Characterization of CCX282-B, an Orally Bioavailable Antagonist of the CCR9 Chemokine Receptor, for Treatment of Inflammatory Bowel Disease


ABSTRACT
The chemokine system represents a diverse group of G protein-coupled receptors responsible for orchestrating cell recruitment under both homeostatic and inflammatory conditions. Chemokine receptor 9 (CCR9) is a chemokine receptor known to be central for migration of immune cells into the intestine. Its only ligand, CCL25, is expressed at the mucosal surface of the intestine and is known to be elevated in intestinal inflammation. To date, there are no reports of small-molecule antagonists targeting CCR9. We report, for the first time, the discovery of a small molecule, CCX282-B, which is an orally bioavailable, selective, and potent antagonist of human CCR9. CCX282-B inhibited CCR9-mediated Ca²⁺ mobilization and chemotaxis on Molt-4 cells with IC₅₀ values of 5.4 and 3.4 nM, respectively. In the presence of 100% human serum, CCX282-B inhibited CCR9-mediated chemotaxis with an IC₅₀ of 33 nM, and the addition of α1-acid glycoprotein did not affect its potency. CCX282-B inhibited chemotaxis of primary CCR9-expressing cells to CCL25 with an IC₅₀ of 6.8 nM. CCX282-B was an equipotent inhibitor of CCL25-directed chemotaxis of both splice forms of CCR9 (CCR9A and CCR9B) with IC₅₀ values of 2.8 and 2.6 nM, respectively. CCX282-B also inhibited mouse and rat CCR9-mediated chemotaxis. Inhibition of CCR9 with CCX282-B results in normalization of Crohn’s disease such as histopathology associated with the TNFARE mice. Analysis of the plasma level of drug associated with this improvement provides an understanding of the pharmacokinetic/pharmacodynamic relationship for CCR9 antagonists in the treatment of intestinal inflammation.

Introduction
Chemokine receptors and their ligands are responsible for orchestrating the movement of cells under both homeostatic and inflammatory conditions within and between compartments in the body (Charo and Ransohoff, 2006; Viola and Luster, 2008). Chemokine receptor 9 (CCR9) is expressed on lymphocytes in the circulation and is the key chemokine receptor that enables these cells to home to the intestine (Zabel et al., 1999). CCR9 has only one identified ligand, CCL25 (Zaballos et al., 1999), expression of which is restricted to the thymus and the intestinal epithelium under normal conditions (Wurbel et al., 2000). Although CCL25 is the only identified ligand for CCR9, CCL25 has also been shown to bind to the atypical chemokine receptor CCX-CKR (Gosling et al., 2000). Although one of the main sites of CCL25 expression is the thymus, CCR9 deletion had no major effect on intrathymic T cell development; in addition, CCR9-deficient mice displayed a decrease in the prepro-B cells, with no measurable impact on the mature B cell compartment (Wurbel et al., 2001). Within the intestine, the primary site of CCL25 expression is epithelial cells lining the lumen (Kunkel et al., 2000); as a result, CCR9-positive cells are enriched in both the small intestine and the colon relative to other compartments.
Materials and Methods

Cell Lines and Cultured Primary Cells. Molt-4 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Invitrogen) in a humidified 5% CO2 incubator at 37°C. Stably transfected L1.2 cells expressing either CCR9A or CCR9B were generated as described. Whole-cell RNA was isolated from Molt-4 cells by using a mRNA isolation kit (mMACs; Miltenyi Biotec, Auburn, CA). DNA contamination was removed by DNase digestion via RNase-free columns (Qiagen, Valencia, CA), and cDNA was generated by using a GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). PCR of cDNA samples was performed with a Taq PCR Master Mix kit (Qiagen) and CCR9A or B-form primers (CCR9A: cagttgctggacagcagcatcagtaaaggac; CCR9B: gccacgctggacagcagcatcagtaaaggac). Plasmid DNA was then isolated from overnight bacterial cultures by Maxiprep (Qiagen) and electroporated into L1.2 cells via Gene Pulser (Bio-Rad Laboratories, Hercules, CA). After electroporation, cells were transferred into selection medium (800 µg/ml), and stable cells were generated. Receptor expression was confirmed by functional responses (see Fig. 2A).

