Danirixin: A Reversible and Selective Antagonist of the CXC Chemokine Receptor 2

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ABSTRACT

CXC chemokine receptor 2 (CXCR2) is a key receptor in the chemotaxis of neutrophils to sites of inflammation. The studies reported here describe the pharmacological characterization of danirixin, a CXCR2 antagonist in the diaryl urea chemical class. Danirixin has high affinity for CXCR2, with a negative log of the 50% inhibitory concentration (pIC50) of 7.9 for binding to Chinese hamster ovary cell (CHO)-expressed human CXCR2, and 78-fold selectivity over binding to CHO-expressed CXCR1. Danirixin is a competitive antagonist against CXCL8 in Ca2+-mobilization assays, with a Kᵦ (the concentration of antagonist that binds 50% of the receptor population) of 6.5 nM and antagonist potency (pA2) of 8.44, and is fully reversible in washout experiments over 180 minutes. In rat and human whole-blood studies assessing neutrophil activation by surface CD11b expression following CXCL2 (rat) or CXCL1 (human) challenge, danirixin blocks the CD11b upregulation with pIC50s of 6.05 and 6.3, respectively. Danirixin dosed orally also blocked the influx of neutrophils into the lung in vivo in rats following aerosol lipopolysaccharide or ozone challenge, with median effective doses (ED50s) of 1.4 and 16 mg/kg respectively. Thus, danirixin would be expected to block chemotaxis in disease states in which neutrophils are increased in response to inflammation, such as pulmonary diseases. In comparison with navarixin, a CXCR2 antagonist from a different chemical class, the binding characterization of danirixin is distinct. These observations may offer insight into the previously observed clinical differences in induction of neutropenia between these compounds.

Introduction

Sustained neutrophil influx to the lung plays a key role of the pathology of diseases such chronic obstructive pulmonary disease (COPD), severe asthma, cystic fibrosis, acute lung injury, and respiratory syncytial virus infection (Wareing et al., 2007; Chapman et al., 2009; Stadtmann and Zarbock, 2012). In these conditions, tissue cells at the site of injury produce ELR+ chemokines (containing a Glu-Leu-Arg motif before the first cysteine), which in turn activate neutrophils through interaction with the G protein-coupled CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2). The ELR+ chemokines are CXCL1 through CXCL3 and CXCL5 through CXCL8. Chronic exposure to activated neutrophils leads to tissue damage either directly through degranulation of proteases or indirectly through additional cell recruitment, and CXCR2 and its ligands have been implicated in disease processes (Qiu et al., 2003; Quint and Wedzicha, 2007; Wareing et al., 2007; Nagarkar et al., 2009). CXCR2 and CXCR1 have overlapping and distinct functions (Hartl et al., 2007; Raghuvanshi et al., 2012). All ELR+ chemokines activate CXCR2, whereas only a subset, namely CXCL6, CXCL8, and potentially CXCL7, activate the CXCR1 receptor. Both CXCR1 and CXCR2 recruit neutrophils to sites of inflammation; CXCR2 may be primarily responsible for the initial recruitment of neutrophils, and CXCR1 appears to be responsible for neutrophil movement and some functions, such as respiratory burst and phospholipase D activation. CXCR1 and CXCR2 are also expressed on a number of other cells involved in inflammation, including monocytes, macrophages, lymphocytes, mast cells, dendritic cells, and endothelial cells. Blockade of CXCR2 alone is therefore hypothesized to be key for the prevention of neutrophil migration to sites of inflammation. CXCR2 antagonists studied to date have varying selectivity with respect to CXCR1, and all block neutrophil migration. Additional consequences of more or less selective compounds, such as host defense functions, have not been studied in detail in the clinic at this time.

