}I]eotaxin, -MCP-4, -MCP-3 and -RANTES. Recently, several small molecule, nonpeptidic CCR3 antagonists have been reported (Naya et al., 2001; Sabroe et al., 2000; Saeki et al., 2001; White et al., 2000). A small molecule, 2-[(6-amino-2-benzothiazolyl)thio]-*N*-[1-[(3,4-dichlorylphenyl)-methyl]-4-piperidinyl]acetamide (Naya et al., 2001) was recently described as a CCR3 selective antagonist (binding IC_{50} = 2.3 nM versus [^{125}I]eotaxin in CCR3/Chinese hamster ovary cells (CHO)) transfectants with negligible affinity for CCR1, and as an antagonist of CCR3 function in human eosinophils by blocking eotaxin-induced increases in calcium and chemotaxis (IC_{50} = 27 and 21 nM, respectively, versus 10 nM eotaxin).

To further explore the pharmacology of CCR3, 2-[(6-amino-2-benzothiazolyl)thio]-*N*-[1-[(3,4-dichloryl)methly]-4-piperidinyl]acetamide, designated Banyu (I), was radiolabeled with tritium. A comparison of the binding isotherms of [^{125}I]eotaxin and [^{3}H]Banyu (I) in both CCR3 transfectants and human peripheral blood eosinophils indicated that a minority of the binding sites were eotaxin-binding sites and that the number of [^{3}H]Banyu (I) binding sites in transfectants greatly exceeded that in eosinophils. This overexpression of CCR3 in the transfectant cells suggested that constitutive activity of the receptor might be observable. Activation of G-protein-coupled receptors with agonists stimulates the exchange of GTP for GDP on the active site of the G_{α} protein (Gilman, 1987). This activation can be measured by the binding of [^{35}S]GTPγS, a nonhydrolyzable GTP analog, in the presence of excess GDP (Wieland and Jakobs, 1994). Constitutive activity, as well as agonist and inverse agonist activity, which modulate the spontaneous, precoupling of the receptor with G proteins (Costa and Herz, 1989) can be assessed through the measurement of [^{35}S]GTPγS binding to receptor-membrane preparations. Here, using a [^{35}S]GTPγS exchange assay to measure signaling, it was found that CCR3 has constitutive activity. The spontaneous CCR3 receptor activity in the absence of agonist allowed us to measure inverse efficacy as well as positive efficacy. In so doing, it was observed that Banyu (I) is an inverse agonist. Of the many natural CCR3 ligands, eotaxin, eotaxin-2 and MCP-4 are full agonists, and eotaxin-3, RANTES, MCP-3, vMIP-I and vMIP-II are partial agonists in inducing CCR3 signaling. In contrast, the chemokines CCL18 and I-TAC are antagonists able to block eotaxin-induced signaling through CCR3.

2. Materials and methods

2.1. Reagents

The chemokines used are denoted by the most common names that are currently in use (Zlotnik and Yoshie, 2000). All chemokines, with the exception of guinea pig eotaxin were obtained from R&D Systems (Minneapolis, MN). Guinea pig eotaxin was made synthetically by Gryphon Sciences (South San Francisco, CA), as has been done with other chemokines (Ueda et al., 1997). Membranes from CHO-K1 cells expressing human CCR3 were obtained from Euroscreen (Cat. ES138-M, Brussels, Belgium). All salts for buffers were obtained from Sigma (St. Louis, MO) and all culture medium components from Life Technologies (Rockville, MD) unless otherwise indicated. Fetal bovine serum was obtained from Gemini Bioproducts (Calabasas, CA). [^{125}I]eotaxin was obtained from NEN (Boston, MA). The compound, 2-[(6-amino-2-benzothiazolyl)thio]-*N*-[1-[(3,4-dichloryl)methyl]-4-piperidinyl]acetamide (Naya et al., 2001), was made by Medicinal Chemistry at Schering-Plough Research Institute, and designated Banyu (I) (Fig. 1). Banyu (I) was radiolabeled as follows. Firstly, 4-Boc-amino-[^{3}H]-piperidine (1) was prepared by tristriphenylphosphine ruthenium (II) chloride catalysed exchange with 90 at.% tritiated water. [^{3}H]3, 4-dichlorobenzaldehyde (2) was then prepared by tritium gas exchange with Crabtree's catalyst. Compounds (1) and (2) were then coupled via a reductive amination and the Boc group next removed by treatment with 4 M HCl in dioxane to form [^{3}H]*N*-(3,4-dichlorobenzyl)-4-aminopiperidine. This compound was then coupled with 6-Benzothiazolecarbamic acid, 2-((carboxymethyl)thio)-, 1,1-dimethylethyl ester, lithium salt under standard DEC/HOBT conditions and finally the Boc group was removed using 4 M HCl in dioxane to generate [^{3}H]Banyu (I). The crude product was purified by reverse

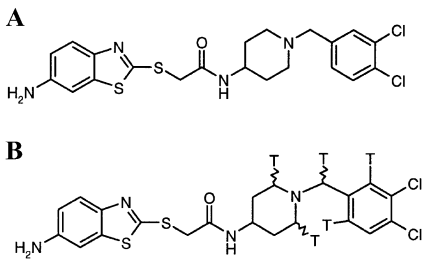

Fig. 1. (A) The structure of 2-[(6-amino-2-benzothiazolyl)thio]-*N*-[1-[(3,4-dichloryl)methyl]-4-piperidinyl]acetamide, Banyu (I). (B) [^{3}H]Banyu (I). T, tritium.

phase high performance liquid chromatography (HPLC) to yield 29 mCi at a specific activity of 31.2 Ci/mmol and a radiochemical purity of >98%.