Retinoic acid-stimulated T cells were generated as follows. PBMCs were obtained by density centrifugation using Ficoll (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK) of fresh human whole blood. Freshly isolated PBMCs were washed with phosphate-buffered saline and resuspended in RPMI medium 1640 supplemented with 10% fetal calf serum in a humidified 5% CO2 incubator at 37°C and cultured for 1 h to remove adherent cells. After this period nonadherent cells were collected and placed in culture on flasks precoated with anti-CD3 and anti-CD28 (1 µg/ml of each overnight; R&D Systems, Minneapolis, MN) supplemented with IL-12 (1 ng/ml; R&D Systems) and retinoic acid (Sigma-Aldrich, St Louis, MO) (1 µM). Expression of CCR9 was determined by chemotactic responses and staining with anti-CCR9 mAb (clone 248621; R&D Systems).

For selectivity profiling using intracellular calcium release, activated T lymphocytes were prepared by culturing human PBMCs isolated from buffy coats (Stanford University Blood Center, Palo Alto, CA) for 21 days in the presence of 10 ng/ml of human IL-2 (R&D Systems).

Mouse thymocytes were isolated from 3-week-old C57BL/6 mice. Rat thymocytes were isolated from 3- to 6-week-old Sprague-Dawley rats. When determining compound IC50 values, unless otherwise stated, all assays were run against the determined EC50 value of CCL25.

Calcium Mobilization Assays. Cells were loaded with 2 µM indo-1/AM dye (Molecular Probes, Carlsbad, CA) in culture medium, then washed with HBSS and resuspended at 107/ml in HBSS containing 0.1% BSA (Sigma-Aldrich). Cytosolic calcium responses were determined with a fluorometer (Photon Technology International, Lawrenceville, NJ) with excitation at 350 nm and dual emission at 400 and 490 nm. Alternatively, a FLIPR machine (Molecular Devices, Sunnyvale, CA) was used to measure intracellular calcium mobilization, in which case cells were loaded with the Fluor-4 AM (Invitrogen). In any given experiment, the CCL25 EC50 was determined before the generation of a compound IC50 value.

Mountain Peak Assay. Lymphocytes were isolated fromuffy coats (Stanford University Blood Bank) and activated with phytohemagglutinin (Sigma-Aldrich) for 3 days then cultured in the presence of IL-2 for another 3 weeks. On the day of the assay, the cells were loaded with Indo-1/AM (3 µg/ml) on a rotating platform in the dark for 45 min in culture medium. After this incubation, cells were centrifuged and resuspended in HBSS. One million cells were used for each trace, and cells were sequentially challenged with a series of chemokines in the presence of either 0.1% DMSO or 10 µM CXC282-B. Additional selectivity data are provided in Supplemental Table 1.

Chemotaxis Assays. Assays were carried out by using ChemotFX (NeuroProbe, Gaithersburg, MD) chemotaxis chambers. A 5-µm pore-sized polycarbonate membrane was used for Molt-4, L1.2, and primary lymphocyte assays, whereas thymocyte assays from both mouse and rat used a 3-µm pore size. Cells were harvested by
centrifugation and resuspended in chemotaxis buffer consisting of HBSS with 0.1% BSA at a density of \(10^7\) cells/ml. For assays designed to determine the compound potency in the presence of human serum, cells were resuspended in 100% human AB serum from pooled donors (Sigma-Aldrich). Equal volumes of cell suspension and diluted compound were mixed and incubated for 10 min at room temperature. Twenty microliters of the mixture was transferred to the upper chamber of the chemotaxis chamber. After a 120-min incubation at 37°C, the assay was terminated by removal of cell drops from the top of the filter. Migration signal was determined by adding 5 \(\mu\)l of CyQUANT solution (Invitrogen) to each well in the lower chemotaxis chamber and measuring the intensity of fluorescence on a SpectraFluor Plus plate reader (Tecan, Grödig, Austria).

