Characterization of CCX140-B, an orally bioavailable antagonist of the CCR2 chemokine receptor, for the treatment of type 2 diabetes and associated complications


ChemoCentryx, Inc.; 850 Maude Avenue, Mountain View, CA 94043 [TJS, AK, ZM, BNZ, RB, HW, TB, DJD, PB, DJ, SM, LSE, LS, AP, SU, ZW, JCJ, TJS] and

Gladstone Institute of Cardiovascular Disease, Gladstone Research Institute, San Francisco, CA 94158 [LB, CT, IFC]
Running Title Page:

a) Running Title: Characterization of the CCR2 Antagonist CCX140-B

b) Corresponding Author:

Timothy J Sullivan, Ph.D.; ChemoCentryx, Inc.; 850 Maude Avenue, Mountain View, CA 94043

Phone: (650) 210-2933; email: tsullivan@chemocentryx.com

c) Number of:

Text Pages: 33
Tables: 2
Figures: 7
References: 38
Words in the Abstract: 224
Words in the Introduction: 503
Words in the Discussion: 1125

d) List of nonstandard abbreviations used in the paper: none used

e) Recommended section assignment: Endocrine and Diabetes
Abstract

The chemokine receptor CCR2 is central for migration of certain populations of monocytes and T cells into sites of inflammation. We report the discovery of a novel small molecule, CCX140-B, that is an orally bioavailable, selective and potent antagonist of human CCR2. CCX140-B inhibited CCR2-mediated Ca^{2+} mobilization and chemotaxis in THP-1 cells with IC_{50} values of 10 and 3.3 nM, respectively. CCX140-B inhibited chemotaxis of primary CCR2-expressing human monocytes towards CCL2 (MCP-1) with an IC_{50} of 8 nM. Human CCR2 knock-in mice were used to assess the in vivo effects of CCX140-B, which does not interact with mouse CCR2. Treatment of these mice with CCX140-B resulted in reduced recruitment of peripheral monocytes in a sterile peritonitis model and normalization of insulin resistance in diet induced obese (DIO) transgenic mice. Unlike other CCR2 antagonists, CCX140-B had no effect on plasma CCL2 levels or blood monocyte numbers in mice. In two Phase 1 clinical trials, CCX140-B was administered to healthy human volunteers and found to be well tolerated and to have a linear pharmacokinetic profile with a T_{1/2} of 48 – 60 hours. Similar to the findings in mice, there were no effects on plasma CCL2 levels or blood monocyte numbers. This study identifies the doses required to effectively block CCR2 on blood monocytes and has informed the doses selected in various ongoing Phase 2 clinical trials.
Introduction

The movement of cells throughout the body under homeostatic and inflammatory conditions is exquisitely orchestrated by the actions of chemokines and chemokine receptors (Charo and Ransohoff, 2006; Viola and Luster, 2008). C-C Chemokine receptor 2 (CCR2) is primarily expressed on monocytes in the circulation, although expression can also be seen on certain T cell populations and possibly on non-immune cells such as adipocytes and podocytes. CCR2 has four known ligands, CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), and CCL13 (MCP-4). CCL2 is the most selective of these chemokines for CCR2 and thus has been the most broadly studied CCR2 ligand.

CCR2 has been implicated in the pathogenesis of type 2 diabetes (T2D) and its associated co-morbidities, particularly diabetic nephropathy and neuropathy. For decades, the presence of systemic markers of inflammation has been known to increase with obesity. The adipose tissue has been shown to produce multiple inflammatory cytokines, including TNFα, IL-6, and CCL2, expression levels of which correlate with the degree of adiposity (reviewed in Ferrante, 2007). Several of these mediators have been shown to impair insulin sensitivity. For example, CCL2 impairs insulin-stimulated glucose uptake in human adipocytes and skeletal muscle cells, providing a link between inflamed adipose tissue and insulin resistance (Sell et al., 2006; Sartipy et al., 2003). CCL2 is overexpressed in obese rodents (Sartipy et al.; 2003, Takahashi et al., 2003) as well as obese humans (Christiansen et al., 2005; Kim et al., 2006). Different depots of adipose tissue, such as visceral, subcutaneous, and epicardial adipose tissues, show increased expression of CCL2 in obese patients (Bruun et al., 2005; Malavazos et al., 2005). A
clinical study identified elevated CCL2 levels in cardiac surgery patients as a risk factor for post-operative insulin resistance (Kremen et al., 2006).

Other studies have defined adipose tissue macrophages as the primary source of pro-inflammatory mediators (Wellen and Hotamisligil, 2003). In fact, the number of adipose tissue macrophages increases with increasing adiposity (Weisberg et al., 2003). Macrophage number in adipose tissue is dynamic and weight loss or gain rapidly changes the macrophage content of adipose tissue (Cancello et al., 2005). Studies using rodent models of insulin resistance and Type 2 diabetes have indicated that the CCR2:CCL2 axis is a primary control point for the entry of inflammatory macrophages into the adipose tissue of obese subjects (Neels and Olefsky, 2006). CCR2-deficient mice have reduced macrophage numbers in adipose tissue depots and display significantly improved metabolic parameters relative to wild-type counterparts (Weisberg et al., 2006).