2.2. Cell culture

CREM3 cells, in which the human CCR3 receptor is stably expressed in the rat Y3 cell line (Dairaghi et al., 1997; Umland et al., 2000), were cultured in Dulbecco's modified Eagle's Medium high glucose containing 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mg/ml G418 (Gemini Bioproducts). Cells were cultured in fresh medium every 2 days. The cells were used for intact cell receptor binding measurements 48 h after reculture in fresh medium and were removed from flasks by treatment with EDTA for 10 min at room temperature.

2.3. Eosinophil preparation

Human eosinophils were obtained from citrate-treated fresh whole blood drawn from normal volunteers. After removal of erythrocytes by Dextran sedimentation (4.5% Dextran T-500, Pharmacia Biotech, Uppsala, Sweden), leukocytes were isolated by Percoll (Pharmacia Biotech) density gradient (~ 1.080 g/l) centrifugation at room temperature. Following hypotonic lysis of residual erythrocytes from the collected granulocyte layer, neutrophils were removed using anti-CD16-coated magnetic beads (2×10^7 cells/mg beads). Anti-CD16 beads were prepared by mixing goat anti-mouse IgG Biospheres (1 mg/ml; Biosource International, Camarillo, CA) with mouse anti-human CD16 monoclonal antibody (5 μg/ml; PharMingen, San Diego, CA) for 30 min at 4 °C, followed by washing to remove unbound antibody. Purity was assessed by differential counts using Diff-Quick stained cytocentrifuge cell preparations and averaged ≥98% eosinophils.

2.4. Receptor binding analysis

Whole cell receptor binding analysis was done in RPMI binding buffer (RPMI 1640 medium, 25 mM HEPES, 0.2% bovine serum albumin, 0.1% sodium azide, and 0.08% CHAPS (White et al., 2000). CREM3 cells (Dairaghi et al., 1997; Umland et al., 2000) and CCR3 expressing CHO-K1 cells (Euroscreen) or human eosinophils were added to Costar 96 deep-well plates (Fisher Scientific, 180,000–300,000 per well in 160 μl). For competition binding, [^{125}I]eotaxin or [^{3}H]Banyu (I) (20 μl/well) were added to a final concentration of 0.1 or 5 nM respectively, followed by various concentrations of compound or chemokine (in 20 μl); each concentration was done in triplicate or quadruplicate. Nonradiolabeled Banyu (I) was dissolved in 100% dimethyl sulfoxide to a concentration of 10 mM and serially diluted in the binding buffer. For saturation binding, various concentrations of [^{125}I]eotaxin or [^{3}H]Banyu (I) (in 40 μl) were added to the cells to determine total binding. Non-specific binding of [^{3}H]Banyu (I) or [^{125}I]eotaxin was determined using nonradiolabeled Banyu (I) (10 μM) or 100 nM of eotaxin, respectively. The total volume per well was 200 μl. Assay plates were shaken for 1 to 2 h at room temperature. Cells were harvested onto 96-well GF/C unifilter plates (Unfilter-96 Harvester, Packard Instrument, Meriden, CT) and washed with ice-cold 500 mM NaCl. The Unifilter plates were air-dried overnight at RT, scintillation cocktail was added (Microscint-20, Packard) and the plates were counted (Top-Counter HTS, Packard). Binding curves were analyzed by nonlinear regression using Graphpad Prism (GraphPad Software, San Diego, CA) and specific binding determined after the subtraction of nonspecific binding.

CREM3 (see below) or CHO membrane (Euroscreen) filtration assays were done in binding buffer (25 mM HEPES, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% bovine serum albumin, pH 7.6) using 20 μg membrane per well and the conditions described above.

The CREM3 membrane scintillation proximity assay (SPA) bead-binding assay was done as described (Dairaghi et al., 1997) with the following modifications. Membranes were prepared by MDS Pharma Services (Bothell, WA) as follows. Cells (Dairaghi et al., 1997; Umland et al., 2000) were grown under selection (1 mg/ml G418) in 50-stack cube bioreactors. Cells were removed from the bioreactors with trypsin, resuspended in 20 volumes of cold 2.5 mM Tris–HCl, pH 7.4 containing protease inhibitors (0.3 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 3 μg/ml leupeptin) and incubated on ice for 15 min. The swollen cells were lysed and the cleared lysate was centrifuged (100,000 × *g*, 1 h, 4 °C); the membrane fraction was resuspended in 25 mM HEPES, pH 7.6, 75 mM NaCl, 1 mM EDTA with protease inhibitors. Membranes were precoated with polyvinyl toluene (PVT)–wheat-germ agglutinin (WGA)–SPA beads (PVT-WGA-SPA beads; Amersham Biosciences, Piscataway, NJ) in a ratio of 1:20 by weight in binding buffer and slowly rotated at 4 °C for 1 h. Membrane-bound beads were centrifuged (1500 rpm, 10 min., 4 °C), resuspended in binding buffer and combined with radioligand. Final concentrations per well were 0.05 nM [^{125}I]eotaxin or the indicated concentrations of [^{3}H]Banyu (I), 10 μg membranes, 200 μg PVT–WGA–SPA beads. To each well of a 96-well plate (Wallac Oy, Turku, Finland), 180 μl of radioligand/membranes/beads and 20 μl of cold competitor were added at the indicated concentrations. All conditions were done in triplicate. Plates were shaken briefly and incubated at room temperature for 5 h before counting on a MicroBeta scintillation counter (Wallac). Analysis was done as described above. Values are reported and mean ± S.D. of *n* experiments.