**Radioligand Binding.** Because of the labile nature of CCL25, traditional binding assays using radiolabeled chemokine are not available for CCR9. Cells expressing the appropriate chemokine receptor were incubated at 4°C with 100 pM of \(^{3}H\)CCX807, a small-molecule CCR9 antagonist from a different chemical class than CCX282-B (Charvat et al., 2008; for CCX807 potency and selectivity data, see Supplemental Table 2 and Supplemental Fig. 1) in binding buffer (25 mM HEPES, 140 mM NaCl, 1 mM CaCl\(_2\), 5 mM MgCl\(_2\), 0.2% BSA, pH 7.1) in the presence of CCX282-B at various concentrations. Nonspecific binding was determined by including >100-fold excess of nonlabeled CCX807. After a 3-h incubation, cells were aspirated onto polyethyleneimine-treated G/P glass filters (PerkinElmer Life and Analytical Sciences, Waltham, MA) with a cell harvester (PerkinElmer Life and Analytical Sciences) and washed twice with washing buffer (25 mM HEPES, 500 mM NaCl, 1 mM CaCl\(_2\), 5 mM MgCl\(_2\), pH 7.1). Scintillant (20 \(\mu\)l of MicroScint-20; PerkinElmer Life and Analytical Sciences) was added to the filters, and the counts per minute were measured on a Topcount Scintillation counter (PerkinElmer Life and Analytical Sciences).

**Compounds.** CCX282-B and \(^{3}H\)CCX807 were provided by Dr. J. Powers, Medicinal Chemistry Department, Chemocentrix (Mountain View, CA).

**Animal Care.** Animals were purchased and housed in accordance with Institutional Animal Care and Use Committee guidelines and requirements of the relevant regulatory agencies. C57BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Portage, MI), all of which were housed at the Chemocentrix animal facility and treated in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals of the National Research Council. TNFAARE mice were bred, housed under specific pathogen-free conditions, and used at the Biomedical Sciences Research Centre Alexander Fleming (Athens, Greece).

**Effects of CCX282-B in the TNF\(^{\text{ARE}}\) Mouse Model of Terminal Ileitis.** Deletion of the TNF \(\text{AU-rich} (\text{ARE})\) elements from the mouse genome affects the mechanisms for TNF-alpha mRNA destabilization and translation repression. As a result, the TNF-\(\text{ARE}\) mice have increased TNF-alpha levels and develop a pathology similar to that of Crohn's disease (Kontoyiannis et al., 1999). In this study, CCX282-B was formulated as a suspension in 10% Cremophor EL (Sigma-Aldrich) at drug concentrations of 2.5 or 12.5 mg/ml for the 10 or 50 mg/kg doses, respectively. Compounds or appropriate vehicle controls were administered by subcutaneous injections of 4 ml/kg every other day. Dosing was started when the animals were 2 weeks of age (n = 10 per group) and continued for 10 weeks. At the end of the study, animals were sacrificed by CO\(_2\) inhalation, their intestines were dissected, divided into jejunum, ileum, terminal ileum, and colon, and the segments were individually formalin-fixed. These tissue samples were then stored in phosphate-buffered saline. Histological analyses of these samples were performed in a blinded manner at the Royal Free Gastroenterology Laboratory (London, UK). Tissues were orientated in a "Swiss-roll" fashion, paraffin-embedded, and transversely sectioned in longitudinal sections to provide histological views of the entire segment of the intestine.