Here we present data detailing the preclinical and clinical characterization of CCX140-B, a novel small molecule antagonist of CCR2 (Basak et al., 2008), for which evidence of clinical efficacy in type 2 diabetes has been recently disclosed (Hanefeld et al., 2011). Its effects on CCR2-mediated functional endpoints, under fully physiological conditions, have been fully characterized in vitro. We also describe the therapeutic benefit of this compound in a mouse model of obesity-driven hyperglycemia and insulin resistance. In addition, we describe key aspects of the Phase 1 clinical evaluation of CCX140-B in healthy human volunteers.
Methods

Compounds

CCX140-B (Basak et al., 2008) for non-clinical work was obtained from the Medicinal Chemistry department at ChemoCentryx, Inc. (Mountain View, CA). Material for the clinical study was prepared under Good Manufacturing Practices and was provided to the clinical site by ChemoCentryx.

In vitro potency assessment

Cells and reagents. THP-1 and HEK293 cells were from ATCC (Rockville, MD). Human monocytes were isolated from healthy volunteer LRS chambers (Stanford Blood Center, Palo Alto, CA) using MACS separation reagents (Miltenyi Biotec, Auburn, CA). Stably transfected HEK293 cells expressing human CCR2B were generated as follows: whole-cell RNA was isolated from THP-1 cells using an mRNA isolation kit (mMACs; Miltenyi Biotec, Auburn, CA). DNA contamination was removed by DNase digestion via RNeasy columns (Qiagen Inc., Valencia, CA) and cDNA was generated using GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). PCR of cDNA samples was performed using Taq PCR Master Mix kit (Qiagen Inc., Valencia, CA) and CCR2B primers: ccagtagcggcgcctactcgttctcg - forward; caatcagcggccgcctctttaac cagccgagac - reverse). Plasmid DNA was then isolated from overnight bacterial cultures by Maxiprep (Qiagen Inc., Valencia, CA) and electroporated into HEK293 cells via Gene Pulser (BioRad Laboratories, Hercules, CA). Following electroporation, cells were transferred into selection medium (800 µg/mL) and stable-expression cells were generated. CCR2B expression was confirmed by flow cytometry (monoclonal antibody clone 48607, R&D Systems, Minneapolis, MN) and cell based assays. CCX140-B
(Basak et al. 2008), the sodium salt of CCX140, was prepared at ChemoCentryx (Mountain View, CA). Recombinant chemokines were from R&D Systems (Minneapolis, MN). [125I]-CCL2 was from PerkinElmer (Boston, MA). Human serum was from Bioreclamation (Hicksville, NY). Phycoerythrin (PE)-conjugated anti-human CD14 mAb was from BD Biosciences (San Jose, CA).

**In vitro assays.** Chemotaxis, calcium mobilization, and radioligand binding assays were conducted as previously described (Walters et al., 2010).

**Data analysis.** Inhibition values (IC\textsubscript{50}) were calculated using non-linear regression with a one-site competition model (GraphPad Prism, GraphPad Software, La Jolla, CA). \( pA_2 \) 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).

**Effects of CCX140-B in the thioglycollate induced mouse model of sterile peritonitis**

In this study, CCX140-B was formulated as a solution in 1% hydroxypropyl methylcellulose\textsuperscript{®} (Sigma-Aldrich, St Louis, USA) at drug concentrations of 0.3 mg/mL, 1 mg/mL or 3 mg/mL, for the 3 mg/kg, 10 mg/kg or 30 mg/kg doses. Mice were dosed orally (PO) twice daily for the duration of the study. Dosing began 1 hour prior to thioglycollate challenge. Mice were challenged intra-peritoneally (IP) with thioglycollate on day 0. Peritoneal macrophages were harvested 3 days after thioglycollate challenge by lavage with 5 mL of ice cold phosphate buffered saline (PBS).
Effects of CCX140-B in hyperglycemic diet induced obese (DIO) mice

Male human CCR2 knock-in mice were placed on a high fat diet (D12492, 60 kCal% fat, Research Diets, New Brunswick, NJ) or lean control diet (D12450B, 10 kCal% fat, Research Diets, New Brunswick, NJ) at 6 weeks of age and maintained on that diet for the duration of the study. Mice were generally sufficiently diabetic to begin therapeutic intervention after about 24-26 weeks on the high fat diet. Blood glucose and insulin levels were determined after an overnight fast (14 – 16 hours). Insulin sensitivity was determined by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). HOMA-IR was calculated according to the following formula: HOMA-IR = Glucose (mg/dL) x Insulin (uU/mL)/405 (Matthews et al., 1985). CCX140-B was formulated as a solution in 1% hydroxypropyl methylcellulose® (Sigma-Aldrich, St Louis, USA) at a drug concentration of 3 mg/mL, for the 30 mg/kg dose. Compound or appropriate vehicle control were administered by oral gavage twice per day. Dosing was started when the animals were 26 weeks of age (body weights between 45–55 g/mouse; n = 10 per group) and continued for 2 weeks. Body weights were measured prior to initiation of treatment and weekly thereafter. At the end of the study, animals were sacrificed by CO2 inhalation and the epididymal fat pads were isolated. The fat pads were minced and shaken for 30 min at 37 °C in 25 mL of buffer (DMEM containing 5% fatty acid-free BSA and 0.01% benzonase) with 1 mg/mg collagenase. The material was filtered through a 70-μm nylon strainer and centrifuged at 400 x g for 5 min. The supernatant was discarded and the cells were suspended in 40 mL of buffer and centrifuged again. The supernatant was discarded and the cells were suspended and incubated for 2 min in 1 mL of Red Blood Cell Lysing Buffer (BD Biosciences, San Jose, CA), after which 25 mL of buffer was added and the
cells were centrifuged again. The supernatant was discarded and the cell populations were analyzed by flow cytometry.