2.5. [^{35}S]-GTPγS binding

Each reaction was set up in quadruplicate by adding the reagents in assay buffer (20 mM HEPES, pH 7.4, 50 mM

NaCl, 3 μM GDP, 3 mM $MgCl_2$, 10 μg/ml saponin) in the following order to NEN Basic FlashPlate® microplates at the indicated final concentration: (1) CCR3/CHO-K1 membranes (Euroscreen), 20 μg protein/well in 160 μl; (2) the indicated concentrations of chemokines or Banyu (I) (or 1 μM GTPγS for nonspecific binding), 20 μl/well; (3) 0.1 nM [^{35}S]GTPγS (NEN), 20 μl/well for a final total volume of 200 μl. Where indicated the assay buffer contained 10 mM NaCl and 1 μM GDP. After 30 min at room temperature including 2 min of slow shaking on a plate shaker, the plates were centrifuged for 5 min at 2500 rpm at 4 °C in a tabletop Sorvall centrifuge (Dupont, Wilmington, DE), and counted immediately with a Packard TopCount. The percent increase over basal binding of [^{35}S]GTPγS was calculated as follows: 100 × [[(mean total sample cpm − nonspecific binding cpm) − mean basal cpm] ÷ mean basal cpm]. Basal cpm was defined as the mean cpm in the absence of chemokine minus the mean nonspecific binding cpm. Half-maximal effective concentrations (EC_{50}, the concentration of agonist required to give 50% of its own maximal stimulation) were calculated using nonlinear regression with GraphPad Prism The maximal increase over basal binding of [^{35}S]GTPγS (E_{max}) achieved for each chemokine or drug is expressed as a percentage of the maximal eotaxin response tested in the same experiment. Values are reported as mean ± S.D. of n experiments.

2.6. Intracellular calcium assay

Intracellular calcium levels were measured using a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale CA) as previously described (Umland et al., 2000). CREM3 cells were cultured overnight at 20,000 cells/well in 96 well black-wall clear bottom plates (Packard), precoated with 100 μg/ml poly D-lysine hydrobromide (Sigma). Adherent CREM3 cells were loaded with 4 μM Fluo-3 AM (Molecular Probes, Eugene, OR), in pH 7.4 Hank's Balanced Salt Solution (HBSS) without phenol red, containing 20 mM HEPES, 0.5% FBS, 2.5 mM probenecid (Sigma), and 0.04% pluronic acid (Molecular Probes) for 1 h at 37 °C. Adherent cells were washed with wash buffer/diluent (pH 7.4 HBSS without phenol red, 20 mM HEPES, 0.5% bovine serum albumin, and 2.5 mM probenecid) by an automated Denley CellWasher (Labsystemes Oy, Helsinki, Finland); after the final wash, fluid was aspirated to a level of 100 μl. Chemokines were diluted in wash buffer/diluent at 4-fold the final concentration and added in a volume of 50 μl/well. Banyu (I) was resuspended in dimethyl sulfoxide and diluted in wash buffer/diluent to 3-fold the final concentration and was titrated in half-log dilutions over 3.5 logs. Solvent control groups, containing concentrations equivalent to that in the highest concentration of Banyu (I), were included. Cells and chemokines were maintained at 37 °C throughout all calcium measurements. Fluorescence data was collected at 1-s interval for 60 s, followed by collection at 2-s intervals for 60 s. Background fluorescence was quantitated in wells containing cells but no chemokines and was subtracted from all experimental samples. All conditions were done in quadruplicate. Nonlinear regression analysis using GraphPad Prism was used to calculate EC_{50} or IC_{50} values. Values are reported as mean ± S.D. of n experiments.

3. Results

Banyu (I) (Fig. 1), a reported CCR3 antagonist, was evaluated for affinity and functional antagonism with human eosinophils and Y3 cells stably expressing human CCR3 (CREM3), since transfectant cell background, receptor number, G protein-coupling and methods were likely to differ from those previously used to evaluate this small molecule inhibitor (Saeki et al., 2001). Banyu (I) displaced labeled eotaxin with a K_i of 3.5 ± 1.6 nM (n = 7, Fig. 2A) and inhibited both eotaxin-induced calcium responses (IC_{50} = 15.6 ± 7.9 nM, n = 15, Fig. 2B) in CREM3 cells and eotaxin-induced chemotaxis of human eosinophils (data not shown). These values are consistent with those previously described for this compound. In the same systems, the previously described CCR3 antagonist chemokines I-TAC, IP-10, MIG (Loetscher et al., 2001), and CCL18 (Nibbs et al., 2000) were also tested. K_i values of 8.8 ± 1.1 nM (n = 4)

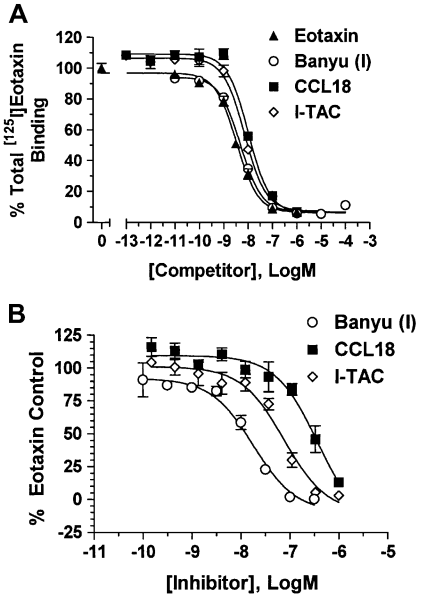

Fig. 2. Banyu (I) and CCL18 and I-TAC are inhibitors of eotaxin binding and function. (A) Competition binding by filtration with [^{125}I]eotaxin and CREM3 transfectants as described in Materials and methods. In the experiment shown, the K_i values for eotaxin, Banyu (I), CCL18 and I-TAC are 3.42, 4.88, 10.34 and 7.15 nM, respectively. (B) Banyu (I), CCL18 and I-TAC inhibit eotaxin-induced calcium mobilization in CREM3 transfectants. The control response was determined using eotaxin at a concentration of 2 nM, which is equivalent to the eotaxin EC_{50} (2.61 ± 1.66, n = 16). In the experiment shown, the IC_{50} of Banyu (I), CCL18 and I-TAC = 11.7, 287.3 and 76.2 nM, respectively, and the eotaxin EC_{50} = 3.8 nM.