Hematoxylin and eosin-stained sections were examined microscopically for signs of inflammation. Tissues from 13-week-old wild-type mice were also processed and used to establish a baseline control. The severity of ileitis was scored by using a semiquantitative scoring system: 0, lack of inflammation (normal), with no excess of inflammatory cells in the lamina propria or separation of muscularis mucosa and muscularis propria; 1, mild inflammation with scattered inflammatory cells seen in the lamina propria between the crypt bases and muscularis mucosa and muscularis propria; 2, moderate inflammation where numerous inflammatory cells are seen in the lamina propria between the crypt bases and muscularis mucosa and muscularis propria are separated by inflammatory cells; 3, severe inflammation, where numerous inflammatory cells are seen in the lamina propria and there are inflammatory cells between muscularis mucosa and muscularis propria and between circular and longitudinal layers of the muscularis propria; and 4, very severe inflammation in which, in addition to the observations associated with severe inflammation, inflammatory cells can be found on serosal surfaces.

**Statistical Methods.** A\(_2\) values for assessment of potency in chemotaxis assays were calculated from the following equation:

\[
pA_2 = p[\text{drug}(M)] - p[(A'/A - 1)]
\]

where \(A\) reflects the potency of the agonist in the absence of the antagonist and \(A'\) reflects the potency of the agonist in the presence of antagonist at a given concentration (M). \(EC_{50}\) and \(IC_{50}\) values were calculated by using Prism 4 (GraphPad Software Inc., San Diego, CA).

**Results**

**CCX282-B Inhibits CCL25-Induced Calcium Mobilization and Chemotaxis in Molt-4 Cells.** The Molt-4 T-cell line endogenously expresses CCR9. Stimulation of Molt-4 cells with CCL25 results in release of Ca\(^{2+}\) from intracellular stores (Fig. 1A). CCL25 induced a bell-shaped chemotaxis dose response typical of chemokines (Fig. 1B). It is noteworthy that CCL25 has a lower \(EC_{50}\) value when the chemotaxis assay is run in 100% human AB serum relative to the assays run in 0.1% BSA/HBSS. CCX282-B inhibited CCL25-induced calcium mobilization with an \(IC_{50}\) value of 5.4 ± 0.7 nM (n = 57; Fig. 1C). CCX282-B is also a potent inhibitor of CCL25-induced Molt-4 chemotaxis with an \(IC_{50}\) of 3.5 ± 0.3 nM (n = 385; Fig. 1D). To gain an understanding of the potency of CCX282-B in a relevant physiological medium, the Molt-4 chemotaxis assay was run in 100% human AB serum. In this system, CCX282-B inhibited Molt-4 migration with an \(IC_{50}\) of 33.4 ± 1.3 nM (n = 118; Fig. 1E). In inflammatory disorders, especially those chronic in nature such as Crohn's disease, the levels of \(\alpha\)-acid glycoprotein (AAG) are known to increase. This acute-phase protein is responsible for increased nonspecific binding of some drugs. This makes the assessment of compound potency in the presence of relevant concentrations of AAG potentially important. The ability of CCX282-B to inhibit CCR9-mediated chemotaxis was not affected by the addition of 5 mg/ml AAG (Fig. 1F).

Although radiolabeled CCL25 is not available it is possible to determine the binding of CCX282-B to CCR9 by direct competition with a tritiated small-molecule CCR9 antagonist (\(^{3}H\)CCX807; \(K_d\) 20 nM) as the tracer (10 nM). CCX282-B inhibited the binding of \(^{3}H\)CCX807 to Molt-4 cells with an \(IC_{50}\) of 6 nM (Fig. 1G).