Challenges in studying ELR+ chemokine-mediated neutrophil function in rodents are ligand and receptor functions of...
CXCR1 homologs that are distinct from human. In rats, a homologous CXCR1 receptor gene has been identified; however, it does not appear to be expressed. In mice, a functional CXCR1 ortholog may exist (Fan et al., 2007); however, its function and expression have not been sufficiently characterized to draw firm conclusions, and ligand pairing is therefore unknown. In addition, the rat and human ELR+ chemokines are distinct; for example, mice and rats lack a CXCL8 ortholog and it is thought that neutrophil chemotaxis in rodents is mediated by CXCL1 [or cytokine-induced neutrophil chemotactic attractant 1 (CINC-1) in rat] and 2 (or macrophage inflammatory protein 2 in rat) (Fan et al., 2007). CINC-1 levels are upregulated by inflammatory stimuli such as lipopolysaccharide (LPS), ozone, or cigarette smoke. (Haddad et al., 1996; Haddad et al., 2002; Thatcher et al., 2005). In contrast, human LPS challenge upregulates CXCL8 but not CXCL1 (Aul et al., 2012). Thus, in rodent models of inflammation, the functions of ELR+ chemokine signaling through these receptors may be significantly different from that observed in humans.

An additional function of CXCR2 has been illuminated from recent human genetic data showing that low frequency missense mutations in CXCR2 are associated with reduced blood leukocyte count. In addition, individuals with frameshift mutations in the CXCR2 gene suffer from myelokathexis or retention of neutrophils in the bone marrow (Auer et al., 2014). CXCR2+/− mice also exhibit this phenotype (Eash et al., 2010). This evidence underscores the importance of CXCR2 in the process of neutrophil migration from the bone marrow.

Numerous small-molecule antagonists of CXCR2 in several chemical series have been reported in the literature (Dwyer and Yu, 2014; compound numbers below are from this reference). Of these, four have been tested in patients with respiratory diseases (cystic fibrosis, asthma, COPD, and bronchiectasis): elubrixin and SB-332235 (Busch-Petersen, 2006). The danirixin. This compound belongs to the diaryl urea class of (compound 4; SB-656933), navarixin (compound 11; MK-7123, (cystic fibrosis, asthma, COPD, and bronchiectasis): elubrixin these, four have been tested in patients with respiratory diseases (Yu, 2014; compound numbers below are from this reference). Of

Materials and Methods

Compounds

Danirixin [3-[4-chloro-2-hydroxy-3-[(3S)-piperidine-3-sulfonyl]-phenyl]-1-(3-fluoro-2-methylphenyl)urea] was synthesized as either freebase (identified as GSK1325756B) or hydrochloride salt (identified as GSK1325756A) by the GSK Respiratory Therapy Area Unit medicinal chemistry group (King of Prussia, PA), as described in Busch-Petersen (2007). Navirixin was prepared following previously reported methods (Dwyer et al., 2006).

Cell Culture

Each of the receptors CXCR1 and CXCR2 was stably expressed with Gs16 in Chinese hamster ovary cells (CHO-K1). CHO-K1 cells were grown to 80% confluence in Dulbecco’s modified Eagle’s medium/F12 (Ham’s) media, 1:1, with 10% fetal calf serum, 2 mM L-glutamine and 0.2 mg/ml G418, at 37°C in a 5% CO₂ incubator.

Scintillation Proximity Binding Assays (CXCR2 and CXCR1)

Membranes were prepared from frozen CHO-K1 cell pellets using a hypotonic buffer containing protease inhibitors as previously described (Sarau et al., 1997; Podolin et al., 2002). Assays were conducted using scintillation proximity with wheat germ agglutinin beads (GE Healthcare Life Sciences, Marlborough, MA). Briefly, membranes were incubated with beads in binding buffer at 4°C for 30 minutes. Compounds were diluted 1:20 from 100% dimethyl sulfoxide (DMSO) solutions in a final volume of 100 μl containing binding buffer, beads, and 0.225 mM (125I) CXCL8 in Packard OptiPlates (Packard Bioscience Company, Meriden, CT). After a 45-minute incubation, the plates were centrifuged at 2000 rpm for 5 minutes and counted in a Packard TopCount. Specific binding was determined by subtracting the counts observed at 30 μM of CXCR2 or CXCR1 antagonist (nonspecific binding) from the total binding counts of CXCR2 or CXCR1, respectively. Curves were generated using GraphPad Prism (v4.0 or later; GraphPad, LaJolla, CA) and IC₅₀ was determined as the concentration required to displace 50% of the specific binding.