Table 1
Comparison of eotaxin and Banyu (I) binding sites in eosinophils and CREM3 transfectants

		[^{125}I]Eotaxin	[^{3}H]Banyu (I)	% Eotaxin sites[a]
Eosinophils	K_d[b]	1.69 ± 0.48	4.34 ± 1.84	
	Sites × 10^4/cell	9.46 ± 3.12	26.91 ± 9.05	35
CREM3	K_d	4.97 ± 1.33	5.04 ± 0.40	
	Sites × 10^4/cell	18.96 ± 8.80	141.18 ± 23.58	13

[a] The % eotaxin sites were calculated as the ratio of eotaxin-binding sites per cell ÷ Banyu (I) binding sites per cell determined by [^{3}H]-Banyu (I).

[b] K_d (nM) and binding sites per cell (CREM3 transfectant or human eosinophil) were determined by saturation binding using [^{125}I]eotaxin or [^{3}H]Banyu (I) as the radioligand. Values are mean ± S.D. for three experiments.

for CCL18 and 9.1 ± 1.8 nM (n = 3) for I-TAC (Fig. 2A) were obtained by intact cell competition binding. IP-10 and MIG were approximately 10- to 100-fold less potent, respectively (data not shown) and because of their low affinity were not pursued further. Both CCL18 and I-TAC inhibited eotaxin-induced calcium responses (IC_{50} = 135.8 ± 103 and 47.2 ± 19.9 nM, n = 4, respectively, Fig. 2B).

The affinity of Banyu (I) for CCR3 on CREM3 cells and human peripheral blood eosinophils was determined by saturation binding using the tritiated form. The affinity of [^{3}H]Banyu (I) was identical for both the transfectant expressed CCR3 (K_d = 5.0 ± 0.4 nM, n = 3, Table 1) and the native receptor on human eosinophils (K_d = 4.3 ± 1.8 nM, n = 3, Table 1). These values were similar to the K_i of the unlabeled compound for displacing [^{125}I]eotaxin (K_i = 3.5 ± 1.6 nM, n = 7) in transfectants indicating that the radiolabel did not affect the affinity of the compound. [^{3}H]Banyu (I) displayed minimal specific binding to Y3 cells, the cell type used to establish the stable CCR3-expressing CREM3 cell line (data not shown), indicating specificity for CCR3 in these cells. As expected, in both eosinophils and CREM3 cells, the number of binding sites detected using the radiolabeled antagonist was greater than the number of eotaxin-binding sites, the latter presumably reflecting active state, G protein-coupled receptors only (Table 1). The number of binding sites detected using the radiolabeled antagonist was 5.2-fold greater in the transfectants than the eosinophils, while the number of eotaxin-binding sites was only 2.0-fold higher in the transfectants (Table 1). Interestingly, the proportion of eotaxin: Banyu (I) binding sites was 3-fold greater in the eosinophils compared to the transfectants.

The results described above comparing the binding of [^{3}H]Banyu (I) and [^{125}I]eotaxin indicated that CCR3 was present in the CREM3 transfectants and human eosinophils in two states: those conformationally inactive or uncoupled and those that are eotaxin-stabilized, G protein-coupled. To distinguish these receptor states, additional saturation binding analyses with membranes from CREM3 and CCR3-CHO-K1 cells were done using [^{3}H]Banyu (I) as the radioligand and the filtration method in the presence and absence of cold GTPγS, which promotes the dissociation of G proteins and receptors. No significant changes in K_d or B_{max} were observed in the presence and absence of GTPγS (CHO-K1: K_d = 4.3 vs. 4.7 nM and B_{max} = 10.6 vs. 11.3 pmol/mg in the absence versus the presence of 100 μM GTPγS).

The overexpression of the CCR3 receptor in the transfectants compared to eosinophils suggested that transfectant membrane-based signaling might be observable and useful for ligand characterization. To test this, a [^{35}S]GTPγS binding assay was developed using membranes from CHO-K1 cells expressing CCR3. The capacity of all known CCR3 ligands to stimulate [^{35}S]GTPγS binding was compared (Fig. 3). Table 2 summarizes the potency and efficacy of these ligands. Eotaxin, eotaxin-2, and MCP-4 were agonists (Fig. 3A) with similar maximal efficacy (E_{max} = 95–115%) and potency (Table 2, EC_{50} = 3.1–3.6 nM). Eotaxin-3, MCP-3 and RANTES were less potent and less efficacious agonists. The rank order of potency was MCP-4 = eotaxin-2 = eotaxin>eotaxin-3 = MCP-3 ≥ RANTES. Both guinea pig and mouse eotaxin (Fig. 3B) were partial agonists (E_{max} = 52%); mouse eotaxin was 7-fold more potent than guinea pig eotaxin. The virally encoded chemokines, vMIP-I and vMIP-II (Fig. 3B) were partial agonists (E_{max} = 40% and 51%, respectively) only weakly able to activate CCR3 with vMIP-II being 25-fold more potent (EC_{50} = 3.7 nM) than