Animal Care

Animals were purchased and housed in accordance with institutional IACUC guidelines and requirements of the relevant regulatory agencies. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). CCR2+/− mice were generated as described (Boring et al., 1997). Human CCR2 knock-in mice were created using homologous recombination in ES cells to replace the entire murine CCR2 gene with human CCR2, under the control of the endogenous murine CCR2 promoter. The neomycin resistance gene was subsequently spliced out using Cre-recombinase. All mice were housed at the ChemoCentryx 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.

Phase 1 Clinical Trials

Double-blind, randomized, placebo-controlled, single-ascending-dose and multiple-ascending-dose Phase 1 studies were conducted to evaluate the safety, tolerability, and PK properties of CCX140-B in healthy subjects.

Single-Ascending-Dose Study. Within each of 7 serial cohorts, healthy subjects (n=8) were randomized to receive a single dose of CCX140-B (n=5) or placebo (n=3). Doses tested were 0.05, 0.1, 0.3, 0.6, 1, 3, and 10 mg of CCX140-B.

Multiple-Ascending-Dose Study. Within each of 4 serial cohorts, healthy subjects were randomized to CCX140-B or placebo. Doses tested were 0.6 mg q.d., 2 mg q.d., 5 mg
q.d., and 10 mg q.d. Subjects in the 0.6 mg and 2 mg q.d. cohorts (n=6) were randomized to CCX140-B (n=5) or placebo (n=1) daily for 7 days. Subjects in the 5 mg and 10 mg q.d. cohorts (n=10) were randomized to CCX140-B (n=8) or placebo (n=2) daily for 10 days.

All subjects in both studies were in good general health prior to enrolling in the study and gave written Informed Consent to participate in the studies prior to any study procedures being performed. Study protocols were approved by the Ethics Committee of the Two Basels and by Swissmedic (Switzerland). The studies were conducted at Covance (Basel, Switzerland) in accordance with the Declaration of Helsinki, and Good Clinical Practice (GCP) and International Conference on Harmonization (ICH) guidelines.

**Safety and tolerability assessments.** Standard clinical methods were used, including physical examinations, vital signs, 12-lead ECG, and clinical laboratory measurements, throughout both studies. Adverse events were recorded for the entire study duration. Investigators assessed all adverse events for severity, duration, outcome, and possible relationship to study medication.

**Pharmacokinetic assessment.** Plasma samples were collected at predetermined timepoints. Blood samples (8 mL) were collected into EDTA tubes, gently mixed and kept on wet ice until centrifuged (within 30-min) at 2,000 $g$, in a refrigerated centrifuge, for 10 min. Resulting plasma was stored (-80 °C) until analysis. Following protein precipitation, supernatant solutions were analyzed by HPLC-MS/MS using a validated method (nominal plasma concentration range: 1-1,000 ng/mL). PK values were generated using non-compartmental analysis with WinNonlin™ Professional v.5.2 (Pharsight, Mountain View, CA).
**Plasma MCP-1 assessment.** Plasma samples were collected at predetermined timepoints. Blood samples (8 mL) were collected into EDTA tubes, gently mixed and kept on wet ice until centrifuged (within 30 min) at 2,000 g, in a refrigerated centrifuge, for 10-min. Resulting plasma was stored (-80 °C) until analysis. MCP-1 levels were determined by Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer’s protocol.

**Blood Monocyte Assessment.** Blood samples (50 mL) were collected into EDTA tubes, gently mixed and kept on wet ice. PBMC were isolated via density gradient centrifugation. Isolated cells were stored (-80 °C) until analysis. Monocyte populations were determined by flow cytometry using antibodies to CD14 and CD16 (BD Biosciences, San Jose, CA)

**Results**

**CCX140-B inhibits CCL2-induced chemotaxis and calcium mobilization in THP-1 cells and inhibits radioligand binding in HEK293-CCR2B cells**

The THP-1 human monocytic cell line endogenously expresses CCR2. CCL2 induces chemotaxis of THP-1 cells with an EC_{50} of 0.1–0.5 nM (data not shown). CCX140-B is a potent inhibitor of CCL2-induced THP-1 chemotaxis with an average IC_{50} of 3.3 nM (n=158, Figure 1A). In order to test the potency of CCX140-B under more physiologically relevant conditions, the THP-1 chemotaxis assay was run in the presence of 4.5% human serum albumin. Under these conditions, CCX140-B inhibited the CCL2-induced THP-1 chemotaxis with an IC_{50} of 300 nM (n=30, Figure 1B). In chronic inflammatory disorders, the levels of α1-acid glycoprotein (AAG) become elevated (Nakamura et al., 1993). This acute phase protein has been shown to increase the non-
specific binding of some drugs. For this reason, the potency of CCX140-B was assessed with increasing amounts of AAG in chemotaxis assays. The ability of CCX140-B to inhibit CCR2-mediated chemotaxis was not impacted by the addition of either 1 or 5 mg/mL AAG (Figure 1C). The ability of CCX140-B to directly compete with $[^{125}\text{I}]$-CCL2 binding to human CCR2 was tested using stably transfected HEK293-CCR2B cells; in this assay, CCX140-B displayed an IC$_{50}$ of 7 nM (n=107, Figure 1D). Stimulation of THP-1 cells with CCL2 results in release of Ca$^{2+}$ from intracellular stores. CCX140-B inhibited CCL2-induced calcium mobilization with an IC$_{50}$ value of 10 nM (n=4; Figure 1E).