Ca²⁺-Mobilization Assays

CHO-K1 cells expressing each receptor were cultured and Ca²⁺-mobilization assays performed in a standard protocol as previously described (Podolin et al., 2002). Compound potency as pA₂ was calculated as the ratio of equiactive CXCL8 responses in the presence and absence of a single concentration of antagonist using the formula log(DR) – 1) – log(B), where DR is the dose ratio of EC₅₀ of CXCL8 in the presence and absence of compound at concentration B. Further characterization of the pattern of antagonism was done using classic Schild analysis from a series of curves at a single compound concentration and a range of CXCL8 concentrations. Unconstrained fits included unity within the 95% confidence limit (CL) for the slope, so values reported are from a fit where slope was constrained to 1. pA₂ is the antagonist concentration which causes a two fold shift in concentration-response curve for an agonist (representing antagonist potency); it is the negative logarithm of the Kᵦ which is the concentration of the antagonist that binds 50% of the receptor population. Compound reversibility was evaluated in washout studies. For the preincubation steps, cells were incubated with compound containing media and washout cells, or vehicle containing media and nonwashout cells, and incubated for 60 minutes at 37°C; medium was aspirated and compound or vehicle re-added for 20 minutes, and then for a further 10 minutes after an additional wash (compound treatment of 90 minutes total). For the washout steps, cells were washed with Krebs-Ringer-HEPES buffer by aspiration, followed by a 30-minute incubation in fresh media a total of three times (90 minutes total). Cells were then loaded with 4 μM Fluo-4 AM and incubated for 60 minutes. Fluo-4 media was subsequently aspirated, cells were retreated as appropriate, and incubated for 30 minutes, for a total washout period of 180 minutes, and the standard challenge protocol was followed.

Effect on Agonist-Induced Neutrophil CD11b Expression in Whole Blood

Rat in vitro assay: Fresh rat blood obtained by cardiac puncture into an EDTA tube was incubated with danirixin (10 μM added to final concentration 0.03–10 μM and 0.1% DMSO) and rat CXCL2 (10 nM final; PeproTech, Rocky Hill, NJ) at 37°C for 30 minutes. The samples were iced for 10 minutes and then labeled with a fluorescein isothiocyanate (FITC)-tagged CD11b-specific antibody (Antigenix America Inc., Huntington Station, NY). The red blood cells (RBCs) were lysed and the leukocytes were washed and fixed. For flow cytometric analysis, LDS-751 was added to label granulocytes and further exclude any unlysed RBCs. Briefly, threshold in the LDS-751 detector was increased to eliminate RBCs and then an analysis gate was placed around the granulocyte population in the forward scatter/side scatter plot. The FITC mean fluorescence intensity (MFI) of this population was then assessed as CD11b.
expression. A similar assay has been described previously for human blood (Davis et al., 2000).

**Rat Ex Vivo Assay**

Following oral dosing of GSK1325756B to male Lewis rats (n = 5 rats per group, see in vivo studies for details), whole blood was obtained by cardiac puncture 1 hour postdosing. Rat pharmacokinetic studies demonstrated a T\text{max} of 0.25 hours. Therefore, for efficacy and logistical reasons, 1 hour postdose was selected as the optimal time point. Whole blood collected in EDTA tubes was treated with CXCL2 for 20 minutes at 37°C and then the cells were labeled with a FITC-tagged CD11b-specific antibody (Antigenix America). Analysis by flow cytometry was performed as described above.