**CCX282-B Inhibits CCL25-Induced Chemotaxis Mediated by CCR9A and CCR9B.** Two CCR9 gene product...
variants (CCR9A and CCR9B), resulting from differential mRNA splicing and different translation starting points, have been described in humans. CCR9A contains an extended N terminus of 12 amino acids, relative to CCR9B, has been described as displaying greater sensitivity toward CCL25 in chemotaxis, and is more abundantly expressed than the CCR9B form (Yu et al., 2000). Although any possible differences in biological or pathological roles remain to be elucidated for both forms of the CCR9 receptor, we set out to evaluate the potency of CCX282-B individually on both splice forms. To this effect, Baf-3 cells were transfected to express either CCR9A or CCR9B. In concordance with literature reports, we observed that the potency of CCL25 for CCR9A (EC$_{50}$; 31 ± 3.4 nM) is greater than for CCR9B (EC$_{50}$; 89 ± 3.1 nM) (Fig. 2A). CCX282-B inhibited the chemotaxis of Baf-3/CCR9A cells with an IC$_{50}$ of 2.8 ± 1.1 nM (n = 4; Fig. 2B) and inhibited the chemotaxis of Baf-3/CCR9B cells with an IC$_{50}$ of 2.6 ± 0.7 nM (n = 4; Fig. 2C). We also confirmed the presence of both splice forms on Molt-4 and primary lymphocytes (Fig. 2D).

CCX282-B Inhibits CCL25-Induced Chemotaxis of RA-Cultured Human T Cells. Assessment of compound activity on primary cells and under fully physiological conditions (i.e., serum or blood) is an important aspect of the characterization of any potential drug. In PBMCs from healthy donors, the proportion of CCR9-expressing cells is very low, typically between 2 and 4% of all CD3$^+$ cells (unpublished data). This low proportion meant that assessment of compound potencies on freshly isolated T cells was not practical. We used an approach based on methods detailed by Sigmundsdóttir et al., (2007) in which T cells were isolated from PBMCs and cultured for 8 days in the presence of all-trans retinoic acid, which is known to be central in the up-regulation of CCR9. After 8 days of culture under these conditions, the frequency of CCR9-expressing cells had increased dramatically (Fig. 3A) as assessed by cell surface antibody staining. The RA-cultured cells displayed a chemotactic response to CCL25, both in 0.1% BSA/HBSS and 100% human AB serum assays, in line with that observed in Molt-4 cells, resulting in EC$_{50}$ values of 80 and 20 nM, respectively.

![Graphs and diagrams](https://via.placeholder.com/150)
Fig. 2. CCX282-B inhibits CCL25-induced chemotaxis mediated by CCR9A and CCR9B. Chemotaxis assays were conducted by using Baf-3/CCR9A or Baf-3/CCR9B cells incubated in 0.1% BSA/HBSS at 37°C for 90 min. Cells migrating to CCL25 were detected by using CyQUANT dye. A, CCL25 induces Baf-3/CCR9A chemotaxis with an EC50 of 31 nM (aqua circles) and Baf-3/CCR9B chemotaxis with an EC50 of 89 nM (E).

B, CCX282-B inhibits CCL25-induced chemotaxis of Baf-3/CCR9A with an IC50 of 2.8 nM. C, CCX282-B inhibits CCL25 induced chemotaxis of Baf-3/CCR9B with an IC50 of 2.6 nM. D, RA-stimulated human T cells (primary cells) and Molt-4 cells express both CCR9A (326 base pairs) and CCR9B (277 base pairs) by RT-PCR. β-Actin loading controls are shown on the right.