**CCX140-B inhibits CCL2-induced chemotaxis, calcium mobilization and radioligand binding in primary human monocytes**

Assessment of compound activity on primary cells and under physiologically relevant conditions (i.e., serum or blood) is an important part of the characterization of potential drugs (Walters et al., 2010; Dairaghi et al., 2011; Schall and Proudfoot, 2011). CCR2 is primarily expressed on the monocytic cell population in blood, particularly in the CD14+ compartment. CCL2 induces chemotaxis of CD14+ primary monocytes with an EC$_{50}$ of 0.1–0.5 nM (data not shown). CCX140-B is a potent inhibitor of CCL2-induced monocyte chemotaxis with an IC$_{50}$ of 8 nM (n=2, Figure 2A). In order to test the potency of CCX140-B under more physiologically relevant conditions, the monocyte chemotaxis assay was run in the presence of 100% human AB serum. Under these conditions CCX140-B inhibited the CCL2-induced primary monocyte chemotaxis with an IC$_{50}$ of 200 nM (n=2, Figure 2B). Stimulation of primary CD14+ monocytes cells with CCL2
results in release of Ca\(^{2+}\) from intracellular stores. CCX140-B inhibited CCL2-induced calcium mobilization with an IC\(_{50}\) value of 3 nM (n=2; Figure 2C). CCX140-B inhibited the binding of \([^{125}\text{I}]\)-CCL2 to human monocytes with an IC\(_{50}\) of 17 nM (n=6, Figure 2D). In order to gain a more clear understanding of the affinity of CCX140 for CCR2, saturation binding was performed using \([^3\text{H}]\)-CCX140 on primary CD14+ human monocytes. This study indicated that CCX140 has an average K\(_d\) of 2.3 nM towards human CCR2 (n=2, Figure 2E).

**CCX140-B inhibits chemotaxis induced by all CCR2 ligands in primary monocytes**

It is important to assess the efficacy of any CCR2 antagonist against all known CCR2-activating chemokines, which in man includes four proteins: CCL2 (MCP-1), CCL7 (MCP-2), CCL8 (MCP-3), and CCL13 (MCP-4). Chemotaxis assays with human monocytes in 100% human serum displayed a typical bell-shaped dose-response to each of these four chemokines (Figure 3A-D). A marked right shift (about 5-fold) and general reduction in the height of the dose-response curves were observed in the presence of 1 µM CCX140-B in human serum. This shift represents approximately 80% CCR2 blockade. At higher concentrations of CCX140-B (e.g., 10 µM) the chemokine dose response curves continued to shift to the right (~30–100 fold shifts) for CCL2, CCL7, and CCL13). Interestingly, the CCL8 dose response did not continue to right shift to the same extent, perhaps reflecting the selectivity of CCX140-B for CCR2, as CCL8 can also signal through CCR1, which is also expressed on blood monocytes. The potency of CCX140-B is unchanged across all members of the MCP family, indicating that effective
blockade of CCR2 function should be expected in vivo in response to any of the major CCR2 ligands.

**Preclinical pharmacokinetic profile of CCX140-B**

The pharmacokinetic behavior of CCX140-B was tested in mouse, rat, and dog (Table 1). CCX140-B showed excellent oral bioavailability in all preclinical species tested. Plasma clearance values were also low across species, with dogs showing very low clearance rates. CCX140-B displays high exposure in all species at relatively low nominal doses and it appears to be primarily restricted to the blood compartment, as illustrated by its low volume of distribution.

**Selectivity profile of CCX140-B against a broad panel of chemokine receptors and CYP enzymes**

A broad panel of chemokine receptor selectivity was undertaken using both radioligand binding and chemotaxis assays. CCX140-B did not show any significant inhibition of any of the receptors tested (Table 2). CCX140-B was also tested at 10 μM against the major CYP isoforms and was found to lack any inhibitory activity in enzymatic assays (Table 2). Assessment of inhibition of the hERG potassium channel activity by patch clamp technique showed that CCX140-B lacked inhibition of hERG at concentrations up to 100 μM (Table 2). CCX140-B was evaluated at 10 μM in screens for interaction with 142 various biologically important receptors and found to lack activity against any of them (Supplemental Table 1).
Characterization of human CCR2 knock-in (hCCR2 KI) mice

Due to the fact that CCX140-B does not bind with high affinity to mouse CCR2, transgenic mice were generated by homologous recombination which express human CCR2 under the control of endogenous genetic elements (hCCR2 KI mice). As shown in Figure 4A, the entire mouse CCR2 coding region was replaced with the human CCR2 coding region, while the endogenous mouse CCR2 control elements remain intact. Following recombination, the neomycin resistance gene, which was flanked by loxP sites, was removed via Cre-mediated recombination. After generating the hCCR2 KI mice, it was important to characterize their monocyte inflammatory response. Mice were challenged with thioglycollate in a sterile peritonitis model and the recruitment of leukocytes into the peritoneal space was assessed 3 days after challenge. CCR2-/- mice displayed a characteristic reduction in total peritoneal leukocyte numbers after challenge, while the hCCR2 KI mice exhibited a response equal to wild-type mice (Figure 4B).