**Human In Vitro Assay**

Whole blood was obtained from consenting donors into EDTA tubes and incubated with danirixin for 10 minutes prior to treatment with human CXCR1 (10 nM final; PeproTech) for a further 10 minutes, all at 37°C. Following a light fixation on ice, cells were labeled with a FITC-tagged CD11b-specific antibody (Dako/Agilent Technologies, Santa Clara, CA) and a PE-tagged CD16 antibody (Dako/Agilent Technologies, Santa Clara, CA). For flow cytometric analysis, LDS-751 was added to label nucleated leukocytes and eliminate RBCs from analysis. Briefly, threshold in the LDS-751 detector was increased to exclude RBCs and then an analysis gate was placed around the granulocyte population in the forward scatter/side scatter plot. A second analysis gate was then placed around the CD16+ neutrophils within the granulocyte gate. The FITC MFI of this population was then assessed as CD11b expression.

**Data Analysis**

Percent inhibition was calculated by subtracting each value of the percentage of positive control (value with CXCL2 alone) from 100. For ex vivo studies, the maximal response was defined as the sample value from animals treated with vehicle alone and stimulated with 100 nM CXCL2. ED\text{50} was defined as concentration of CXCL2 required to induce 50% of the maximal response and was calculated using GraphPad Prism v4.0 or later by plotting neutrophil counts against log[drug conc.] and interpolating the value from half of the neutrophil count of the vehicle control group.

**Blood Neutrophils in the Rat**

Danirixin was given to rats (12 per sex per group) as a suspension at 0 (vehicle), 25, 50, 150, or 500 mg/kg per day once daily for 26 weeks by oral gavage (20% PEG and 16% (40% w/v, hydroxypropyl 2-cyclohexyl) sucrose (PEG 400) in water, and animals were dosed by oral gavage.

**In Vivo Studies in the Rat**

All studies were conducted in accordance with the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee at GlaxoSmithKline facilities, where the work was performed. Male Lewis rats (275–400 g; Charles River Laboratories, Portage, MI) were used in all studies. Compound was dosed in a vehicle of 1% DMSO, 20% polyethylene glycol (PEG) 400) in water, and animals were dosed by oral gavage.

**Lipopolysaccharide Challenge**

Rats were dosed 30 minutes prior to LPS exposure, which consisted of 0.1 mg/ml solution of *Escherichia coli* serotype O26:B6 LPS aerosol delivered via nebulizer at 4 l/min for 20 minutes into an anesthesia-induction chamber. Four hours later, the rats were euthanized with pentobarbital (2.6 g/kg, i.p.) and bronchoalveolar lavage (BAL) collected by a total 21-ml wash with Dulbecco’s phosphate-buffered saline, in seven 3-ml washes. Total counts and neutrophil counts were obtained as previously described (Underwood et al., 2000). Absolute neutrophils with saline exposure were typically very low and not routinely measured to reduce animal use (see Haddad et al., 2002, Nicholls et al., 2015) for typical results; neutrophil counts in saline controls were <1% of total. Absolute neutrophil counts following LPS exposure in vehicle-treated rats in the various studies were: 30.8 × 10⁶ ± 3.6 for the dose response study; 36.1 × 10⁶ ± 9 and 46 × 10⁵ ± 12.7 for the fasted and fed rats, respectively, and 22.8 × 10⁵ ± 3.7 in the repeat dose study.

**Ozone Challenge.** Animals were exposed to ozone in a purpose-built exposure chamber for a period of 3 hours. Ozone was generated (model HTU500-G; Azco Industries, Limited, Langley, B.C., Canada) and fed into the chamber at a rate of 0.5 l/min mixed with laboratory air at 10 l/min. The ozone concentration was monitored throughout the exposure period and maintained at 3 ppm through the use of an ozone analyzer (Model 450; Advanced Instrument, Inc., Norwood, MA). At 4 hours following the completion of the ozone exposure, BAL was collected and neutrophil counts determined as described above.

**Data Analysis.** Data are expressed as the mean ± standard error of the mean for total and differential cell counts in BAL fluid. Statistical analysis of cell counts was performed using a one-way analysis of variance with differences between groups evaluated employing Bonferroni’s correction, with P values less than 0.05 considered significant. ED\text{50} values were determined using GraphPad Prism v4.0 or later by plotting neutrophil counts against log[drug conc.] and interpolating the value from half of the neutrophil count of the vehicle control group.