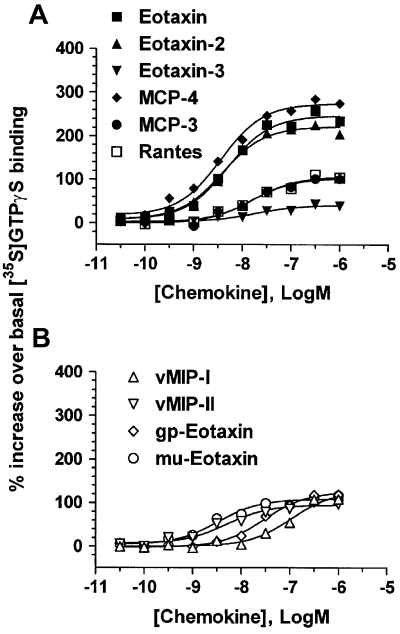

Fig. 3. Chemokine induction of [^{35}S]GTPγS binding to CCR3-CHO-K1 membranes. (A) Eotaxins 1–3, MCP-4, MCP-3, RANTES, or (B) vMIP-I or -II, or guinea pig (gp) or mouse (mu) eotaxin were added at the indicted concentrations to the reaction. The mean % increase over basal ± S.E.M. of quadruplicates was calculated after the subtraction of nonspecific binding as described in Materials and methods. The experiment shown is representative of two to five independent experiments for each chemokine. Table 2 summarizes the EC_{50} and E_{max}% for all experiments.

Table 2
Chemokine-induction of [^{35}S]GTPγS binding[a]

	EC_{50} (nM)	E_{max}% at 1 μM[b]
Eotaxin	3.6 ± 1.1[c]	100
Eotaxin-2	3.3 ± 0.8	94.9 ± 7.0
Eotaxin-3	14.0 ± 6.4	14.8 ± 2.5
MCP-4	3.1 ± 0.7	115.4 ± 6.1
RANTES	17.8 ± 0.8	50.1 ± 9.9
MCP-3	14.4 ± 0.6	49.3 ± 10.7
vMIP-I	90.5 ± 24.3	51.4 ± 0.4
vMIP-II	3.7 ± 1.9	39.7 ± 1.5
gp eotaxin	22.6 ± 9.6	52.0 ± 1.7
mu eotaxin	3.3 ± 0.1	52.5 ± 11.9

[a] The [^{35}S]GTPγS binding assay was done using 3 μM GDP and 50 mM NaCl.
[b] The maximal % increase in [^{35}S]GTPγS binding over basal binding (E_{max}) is expressed as a percentage of the maximal eotaxin response which is set to 100%.
[c] Values are means ± S.D., $n=2$, except eotaxin, $n=5$.

vMIP-I ($EC_{50}=91$ nM). CCL18 and I-TAC had negligible activity even when tested at high concentrations (300–1000 nM, data not shown).

The ability of Banyu (I), I-TAC and CCL18 to inhibit eotaxin-induced signaling was also tested. Banyu (I) inhibited the eotaxin-induced binding of [^{35}S]GTPγS in a dose-dependent manner (Fig. 4A). Its activity ($IC_{50}=16.1\pm3.4$ nM, $n=2$) was similar to its inhibition of eotaxin-induced increases in intracellular calcium in CREM3 cells (Fig. 2B) and eosinophil chemotaxis (Saeki et al., 2001). CCL18 ($IC_{50}=8.6\pm2.4$ nM, $n=4$) and I-TAC ($IC_{50}=34.5\pm19.4$ nM, $n=4$) also inhibited eotaxin-induced [^{35}S]GTPγS binding (Fig. 4A) with potency similar to the inhibition of eotaxin-induced intracellular calcium. Complete inhibition of eotaxin-induced [^{35}S]GTPγS binding was attained by CCL18 but not I-TAC.

In the above studies, negative % increase over basal values were obtained in [^{35}S]GTPγS binding over the 10^{-7} to 10^{-5} M concentration range of Banyu (I) (Fig. 4A). This suggested the presence of constitutive receptor activity in the CCR3 overexpressing transfectants, which was inhibited by Banyu (I). To explore this, the concentrations of NaCl and GDP were altered to increase the level of constitutive activity (Kenakin, 2001a). Parameters of basal activity and eotaxin potency were compared to those established above using higher GDP and NaCl concentrations (Table 3). Using 1 μM GDP and 10 mM NaCl, baseline cpm increased from 798 to 2321 (290%). In addition, the eotaxin-induced % increase in [^{35}S]GTPγS binding over basal (46%) decreased 3-fold and the potency of eotaxin ($EC_{50}=0.8$ nM) increased 3-fold compared to the values (145% and 2.4 nM) obtained with the higher salt and GDP conditions (Fig. 4B). This would provide an increased window for detection of inverse agonists (Kenakin, 2001a). Therefore, Banyu (I) was tested using the lower salt and GDP concentrations (Fig. 4B). Decreases in basal levels of bound [^{35}S]GTPγS induced by Banyu (I) clearly indicated that this compound was an inverse agonist. Maximal decreases in baseline activity were