Activity of CCX140-B in hCCR2 KI mice

Having established that the hCCR2 KI mice displayed normal monocyte populations and responses to CCR2 ligands, pharmacology experiments were undertaken to determine the activity of CCX140-B in vivo. CCX140-B displays high selectivity for human CCR2 as there is strong inhibition of hCCR2 KI mouse bone marrow derived monocyte chemotaxis induced by mJE (mouse CCL2) (Figure 5A) but no inhibition of similar cells from wild-type control mice (Figure 5B). In concordance with the in vitro results, CCX140-B significantly reduces the peritoneal accumulation of macrophages in hCCR2 KI mice following thioglycollate challenge (Figure 5C) but has no effects on the influx of cells into the peritoneal space of wild type control mice (Figure 5D). CCX140-B (10
mg/kg) demonstrates an approximate 50% reduction in the number of infiltrating cells. The reduction of cellular accumulation in hCCR2 KI mice by CCX140-B is complete at 30 mg/kg, as the cell numbers measured at this dose were indistinguishable from those seen with CCR2−/− mice (data not shown). The trough plasma levels of CCX140 at 30 mg/kg CCX140-B result in ~85% receptor blockade (Supplemental Figure 1) and these levels correlate well with compound levels required to shift 10-fold the in vitro dose response to CCL2 in the presence of 100% serum. Drug levels falling below 50% receptor coverage at trough result in no inhibition of monocyte recruitment. Interestingly, CCX140-B does not alter the population of blood monocytes in hCCR2 KI mice challenged with thioglycollate. Specifically, there is no change in the frequency of 7/4+Ly6G− monocytes following CCX140-B administration (Figure 5E), indicating that the reduction in peritoneal cell accumulation is not the result of diminished release of cells from bone marrow. Additionally, CCX140-B exerts its inhibitory effects without altering the systemic levels of mouse JE (CCL2) (Figure 5F).

**CCX140-B improves insulin sensitivity in obese, diabetic hCCR2 KI mice**

In order to study the effect of CCX140-B on hyperglycemia and insulin resistance in a mouse model of Type 2 diabetes, male hCCR2 KI mice were placed on a high fat diet at 6 weeks of age. The high fat diet delivered 60% of caloric content in the form of fat, while control diet mice received a diet with 10% of the caloric content derived from fat. Mice were randomized to CCX140-B treatment or vehicle when fasting blood glucose levels exceeded 180 mg/dL, typically after 24–26 weeks on high fat diet. Two weeks of CCX140-B treatment significantly reduced both fasting glucose levels (Figure 6A, p<0.005 vs vehicle) as well as fasting insulin levels (Figure 6B, p<0.05 vs. vehicle) in
these mice. CCX140-B nearly normalized blood glucose levels. As a result of the reduction in both fasting glucose and insulin, significant improvement in the insulin sensitivity was seen in treated mice as calculated by the HOMA-IR index (Figure 6C, p<0.05 vs vehicle). The improvements in glycemic control and insulin sensitivity coincided with significant reductions in the number of inflammatory (F4/80+CD11c+) macrophages present in the epididymal fat depots of the mice (Figure 6D, p<0.005 vs vehicle). Similarly to the findings in the thioglycollate studies, CCX140-B improved the diabetic condition of these mice with no change in systemic CCL2 levels (Figure 6E). CCX140-B exerted all beneficial effect without altering body weight in any experimental animals (data not shown).

**Pharmacokinetic profile of CCX140-B in healthy human volunteers**

CCX140-B was well tolerated and displayed a linear dose-exposure profile at all doses (0.05-10 mg) in the single-ascending-dose Phase-1 study (Figure 7A). Average CCX140 plasma levels above 1,000 ng/mL (~2 uM) were reached in this study and the plasma half-life of CCX140 approached 48 hours at the 10 mg dose. Following 10 days of 10 mg CCX140-B (Fig. 7B), steady-state CCX140 plasma levels fluctuated from a peak of 2,800 ± 440 ng/mL (~5,600 ± 880 nM), achieved 2 hours post-dose, to a trough level of 1,770 ± 232 ng/mL (~3,500 ± 460 nM). Repeated dosing of CCX140-B did not result in changes to circulating monocyte numbers at either the 5 or 10 mg doses (Figure 7C). No increases in plasma levels of CCL2 were observed after CCX140-B administration (Figure 7D). The lack of effect of CCX140-B on both monocyte numbers as well as CCL2 levels agrees with the preclinical mouse pharmacology data but differs from recent reports of CCR2 antagonists evaluated in both preclinical and clinical studies (Vergunst...
et al., 2008; Wang et al., 2009; Aiello et al., 2010; Olzinski et al., 2010). No subjects experienced any dose-limiting adverse events, and there were no serious adverse events reported or observed at any of the dose levels or dosing regimens used in this study.

Discussion

CCX140-B is an orally active, potent, and selective antagonist of CCR2 and is one of a small group of chemokine receptor antagonists that have advanced into clinical development. CCX140-B has recently demonstrated efficacy in the reduction of fasting blood glucose and glycated hemoglobin A1C (HbA1C) in type 2 diabetic patients (Hanefeld et al., 2011). In functional assay systems, using both cell lines and primary cells, CCX140-B effectively inhibits CCL2-induced function with single-digit nanomolar potency. When correlating the total exposure levels of a drug with a specific biological outcome, it is important to assess the non-specific binding of the molecule to serum proteins. In order to accurately determine the effective potency of CCX140-B, chemotaxis assays were conducted in the presence of 100% human AB serum. In these assays, CCX140-B retained its ability to inhibit CCR2 on both THP-1 and primary human monocytes. Additional serum proteins, such as α1-acid glycoprotein (AAG), are increased in the circulation of patients with inflammatory disorders (Nakamura et al., 1993) and can potentially reduce 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 drug levels required to achieve the desired therapeutic effect (Dairaghi et al., 2011; Schall and Proudfoot 2011). CCX140-B
displayed no loss of potency in 100% human serum supplemented with increasing concentrations of AAG.