![Fig. 1. Antagonism by danirixin and navirixin of CXCL8 at the CXCR2 receptor as assessed by Ca²⁺-flux studies in hCXCR2-expressing CHO cells. (A) Danirixin (B form) was preincubated for 30 minutes and shows competitive antagonism. Results are expressed as mean ± S.E.M.; n = 3. (B) Apparent uncompetitive antagonism by navirixin at the CXCR2 receptor under the identical experimental conditions as (A).](image-url)
to assess the reversibility of possible test item-related changes following a 6-week recovery period. Three rats per sex were added at each dose level for toxicokinetic evaluation.

Results

Potency and Kinetic Profile at Cloned Human Receptors. Binding of Danirixin in hCXCR1-CHO and hCXCR2-CHO Cell Membranes

Binding studies were radiolabeled to assess relative affinity to expressed human CXCR1 and CXCR2. Danirixin (B form) competed with [125I] CXCL8 binding to membranes prepared from CHO stably transfected with human CXCR2 or CXCR1 cells, with pIC50s, respectively, of 7.90 ± 0.04 (n = 3; IC50 12.5 nM) and of 6.03 ± 0.05 (n = 3) (IC50 977 nM), or 78-fold higher affinity for CXCR2. Binding affinity for CXCR2 was also assessed in the presence of 0.4% human serum albumin (hSA) to more closely approximate physiologic conditions, and danirixin competed with [125I] CXCL8 (0.23 nM) with a pIC50 of 6.95 (n = 2) (IC50 = 113.5 nM).

Inhibition of CXCL-8-Induced Functional Calcium Mobilization in CHO-K1 hCXCR2 + Goα16 Cells

CHO-K1 cells expressing hCXCR2 + Goα16 were used to directly assess the functional potency of compounds by monitoring Ca2+ flux. Danirixin (B form) potently, competitively, and reversibly inhibited CXCL8-induced calcium mobilization. CXCL8-induced Ca2+-response curves are shown in Fig. 1A in the presence of increasing danirixin concentrations. All danirixin-induced dextral displacement can be surmounted by increasing concentrations of CXCL8, achieving the maximal CXCL8 response with all but the highest concentrations of danirixin (Fig. 1A). This reduction at the high concentrations probably resulted from incomplete equilibrium at the receptor during the rapid Ca2+ flux response. Schild analysis of the concentration curve data gives a slope of 1 (data not shown), as expected for competitive inhibition, a mean pA2 of 8.12 (95% confidence interval (CI) 8.14–8.23), or a KB of 6.5 nM (n = 3). When the assay was performed in the presence of 0.5% hSA, danirixin (B form) had a single concentration pA2 value of 7.52 (95% CI 7.30–7.73) or a roughly 5-fold lower potency in the presence of added protein (data not shown).

The hydrochloride salt, or A form, was evaluated separately and, as expected, had comparable functional potency, with a mean pA2 of 8.25 (95% CI 8.16–8.33) or a KB of 5.6 nM (n = 3). When the assay was performed in the presence of 0.5% hSA, danirixin (B form) had a single concentration pA2 value of 7.54 (95% CI 7.30–7.73) or a roughly 5-fold lower potency in the presence of added protein (data not shown).

Additional studies were done to evaluate reversibility at the receptor by using washout studies. Danirixin B form was incubated with cells expressing hCXCR2 for a total of 90 minutes, and excess drug was washed out and cells allowed to re-equilibrate over a period of 180 minutes. Figure 2A shows that, following the washout of danirixin, CXCL8 agonist response curves overlaid the response curve obtained for the vehicle-treated cells (open symbols). These data indicate that the danirixin interaction with hCXCR2 is completely reversible within 180 minutes over the concentration range used.

Similar Ca2+-mobilization studies were carried out with navarixin to compare and contrast the kinetics and binding of the two compounds. Navarixin kinetics were assessed at both CXCR1 (data not shown) and CXCR2 and they demonstrated reversible, competitive binding at CXCR1, as did danirixin, but somewhat different kinetics at CXCR2, consistent with previous studies suggesting noncompetitive or allosteric binding (Gonsiorek et al., 2007), as shown in Fig. 1B. Again, similar reversibility studies at CXCR2 were carried out with a 90-minute incubation and 180-minute washout period (Fig. 2B). These studies also demonstrated that navarixin could be completely removed from the receptor by washing of the cells.