Fig. 3. CCX282-B inhibits CCL25-induced chemotaxis of RA-stimulated human T cells. For chemotaxis assays RA-stimulated human T cells were incubated in 0.1% BSA/HBSS or 100% human AB serum as appropriate at 37°C for 90 min. Cells migrating to CCL25 were detected by using CyQUANT dye. A, RA-cultured human T-cells express high levels of CCR9 (left, isotype staining; right, anti-CCR9; number in top right reflects the percentage of CCR9-positive cells). B, CCL25 induces chemotaxis of RA-cultured human T cells with an EC50 of 20 nM in 100% human AB serum (aqua circles) and an EC50 of 80 nM in 0.1% BSA/HBSS (○). C and D, CCX282-B is a potent inhibitor of CCL25-induced chemotaxis of RA-cultured human T cells with an IC50 of 6.8 nM (C) and an IC50 of 141 nM in the presence of 100% human AB serum (D). E, CCX282-B (Closed purple circles, 0.1% DMSO; black circles, 100 nM CCX282-B; green circles, 300 nM CCX282-B; purple circles, 1000 nM CCX282-B; red circles, 3000 nM CCX282-B) has an A2 value of 195 nM (dose required to right-shift 2-fold the CCL25 dose-response curve).
(Fig. 3B). CCX282-B potently inhibited CCL25-induced chemotaxis with an IC\textsubscript{50} value of 6.8 ± 1.7 nM in buffer (n = 12 individual donors; Fig. 3C). Assessment of the ability of CCX282-B to inhibit RA-cultured cell CCL25-mediated chemotaxis in 100% human AB serum resulted in an IC\textsubscript{50} of 141 ± 13 nM (n = 17 individual donors; Fig. 3D). By using the chemotaxis bell-shaped curve resulting from a concentration response to CCL25, it is possible to calculate the potency of the compound in terms of its A\textsubscript{2} value. On human primary RA-cultured T cells in the presence of 100% human AB serum, CCX282-B has an A\textsubscript{2} value of 195 ± 20 nM (n = 17 individual donors; Fig. 3E).

**CCX282-B Inhibits CCL25-Induced Chemotaxis of Mouse and Rat Thymocytes.** To assess the potency of CCX282-B on nonhuman CCR9, freshly isolated thymus cells were used. All assessment of cross-species potency was conducted by using the standard chemotaxis assay. CCX282-B is a potent inhibitor of CCL25-induced mouse and rat thymocyte chemotaxis with IC\textsubscript{50} values of 6.9 ± 2.9 nM (n = 5) and 1.3 ± 0.8 nM (n = 3), respectively. Data are shown in Table 1. The similarity between the extent of nonspecific protein binding in both mouse and human serum, coupled with the similar potency of CCX282-B on mouse and human CCR9, support the use of the serum-adjusted potency values obtained for CCX282-B in the human system to be used as the target values for the mouse system.

**Selectivity Profile of CCX282-B Against a Panel of Chemokine Receptors.** Because the Molt-4 cells also express CXCR4, we tested the selectivity of CCX282-B against this receptor. Molt-4 cells were stimulated sequentially with CCL25 and stromal cell-derived factor-1α. Addition of CCX282-B resulted in complete inhibition of the CCL25-mediated signal, while having no impact on the ability of the cells to mobilize Ca\textsuperscript{2+} in response to stromal cell-derived factor-1α (Fig. 4A). To assess the selectivity of CCX282-B against numerous chemokine receptors in real time IL-2 cultured lymphocytes were stimulated sequentially, in the presence of either 0.1% DMSO (Fig. 4B) or 10 μM CCX282-B (Fig. 4B), allowing a comparison of the two traces and an assessment of the selectivity of CCX282-B. Cells stimulated in the presence of CCX282-B (10 μM) did not show any inhibition of the signal. A broader selection of chemokine receptor selectivity was undertaken using both binding and chemotaxis assays. CCX282-B did not show any significant inhibition of any of the receptors tested (Fig. 4C). CCX282-B was evaluated at 10 μM (n = 2) in screens for interaction with 142 various receptors and found to lack activity against any of them (Supplemental Table 1).