Several literature reports have shown that interfering with CCR2/CCL2 interactions can inhibit the migration of blood monocytes into multiple tissues. Mice deficient for CCR2 or CCL2 display significantly reduced accumulation of peripheral blood monocytes into the peritoneal space following thioglycollate challenge (Boring et al., 1997, Kuziel et al., 1997, Kurihara et al., 1997, Lu et al., 1998). Recent reports have suggested that the reduction in monocyte accumulation in this model is primarily due to significant impairment of monocyte egress from the bone marrow in the absence of CCR2:CCL2 interactions (Serbina and Pamer 2006, Tsou et al., 2007). Since CCX140 does not bind to mouse CCR2, mice were generated in which the mouse CCR2 gene was replaced with human CCR2 for \textit{in vivo} pharmacology experiments. These hCCR2 KI mice displayed no alterations in myeloid cell populations, indicating no developmental defects in the myeloid compartment. Murine CCL2 (JE) effectively activates human CCR2 and the hCCR2 KI mice responded similarly to wild-type mice in response to peritoneal challenge with thioglycollate, making them a suitable model to assess the \textit{in vivo} activity of CCX140-B. Pharmacological inhibition of CCR2 with CCX140-B significantly inhibited monocyte recruitment into the peritoneal space in these transgenic mice to a level equal to that seen in CCR2\textsuperscript{-/-} mice. The exposure required for maximal inhibition related to \~85\% receptor inhibition at trough, based on \textit{in vitro} measures of potency. This clearly indicates that the prior published results with CCR2\textsuperscript{-/-} mice is not the result
of a developmental defect in monocyte function but that CCR2 is the key driver of peripheral monocyte recruitment into the peritoneum following thioglycollate challenge.

The role of inflammation in obesity and type 2 diabetes has become clearer in recent years (Olefsky and Glass, 2010). Monocytes are found in increasing numbers in adipose and hepatic tissues of insulin resistant and obese humans, and this observation is faithfully reproduced in rodent models of type 2 diabetes (Weisberg et al., 2003, Cancello et al., 2005). CCR2 has been implicated as the major chemokine receptor responsible for the accumulation of monocytes in peripheral tissues of obese, diabetic rodents and this reduced monocyte accumulation correlates with improvements in systemic glucose tolerance (Weisberg et al., 2006, Tamura et al., 2008, Yang et al., 2009, Kang et al., 2010). Here we show that inhibition of CCR2 by CCX140-B significantly improves fasting plasma glucose levels in obese, diabetic hCCR2-KI mice. This benefit is rapid, as only 2 weeks of treatment are required for the improved condition. Further, CCX140-B treatment reduces HOMA-IR values, indicating that CCR2 antagonism improves systemic sensitivity to insulin, which correlates with reduced glucose and insulin levels in these diabetic mice. The improvements seen with CCX140-B on insulin sensitivity correlated with significantly reduced numbers of total and inflammatory macrophages in visceral adipose tissue depots. No other infiltrating cells were evaluated in our study, given the central role ascribed to the macrophage by earlier authors. Our results clearly support those findings and implicate the adipose tissue monocyte/macrophage as a cell type that has deleterious effects on adipose tissue function, thereby mediating systemic effects on insulin sensitivity.
Recent reports on several CCR2 antagonists have created the impression that CCR2 inhibition \textit{in vivo} invariably results in the elevation of systemic levels of CCL2. These findings have been reported from numerous groups with disparate molecules and have been seen in rodents, non-human primates, as well as human subjects (Vergunst et al., 2008, Wang et al., 2009, Aiello et al., 2010, Olzinski et al., 2010). CCX140-B does not show this type of behavior \textit{in vivo}. Our results using multiple models in the hCCR2 KI mice clearly demonstrate that robust inhibition of cellular recruitment mediated by CCR2 can occur with no systemic rise in CCL2 levels. Additionally, the administration of clinically relevant doses of CCX140-B to healthy human volunteers results in no alterations in systemic CCL2 levels, even after repeated dosing out to 10 days. Plasma levels achieved in this study exceeded the serum A2 potency of the drug by 30 fold. In fact, there were no elevations seen in any of the CCR2 ligands in these healthy subjects. These results clearly show that robust inhibition of CCR2 function can occur without elevations in ligand levels and may be a desirable feature of a CCR2 receptor antagonist.

The unique pharmacology responsible for the lack of CCL2 elevation or the lack of effects on monocyte numbers after CCX140-B administration is an area of active research in our laboratories. Some of the possibilities that are being addressed include the following: greater CCR2 selectivity of CCX140-B relative to other agents which also inhibit CCR5 to a great extent; unique binding kinetics to albumin and CCR2 when present in blood; some of the unwanted effects produced by other compounds result from a greater degree of inhibition of CCL8 (MCP-3) relative to CCX140-B; differences
among the various compounds in the degree of penetration of certain tissues where the increased CCL2 and decreased monocyte phenotypes originate (perhaps the bone marrow). The results of our ongoing investigations will be presented in due course.