Selectivity: CEREPI Screen and hERG Channel

Binding studies were performed to evaluate the selectivity profile of the A form at 50 targets in the CEREPI panel of receptors, ion channels, and transporters (Eurofins CEREPI, Celle L’Evescault, France). GSK1325756A at a screening concentration of 1 μM did not inhibit binding at any non-CXCR2 target by greater than 21%.

Inhibition of Agonist-Induced CD11b Expression in Whole Blood

Engagement of the CXCR2 receptor on neutrophils by its ligand, the chemoattractant CXCL1, results in several pathways of cellular activation, including the rapid upregulation of CD11b, which, together with CD18, forms the adhesion molecule Mac-1. The ability of an antagonist to block this...
upregulation in rat as well as human whole blood was used as a measure of functional antagonism in a physiologic setting. The identical assay can then be directly translated to a clinical setting (Nicholson et al., 2007; Lazaar et al., 2011).

**Rat In Vitro Assay.** Fresh rat blood was incubated with compound and then challenged with a single concentration of rat CXCL2, followed by a short incubation and assessment of surface CD11b levels by flow cytometry. GSK1325756A caused a concentration-dependent inhibition of rat neutrophil CD11b expression with a pIC50 = 6.05, IC50 890 nM, and maximum inhibition of 104% (n = 4) (data not shown).

**Rat Ex Vivo Assay.** Similar studies were carried out that used rat blood obtained following oral dosing of danirixin B form in Lewis rats. Increasing doses of danirixin caused a dose-dependent rightward shift in CXCL2 concentration-response curves compared with vehicle-treated rats, as shown in Fig. 3. The EC50 for CXCL2-induced neutrophil CD11b expression in vehicle-treated rats was 4.5 nM and this increased to 9.4, 11.9, and 38.1 nM at danirixin doses of 0.3, 3, and 30 mg/kg, respectively. That is, the antagonist present in the blood obtained from rats dosed with compound was sufficient to reduce the effect of the agonist, making it appear less potent at the receptor.

**Human In Vitro Assay.** Similar studies using human whole blood and CXCL1 as the ligand are shown in Fig. 4. Danirixin shows a simple dose response with a pIC50 of 6.41 ± 0.07, IC50 420 nM, whereas the comparator compound navirixin was more potent, with a pIC50 of 6.8, IC50 of 150 nM (data not shown).

**Effect on Neutrophil Influx in Rat Models**

Although there are no animal models of COPD, inhalation of LPS or ozone by humans and rats causes an inflammatory response in the lungs characterized by increases in airway neutrophils. Danirixin was therefore evaluated in several studies in two rat models of neutrophil influx, using LPS and ozone as the challenge agents.

**LPS-Induced Pulmonary Neutrophilia.** The ability of danirixin (B form) to block neutrophil influx to the lung following inhaled LPS challenge was evaluated in fed and fasted Lewis rats, and following multiple doses of danirixin. All studies demonstrated a dose response over the dose range used (1–30 mg/kg, by mouth). In fasted rats, the highest dose, 10 mg/kg, produced a maximum 84% inhibition of neutrophils in BAL fluid, with an ED50 of 1.4 mg/kg as shown in Fig. 5A. In fed rats (Fig. 5B), the maximum inhibition observed was 64% at 10 mg/kg with an ED50 of 2.3 mg/kg. The analysis did incorporate the apparent outlier point at 3 mg/kg, since it did not meet the criteria for omission. Leaving this point out results in an ED50 of 1.2 mg/kg. The possibility of drug accumulation or changes in receptor function following repeated daily dosing of GSK1325756B was also assessed by dosing for 5 days, followed by a single LPS challenge. On day five, rats received the final dose 1 hour prior to LPS aerosol challenge. Rats treated for five consecutive days had a 60% inhibition of neutrophils at the single dose of 3 mg/kg recovered in BAL compared with the vehicle-treated group. A separate group of rats receiving only a single dose of danirixin 1 hour prior to LPS challenge had a 53% inhibition of neutrophils in BAL (Fig. 6). There was no significant difference in the level of neutrophil attenuation between one and five daily doses, and thus no evidence for drug accumulation or change in receptor function.