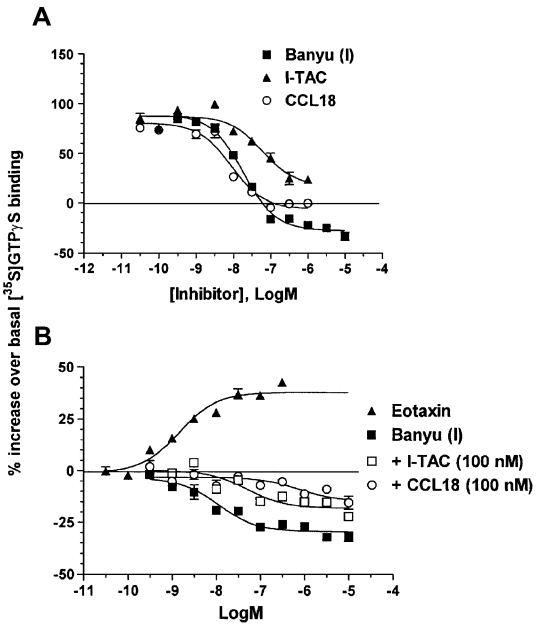

Fig. 4. Effects of Banyu (I), CCL18 and I-TAC on [^{35}S]GTPγS binding to CCR3-CHO-K1 membranes. (A) Eotaxin (3 nM) was first mixed with CCR3-CHO-K1 membranes followed by the addition of Banyu (I), CCL18 or I-TAC and reacted for 10 min prior to the addition of 0.1 nM [^{35}S]GTPγS. In the experiment shown, the IC_{50} values for Banyu (I), CCL18 and I-TAC were 18.5, 8.8 and 60.6 nM, respectively. The horizontal line indicates 0% increase over basal binding. (B) Assay buffer contained 10 mM NaCl and 1 μM GDP. Eotaxin or Banyu (I) at the indicated concentrations, in the absence or presence of 100 nM I-TAC or CCL18, was added to the CCR3-CHO-K1 membranes and reacted for 10 min prior to the addition of 0.1 nM [^{35}S]GTPγS. The mean % increase over basal ± S.E.M. of quadruplicates was calculated after the subtraction of nonspecific binding. The horizontal line indicates 0% increase over basal binding. The maximum mean % increase over basal ± S.E.M. for Banyu (I) in the experiment shown is −29.5 ± 1.0. The eotaxin and Banyu (I) EC_{50} values were 1.44 and 11.26 nM, respectively, and are representative of five independent experiments. The EC_{50} of Banyu (I) in the presence of 100 nM I-TAC or CCL18 were 51.5 or 731.8 nM, respectively. In a second experiment, the EC_{50} of Banyu (I) in the absence and presence of 100 nM I-TAC or CCL18 were 12.0, 121.1 or 76.2 nM, respectively.

17.9 ± 6.7 ($n=5$), and were consistently obtained. The EC_{50} was 11.9 ± 3.9 nM ($n=5$).

The chemokines CCL18 and I-TAC, which had minimal efficacy alone in inducing [^{35}S]GTPγS binding (see above) were similarly ineffective in altering basal [^{35}S]GTPγS binding under conditions of lower NaCl and GDP concen-

Table 3
Eotaxin induction of [^{35}S]GTPγS binding

	Buffer A[a]	Buffer B
Basal (cpm)	798 ± 80	2321 ± 85
Increase over basal (%)[b]	145 ± 18	46 ± 4
EC_{50} (nM)	2.4 ± 0.6	0.8 ± 0.2

[a] Buffer A contains 3 μM GDP/50 mM NaCl. Buffer B contains 1 μM GDP and 10 mM NaCl.
[b] Eotaxin was tested at half-log dilutions starting from 1 μM and diluted over 4.5 log units. % Increase over basal was calculated at the plateau response which occurred between $10^{-8.5}$ and 10^{-6} M eotaxin. The values are from three experiments in which the buffers were directly compared.

trations (data not shown), in contrast to Banyu (I). However, inclusion of 100 nM I-TAC or CCL18, reduced the potency of Banyu (I) in inhibiting basal [^{35}S]GTPγS binding by 5- or 65-fold, respectively (Fig. 4B). In addition, the negative efficacy of Banyu (I) was also decreased. Collectively, this indicates that these chemokines are CCR3 antagonists.

Lastly, the mechanism of Banyu (I) binding was established by several methods. First, Banyu (I) completely inhibited the binding of 0.005, 0.5 and 0.5 nM [^{125}I]eotaxin (Fig. 5A); however, minimal changes in IC_{50} values of Banyu (I) over this 100-fold change in radioligand input were observed (IC_{50} = 65.3, 34.4 and 23.1 nM for 0.005, 0.05 and 0.5 nM [^{125}I]eotaxin, respectively). In addition, the Hill slopes differed significantly from unity at each concentration of radioligand (n_H = − 0.71, − 0.58 and − 0.43 for 0.005, 0.05 and 0.5 nM [^{125}I]eotaxin). Second, the ability of eotaxin to displace [^{3}H]Banyu (I) binding to CREM3 cells was also examined. Eotaxin was a poor competitor of [^{3}H]Banyu (I), only partially displacing bound [^{3}H]Banyu (I) (Fig. 5B). Third, in the signaling assay, the ability of increasing concentrations of eotaxin to overcome the inhibitory effect of Banyu (I) on eotaxin-induced binding of [^{35}S]GTPγS was quantitated (Fig. 5C). Parallel rightward shifts in the eotaxin concentration–response curves in the presence of increasing concentrations of Banyu (I) were observed. In addition, a depression of the maximal responses to eotaxin was observed at all concentrations of Banyu (I). This indicates that the inhibitory activity of Banyu (I) was insurmountable. Thus, Banyu (I) is an allotopic (noncompetitive) CCR3 ligand.