**CCX282-B Ameliorates the Severity of Intestinal Inflammation in the TNF\textsubscript{ΔARE} Mouse Model.** TNF\textsubscript{ΔARE} mice spontaneously develop a syndrome, characterized by severe inflammation of the small intestine, which bears many of the hallmarks associated with human Crohn’s disease (Kontoyiannis et al., 1999). In our study, by 12 weeks of age 60% of mice in the vehicle control group (n = 10) had developed severe/very severe intestinal inflammation, 20% had moderate inflammation, and the remaining 20% scored as normal (Fig. 5A). Treatment with CCX282-B (50 mg/kg twice daily; n = 10) resulted in complete protection from the severe inflammation associated with TNF-α overexpression. Seventy percent of the mice in this treatment group were...
scored as normal, and the remaining 30% were scored as having moderate inflammation. A similar protective effect was also noted with a lower dose of CCX282-B (10 mg/kg twice daily; n = 10). Figure 5B shows a representative image of a CCX282-B-treated animal (left; scored as normal) compared with an untreated control (right; scored as severe). Maximal plasma levels of CCX282-B associated with the dosing in this study were 1253 and 4107 ng/ml for the 10 and 50 mg/kg groups, respectively.

Discussion

CCX282-B is an orally active, potent, and selective antagonist of CCR9 and represents the first molecule of its class advanced into clinical development. CCX282-B has recently demonstrated efficacy in the induction of clinical response and maintenance of clinical remission in Crohn’s disease (Keshav et al., 2009a). In functional assay systems, using both cell lines and primary cells from humans and rodents, CCX282-B effectively inhibits CCL25-induced function with single-digit nanomolar potency. Although numerous other small molecules targeting chemokine receptors display significant cross-species differences in potency, CCX282-B is equally potent on mouse, rat, and human CCR9. In the standard in vitro assays, CCX282-B is at least 10-fold more potent than CCL25 on CCR9. When correlating the circulating level of a drug with a specific biological outcome, it is important to take into account the level of nonspecific binding of the molecule to serum proteins. To determine the degree of “serum” shift with CCX282-B, chemotaxis assays were conducted in the presence of 100% human AB serum. In these assays, CCX282-B retained its ability to inhibit the function of CCR9 on both Molt-4 and primary human lymphocytes. Other serum proteins, such as AAG, are known to be increased in the circulation of patients with inflammatory disorders (Nakamura et al., 1993) and potentially could alter the free fraction of drug available to engage its target. Failure to perform in vitro assessments of potency under pathophysiological conditions could lead to an underestimation of the plasma levels required to achieve the desired therapeutic effect. CCX282-B displayed no loss of potency in 100% human serum and in the presence of increasing concentrations of AAG. Two splice forms of the CCR9 receptor, designated CCR9A and CCR9B, have been described, both of which are capable of responding functionally to CCL25 stimulation in isolation (Yu et al., 2000). Although CCR9A has an additional 12 amino acids at the N-terminal end, both receptors are capable of mobilizing Ca\(^{2+}\) and chemotaxis in response to CCL25. Yu et al. (2000) reported that on cells expressing CCR9 both forms are found on CCR9-expressing cells, with CCR9A being the predominant form. Our results confirmed the presence of both CCR9A and CCR9B on Molt-4 cells; we also observed the presence of both isoforms on primary human lymphocytes. Although the relative contribution of the individual CCR9 isoforms to the biology associated with CCR9 is not known at this stage, we evaluated CCX282-B for its ability to inhibit both forms. In similar fashion to that seen on cells expressing both CCR9 splice forms, CCX282-B was able to inhibit the functional activity of both CCR9A and CCR9B. As CCX282-B represents the first molecule in its class, it was important to understand its selectivity profile. When tested against other chemokine receptors, CCX282-B displayed no activity on any other chemokine receptor, including CCX-CKR, the only other known receptor for CCL25 (Gosling et al., 2000).