In conclusion, CCX140-B is an orally bioavailable, potent and selective CCR2 antagonist that demonstrates clear therapeutic benefit in an obesity driven mouse model of type 2 diabetes. Recently described clinical results from a Phase 2 study in type 2 diabetic patients provide further evidence for the key role of the chemokine receptor CCR2 in the pathophysiology of type 2 diabetes.
Authorship Contributions

*Participated in research design:* Sullivan, Dairaghi, Pennell, Charo, Johnson, Bekker, Schall, and Jaen

*Conducted experiments:* Sullivan, Dairaghi, Z. Miao, Wang, Zhao, Baumgart, Berahovich, Ertl, Seitz, Wei, Tsou, and Boring

*Contributed new reagents of analytic tools:* Krasinski, Pennell, Ungashe, Tsou, Boring, and Charo

*Performed data analysis:* Sullivan, Dairaghi, Wei, Boring, and Charo

*Wrote or contributed to the writing of the manuscript:* Sullivan, Charo, Bekker, Schall, and Jaen
References


Kang YS, Lee MH, Song HK, Ko GJ, Kwon OS, Lim TK, Kim SH, Han SY, Han KH, Lee JE, Han JY, Kim HK, Cha DR (2010) CCR2 antagonism improves insulin resistance,


Figure 1: Effects of CCX140-B on MCP-1 mediated chemotaxis, radioligand binding, and calcium flux in THP-1 and transfected HEK293-CCR2 cells. CCX140-B potently inhibits MCP-1 (0.1 nM) induced chemotaxis of THP-1 cells in buffer (A); in buffer supplemented with 4.5% human serum albumin (B); and in buffer supplemented with 4.5% human serum albumin and increasing amounts of alpha acid glycoprotein(C). CCX140-B potently inhibits binding of $^{125}$I-MCP-1 (50 pM) (D) and inhibits MCP-1 (5 nM) induced calcium flux in HEK293-CCR2 cells (E).

Figure 2: Effects of CCX140-B on CCR2- mediated chemotaxis, calcium flux, and radioligand binding in primary human CD14+ monocytes. CCX140-B inhibits MCP-1 (0.1 nM) induced chemotaxis of primary human CD14+ monocytes in buffer (A) and 100% human serum (B). Calcium flux induced by MCP-1 (5 nM) in buffer is completely inhibited by CCX140-B (C). Binding of $^{125}$I-MCP-1 (50 pM) is also potently inhibited by CCX140-B (D). Saturation binding of $^3$H-CCX140-B is shown in (E).

Figure 3: Effects of CCX140-B on chemotaxis of human monocytes induced by all MCP family members. CCX140-B is equally effective at inhibiting chemotaxis of primary human CD14+ monocytes in 100% human serum towards MCP-1 (A); MCP-2 (B); MCP-3 (C); and MCP-4 (D). CCX140 displays an A$_2$ value of 280 nM for MCP-1, 180 nM for MCP-2, 250 nM for MCP-3, and 280 nM for MCP-4.
Figure 4: Generation of human CCR2 knock-in mice.

(A) The wild-type mouse genome locus, the targeted allele, and the targeted allele after excision of the neomycin resistance gene. The human CCR2 gene replaces the endogenous murine CCR2 gene, and is under the control of the endogenous promoter.

(B) Human CCR2 KI mice recruit leukocytes to the peritoneum in response to instillation of thioglycollate similarly to wild type littermate mice or control C57BL/6 mice. Total peritoneal leukocytes were quantified 3 days after thioglycollate challenge (n=3-6 mice per group).

Figure 5: CCX140-B is highly selective for human CCR2. CCX140-B potently inhibits mouse CCL2 (mJE) induced chemotaxis of primary bone marrow cells from human CCR2 knock-in mice (A) but does not inhibit mouse CCL2 (mJE) induced chemotaxis of primary bone marrow cells from wild type mice (B). CCX140-B inhibits peritoneal accumulation of monocytes in human CCR2 knock-in mice following thioglycollate challenge (C) but has no effect in wild-type mice (D). CCX140-B does not alter frequency of inflammatory blood monocytes (E) or mediate elevation of mouse CCL2 mJE) in human CCR2 knock-in mice (F). * p<0.05; **p<0.005 relative to vehicle treated mice.

Figure 6: CCX140-B improves hyperglycemia and insulin sensitivity in obese, diabetic human CCR2 knock-in mice. Obese, diabetic human CCR2 knock-in mice were treated with vehicle or CCX140-B orally for 14 days and the effects of treatment
were compared to control, lean mice. CCX140-B therapy significantly reduced fasting plasma glucose (A) and fasting plasma insulin (B) relative to vehicle treated mice and also significantly improved insulin sensitivity as determined by HOMA-IR (C). Adipose inflammatory macrophage numbers were reduced by CCX140-B treatment (D). Serum mCCL2 (mJE) levels were unaffected by CCX140-B treatment (E). * p<0.05; **p<0.005 relative to vehicle treated mice.