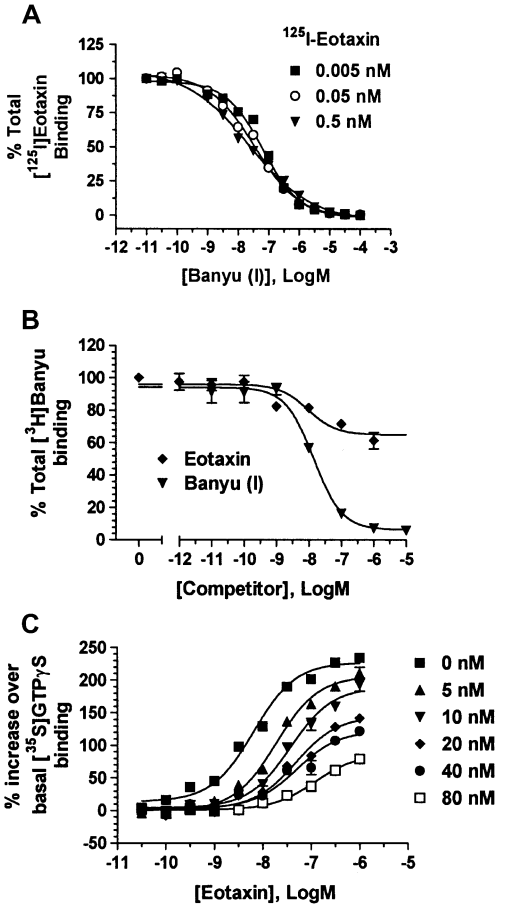

Fig. 5. Banyu (I) is a noncompetitive CCR3 antagonist. (A) Banyu (I) was tested for its ability to displace [^{125}I]eotaxin (0.005, 0.05 or 0.5 nM input) using CREM3 membrane–SPA binding as described in Materials and methods. IC_{50} and n_H values were determined using the sigmoidal dose response, variable slope equation in GraphPad Prism. (B) Receptor binding analysis using CREM3 cells was done with[^{3}H]Banyu (I) as the radioligand (5 nM) and unlabeled eotaxin or Banyu (I) as the unlabeled competitor. Results are expressed as a mean % of total binding ± S.E.M. of quadruplicates, determined in the presence of eotaxin or Banyu (I). In the experiment shown, the K_i of Banyu (I) is 7.8 nM and is representative of three independent experiments (K_i = 6.3 ± 1.6 nM, n = 3). (C) Eotaxin at the indicated concentrations was first mixed with CCR3-CHO-K1 membranes followed by the addition of 5, 10, 20, 40 or 80 nM Banyu (I) and reacted for 10 min prior to the addition of 0.1 nM [^{35}S]GTPγS. The mean % increase over basal ± S.E.M. of quadruplicates was calculated after the subtraction of nonspecific binding as described in Materials and methods. The experiment shown is representative of two independent experiments.

4. Discussion

Pharmacological examination of the CCR3 receptor has been limited to using radiolabeled chemokines, which are agonists. Recently, small molecule, nonpeptidic CCR3 antagonists have been described (Naya et al., 2001; Sabroe et al., 2000; Saeki et al., 2001; White et al., 2000). Here, Banyu (I) (Naya et al., 2001) was tritiated and used as a pharmacological tool. These studies provide the following new insights into the CCR3 receptor. First, Banyu (I) is a potent inhibitor of eotaxin binding. Moreover, it is a noncompetitive ligand, the first described for CCR3, since binding IC_{50} values did not increase predictably despite a 100-fold increase in [^{125}I]eotaxin concentration and inhibition of eotaxin-signaling was not overcome with increasing amounts of eotaxin. Second, CCR3 is able to spontaneously couple to G proteins in CCR3 overexpressing transfectants as revealed by [^{35}S]GTPγS binding. Third, Banyu (I) is an inverse agonist as evidenced by its ability to inhibit constitutive activity of the receptor. Fourth, in contrast, CCL18 and I-TAC are natural neutral antagonists of CCR3, inhibiting eotaxin-induced intracellular calcium increases and [^{35}S]GTPγS binding as well as the Banyu (I)-induced reductions in basal [^{35}S]GTPγS binding, with minimal intrinsic ability to modulate spontaneous coupling of CCR3 to G proteins. Lastly, [^{35}S]GTPγS binding, which measures a membrane-proximal event, is the most sensitive method to quantitate agonist efficacy, identifying eotaxin, eotaxin-2 and MCP-4 as full agonists and eotaxin-3, MCP-3, RANTES, vMIP-I and vMIP-II as partial agonists.

The use of a nonagonist radioligand, such as [^{3}H]Banyu (I), with specificity for CCR3, allowed for the estimation of the total CCR3 binding sites, including those conformationally inactive or uncoupled. This estimate was compared to the number of eotaxin-stabilized G protein-coupled recep-

tors (eotaxin binding sites). As expected, in both CREM3 transfectants and eosinophils, eotaxin binding sites were the minority (13% and 35%, respectively) and CREM3 cells expressed more [^{125}I]eotaxin- and [^{3}H]Banyu (I)-binding sites than did eosinophils. Interestingly, the proportion of [^{125}I]eotaxin binding sites to [^{3}H]Banyu (I) binding sites was 3-fold higher in eosinophils than in transfectants.