Several reports have shown that interfering with the CCR9/CCL25 axis can inhibit the migration of T cells to the intestines (Hosoe et al., 2004; Rivera-Nieves et al., 2006). Increased levels of CCL25 have been reported in preclinical models of inflammatory bowel disease and, in line with this, inhibiting the recruitment of pathogenic T cells to the small intestine has been shown to be beneficial in the resolution of inflammatory disorders such as in the SAMP1/YitFc sponta-
neous murine model of ileitis (Rivera-Nieves et al., 2006). Although these reports have shown that inhibition of CCR9 can be beneficial, this work has been conducted using antibodies to neutralize the interaction between CCR9 and CCL25. However, before the present report no work had been presented on the efficacy of a CCR9 small-molecule antagonist in ameliorating intestinal inflammation. Because of the many similarities between the ileal inflammation observed in the TNF<sup>ΔA10</sup> mouse model (Kontoyiannis et al., 1999) and human Crohn’s disease, this model was chosen to evaluate the efficacy of CCX282-B. Both dose levels of CCX282-B (10 and 50 mg/kg) produced equally effective protection against intestinal inflammation. Thus, whereas 60% of the vehicle-treated control mice were scored as suffering from severe/very severe inflammation, none of the mice in the CCX282-B treated groups experienced such a degree of inflammation. In fact, 70% of the drug-treated mice were free of any histological indication of inflammation, and the remaining 30% experienced only a mild degree of inflammation (all histological scoring was performed in a blinded fashion). It is worth noting that, even though CCX282-B displays excellent oral bioavailability in humans and nonhuman species, in this study drug (or vehicle alone) was administered by the subcutaneous route to assess the systemic effects of the drug and not to create confusion with possible local intestinal nonspecific effects of the drug if it had been given orally.

Doses of CCX282-B for the TNF<sup>ΔA10</sup> mouse study were initially selected to provide a wide range of systemic exposure and, presumably, different degrees of efficacy. It is noteworthy that both doses provided similar and robust prevention of inflammation, suggesting that the exposure associated with the lower dose of 10 mg/kg is sufficient to produce maximal benefit in this model. Peak plasma levels of drug occurred shortly after subcutaneous administration and reached values as high as 1250 ng/ml (2.5 μM) and 4100 ng/ml (8.2 μM) in the 10 and 50 mg/kg cohorts, respectively. Both values are far greater than the concentration of compound (A<sub>10</sub> ~ 1.8 μM) required to produce a 10-fold right-shift of the CCL25 dose response (approximately equivalent to 90% receptor coverage) on primary cells assessed in the presence of 100% serum (Fig. 3E). In fact, at steady state, plasma levels of CCX282-B were estimated to exceed this A<sub>10</sub> value for approximately 12 h each day at the 10 mg/kg dose and for 24 h each day at the 50 mg/kg dose. The results of the mouse study suggest that exceeding 90% CCR9 blockade in the circulation, for at least 50% of the time, is sufficient to effectively block the development of intestinal inflammation in this particular model. It is noteworthy that a similar observation was made in the PROTECT-1 clinical trial in Crohn’s disease patients, in which clinical improvement was associated with plasma levels in excess of the A<sub>10</sub> value for several hours each day (Bekker et al., 2009; Keshav et al., 2009b).

Apostolaki et al. (2008) generated CCR9-deficient TNF<sup>ΔA10</sup> mice and, in contrast to our results, did not see any protective phenotype against intestinal inflammation with those mice. It is interesting to speculate as to why this difference should be observed between pharmacological inhibition and genetic manipulation of CCR9. Although no published data are available detailing the potential compensatory changes that may have occurred in the CCR9 and/or CCL25-deficient mice, there are examples from the chemokine field in which the deletion of a receptor results in increased levels of ligands for alternative receptors (Warmington et al., 1999). A similar, and as yet unobserved, phenomenon in the CCR9(−/−) TNF<sup>ΔA10</sup> mouse could explain the difference observed between pharmacological intervention and the genetic deletion.

In conclusion, CCX282-B is an orally bioavailable, potent, and selective CCR9 antagonist that demonstrates clear therapeutic benefit in a genetic mouse model of intestinal inflammation. Clinical results from a large study in Crohn’s patients provide further evidence for the key role of the chemokine receptor CCR9 in the pathophysiology of inflammatory bowel disease.

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**Characterization of the CCR9 Antagonist CCX282-B**