**Figure 7: Phase 1 clinical pharmacokinetic and pharmacodynamic profile of CCX140-B**. Systemic drug exposures resulting from a Phase 1 single ascending dose study (A) and day 10 of the multiple ascending dose study (B) of CCX140-B in healthy human volunteers. CCX140-B does not alter numbers of circulating monocytes in subjects from the multiple dose Phase 1 study (C). Blood CCL2 (MCP-1) levels are also unaffected by CCX140-B after repeat dosing in the multiple dose Phase 1 study (D).
Table 1. Preclinical pharmacokinetic properties of CCX140-B in mouse, rat, and dog

<table>
<thead>
<tr>
<th></th>
<th>Mouse&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rat&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dog&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intravenous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;[ng.h/mL]</td>
<td>675</td>
<td>3,000</td>
<td>84,400</td>
</tr>
<tr>
<td>CL [mL/min/kg]</td>
<td>12.7</td>
<td>2.8</td>
<td>0.2</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; [h]</td>
<td>1.0</td>
<td>2.7</td>
<td>4.7</td>
</tr>
<tr>
<td>V&lt;sub&gt;dss&lt;/sub&gt; [L/kg]</td>
<td>1.0</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Oral</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;[ng/mL]</td>
<td>1,500</td>
<td>4,380</td>
<td>44,400</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; [h]</td>
<td>0.3</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; [h]</td>
<td>2.8</td>
<td>2.8</td>
<td>8.7</td>
</tr>
<tr>
<td>F [%]</td>
<td>≥100</td>
<td>≥100</td>
<td>≥100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Non-serial blood sampling was used and a composite PK profile was obtained using the mean concentration of 3 animals at each time point;  
<sup>b</sup> PK parameters reported as the group means;  
<sup>c</sup> CCX140-B in water at 0.1 mg/mL;  
<sup>d</sup> CCX140-B in PG/DMA/EtOH (31.6/31.6/36.8) at 0.5 mg/mL;  
<sup>e</sup> CCX140 (neutral form) in PG/DMA/H<sub>2</sub>O (31.6/31.6/36.8) at 1 mg/mL;  
<sup>f</sup> CCX140-B in 1% HPMC at 0.4 mg/mL;  
<sup>g</sup> CCX140-B in 1% HPMC 0.25 mg/mL;  
<sup>h</sup> CCX140 (neutral form) in Tween/1%HPMC (0.5/99.5) at 2 mg/mL.
Table 2. Selectivity of CCX140-B across a broad range of chemokine and chemoeattractant receptors, CYP isoforms, and the hERG channel

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell Type</th>
<th>Assay Format</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>THP-1</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CCR4</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CCR5</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>7</td>
</tr>
<tr>
<td>CCR6</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CCR7</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CCR9</td>
<td>MOLT-4</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CCR12</td>
<td>Neutrophils</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Neutrophils</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Neutrophils</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Activated T lymph</td>
<td>Chemotaxis</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR6</td>
<td>NSO-CXCR6 transf.</td>
<td>Radioligand Binding</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CXCR7</td>
<td>MDA435-CXCR7 transf.</td>
<td>Radioligand Binding</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>C3aR</td>
<td>HEK293-C3aR transf.</td>
<td>Radioligand Binding</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>C5aR</td>
<td>L1.2-C5aR transf.</td>
<td>Radioligand Binding</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>ChemR23</td>
<td>HEK293-ChemR23 transf.</td>
<td>Radioligand Binding</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Human Hepatocyte Microsomes</td>
<td>Enzymatic Activity</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>hERG</td>
<td>HEK293-hERG transf.</td>
<td>Patch Clamp</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>
Figure 2

A

Migration Signal

CCX140 (log [M])

B

Migration Signal

CCX140 (log [M])

C

Calcium Signal

CCX140 (log [M])

D

[^125] MCP-1 [cpm]

CCX140 (log [M])

E

Specific Binding

[^3H]-CCX140 (cpm)

[^3H]-CCX140 Input (nM)

|^ Bound/Free |

0 500 1000 1500

0 200 400 600 800
Figure 3

A

B

C

D

Migration Signal vs. MCP-1 (log [M])

Migration Signal vs. MCP-2 (log [M])

Migration Signal vs. MCP-3 (log [M])

Migration Signal vs. MCP-4 (log [M])

Concentrations: DMSO, 1 uM CCX140-B, 10 uM CCX140-B
Figure 4

A

Human CCR2 Knock-in Vector
Targeted allele after cre excision of neo cassette

Mouse CCR2 Genomic Locus

Targeted allele

Targeted allele after cre excision in vivo

BH = BamHI
H3 = HindIII
K = KpnI
Sm = Smal
Xb = XbaI
Xh = XhoI
TAA = Stop codon

- Prolactin signal sequence.
- Flag epitope tag (DDD).
- Mouse splice acceptor site (SA).
- Loxp sites (#1,#2,#3)
- Primers for homology arm PCR

* Sall and SpeI sites recreated

B

Total Peritoneal Leukocytes (x10^3)

C57BL/6  CCR2 KO  Littermate  hCCR2 KI
Figure 5

A

B

C

D

E

F

Migration Signal vs. mJE (log [M]) for different treatments:
- DMSO
- 100 nM CCX140-B
- 1 uM CCX140-B

Migration Signal vs. mJE (log [M]) for different treatments:
- DMSO
- 1 uM CCX140-B

Total Cells/mL vs. CCX140-B (mg/kg):
- Vehicle
- 3
- 10
- 30

Total Cells/mL vs. CCX140-B (mg/kg):
- Vehicle
- 10
- 30

% 7/4+Ly6G-Blood Monocytes vs. CCX140-B (mg/kg):
- Vehicle
- 10
- 30

CCL2 (pg/mL) vs. CCX140-B (mg/kg):
- Vehicle
- 3
- 10
- 30