The overexpression of CCR3 suggested that CCR3 might spontaneously couple to G proteins, as has been shown for other G protein-coupled chemokine receptors (Chen et al., 2000; Geras-Raaka et al., 1998; Rosenkilde et al., 1999). To investigate this, a [^{35}S]GTPγS exchange assay was used. Increases in [^{35}S]GTPγS binding above basal levels are observed with positive agonists and decreases in [^{35}S]GTPγS binding below basal levels are indicative of agonists with negative efficacy (inverse agonists) that are able to modulate the spontaneous, precoupling of the receptor with G proteins (Costa and Herz, 1989). Because changes in the ionic environment can modulate constitutive receptor activity and the window to detect positive and negative efficacy agonists (Kenakin, 2001a), the behavior of CCR3 under different conditions was examined. With 5-fold and 3-fold lower concentrations of NaCl and GDP, respectively, increased constitutive activity (higher basal cpm), significantly decreased eotaxin-induced maximal binding of [^{35}S]GTPγS and increased potency of eotaxin were observed. Each of these changes is consistent with ionic effects on a constitutively active receptor and would allow the detection of inverse agonist activity (Kenakin, 2001a). Banyu (I) displayed inverse agonist activity by its ability to decrease basal [^{35}S]GTPγS binding. The maximal decrease observed (20%) was small, though consistent, and possibly due to weak intrinsic negative efficacy of this compound or to a low proportion of basal [^{35}S]GTPγS binding due to CCR3.

Interestingly, we were unable to observe either an increase in affinity of [^{3}H]Banyu (I) or a decrease in affinity of small molecule agonist compounds used as competitors of [^{3}H]Banyu (I) (data not shown) in the presence of excess free GTPγS, which causes a shift from the receptor G protein-coupled to the -uncoupled state. This could be due to the low proportion of eotaxin-stabilized CCR3/G protein-coupled complexes in the transfectants (13% of total CCR3 binding sites). This is also likely to be the explanation for the inability of membranes from these cells (CREM3) to support measurable [^{35}S]GTPγS binding. As this was the only cell line available to us, experiments using pertussis toxin treatment (Passador and Iglewski, 1994) of a CCR3 transfectant to observe reduction of basal [^{35}S]GTPγS binding and blockade of the stimulatory effect of eotaxin and the inhibitory effect of Banyu (I) were precluded.

Nevertheless, it is clear that CCR3 can display constitutive activity upon overexpression. This, together with the higher proportion (3-fold) of eotaxin-stabilized G-protein coupled receptors in eosinophils compared to transfectants (Table 1), raises the question as to the existence of precoupled, constitutively active CCR3 receptors in the native state, i.e., in eosinophils. However, the limitations in obtaining sufficient numbers of human peripheral blood eosinophils makes such studies of the native receptor difficult. It is also of interest as to whether precoupled, constitutively active CCR3 receptors in eosinophils play a role in eosinophil-mediated pathology. While other pathologies (De Ligt et al., 2000), particularly proliferation of transformed cells (Kenakin, 2001b) have been associated with the downstream and long-term effects of constitutively active G protein-coupled receptors, this has not been studied in eosinophil-mediated disease.

All of the natural chemokines reported to be CCR3 ligands were also examined for their efficacy in inducing [^{35}S]GTPγS binding. The rank order of potency of CCR3 agonists was MCP-4 = eotaxin-2 = eotaxin>eotaxin-3 = MCP-3 ≥ RANTES, with the most potent three being full agonists. Guinea pig and mouse eotaxin were partial agonists (E_{max} = 52%), indicating species diversity in the eotaxin-CCR3 binding interface. This pattern is similar to that observed for ligand-induced intracellular calcium increases in CREM3 transfectants, where RANTES and MCP-3 were also less potent, efficacious agonists and both guinea pig and mouse eotaxin were weak efficacy agonists (Umland et al., 2000). The virally encoded chemokines, vMIP-I and vMIP-II were also partial CCR3 agonists. This indicates this is a sensitive method to detect agonist activity since we and others (Kledal et al., 1997) were unable to observe a vMIP-II-induced intracellular calcium response in transfected cells. Moreover, the vMIP-II induction of [^{35}S]GTPγS binding in membranes of a CCR3 transfectant confirms previous findings of agonist activity of this viral chemokine in promoting eosinophil chemotaxis (Boshoff et al., 1997). However, in contrast to eosinophils, vMIP-II was a partial agonist in the [^{35}S]GTPγS exchange assay in the membrane system used here. These differences amongst various cells and responses likely reflect differences in the levels of CCR3 receptor expression and receptor reserve (Umland et al., 2000).

Using this sensitive measurement, the CXCR3 ligand, I-TAC did not modulate alone the binding of [^{35}S]GTPγS, yet it potently inhibited eotaxin-induced [^{35}S]GTPγS binding. Moreover, it reduced the potency of the inverse agonist Banyu (I) significantly. This, coupled with its potent affinity (K_i < 10 nM), clearly indicates that I-TAC is a true CCR3 antagonist, not a partial agonist at CCR3 acting by receptor desensitization. The physiological role of I-TAC in vivo is likely to result from its net effect of attracting CXCR3-bearing Th1 cells to sites of inflammation with its inhibiting the migration and activity of CCR3-expressing eosinophils, Th2 cells and basophils (Loetscher et al., 2001).

Similarly, CCL18 lacked activity in modulating basal [^{35}S]GTPγS binding and reduced the potency of the inverse agonist, Banyu (I). This, along with high affinity and antagonism of eotaxin-induced [^{35}S]GTPγS binding being observed at physiologically achievable concentrations (< 10

nM), indicates CCL18 is a potent CCR3 antagonist. Because its cognate receptor is currently not identified, its full physiological function is unknown.

In conclusion, the existence of naturally occurring CCR3 receptor antagonists is indicative of a selective pressure to regulate the location and activity of CCR3-bearing cells. This applies in particular to the eosinophil, the predominant cell expressing CCR3. The development of small molecule inhibitors of CCR3, such as Banyu (I), and their pharmacological characterization using sensitive measurements to define functional antagonism as done here, may provide therapies for eosinophil-mediated diseases.

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