**Effect on Ozone-Induced Pulmonary Neutrophilia.** A single inhaled ozone challenge over 3 hours in rats results in a similar influx of neutrophils to the lung. Danirixin (B form) dosed to fasted Lewis rats 1 hour prior to ozone exposure caused a dose-dependent inhibition of neutrophils recovered in the BAL 4 hours postchallenge. The maximum inhibition of neutrophils was 79% and the ED50 was 16.0 mg/kg (Fig. 7). Although the ED50 is considerably higher than that in the LPS model, similar shifts in potency have been observed with other anti-inflammatory mechanisms, such as p38 inhibition (Williams et al., 2008).

**Correlation of Rat LPS-Induced Neutrophilia and Ex-Vivo CD11b Upregulation**

The relationship between the in vivo LPS-induced neutrophilia with the ex vivo rat CXCL2-induced CD11b upregulation was evaluated. Blood levels for the in vivo LPS-induced neutrophilia were obtained after 5 hours, whereas blood levels for the samples from the ex vivo rat CXCL2-induced CD11b upregulation were obtained after 1 hour. The ex vivo ED50s are plotted in comparison with the inhibition of neutrophils in the BAL over the same dose range as in Fig. 8, showing the same general trend over this dose range, consistent with a common mechanism for the two processes.

Effects on Peripheral Hematology in Preclinical Species

Hematological effects of danirixin in the clinic have previously been described (Miller et al., 2015). In contrast to danirixin, CXCR2 antagonists from other chemical classes
have demonstrated a reduction in peripheral blood neutrophils in humans in clinical studies (Kirsten et al., 2015; Rennard et al., 2015), posing a challenge to development. The preclinical hematologic effects of danirixin were also evaluated as a component of toxicology studies. In pivotal dog studies with a solution formulation, no hematologic effects were evident in dogs given ≤30 mg/kg per day for 7 days; however, mildly increased (≤2-fold) mean total white blood cell and neutrophil counts were noted in male dogs given 30 mg/kg per day following 4 and 39 weeks of dosing. These changes were not considered adverse owing to lack of correlating microscopic findings and mild severity. In 7-day or 4-week studies in rats, increases in mean neutrophil and/or monocyte counts (2- to 3-fold) occurred in females given ≥150 mg/kg per day and males given 1000 mg/kg per day. Effects on total white blood cell and/or neutrophil and monocytes counts were not noted in rats following 26 weeks of dosing at doses up to 500 mg/kg per day. Decreases in one or more red cell mass indices (decreased mean hemoglobin concentration, total red cell count, and hematocrit values) occurred in rats following 7 days of dosing at the highest dose (2000 mg/kg per day) and at doses ≥300 mg/kg per day in dogs.

Discussion

The major findings of the studies described here are that danirixin is a potent antagonist at the CXCR2 receptor, with fully competitive and reversible kinetics at the receptor, as well as significant selectivity with respect to the CXCR1 receptor. In addition, danirixin is an effective inhibitor of...
pulmonary neutrophil influx following LPS or ozone challenge with no loss of activity on repeat dosing in LPS challenge.

Binding and functional assays of receptor antagonism demonstrated that danirixin is able to prevent the binding of CXCL8 to the human receptor in CHO cell membranes and to prevent Ca^{2+}-mobilization in response to CXCL8 in intact CHO cells with comparable potency (pic50 7.9, pA2 8.2). Excess CXCL8 was also able to compete out the danirixin, demonstrating a binding mode that is kinetically equivalent to competitive inhibition. Danirixin was also shown to be fully reversible, completely restoring function after no more than a 180-minute washout period.

Inhibition of the CXCL1-induced CD11b upregulation has already been demonstrated to be a valuable assay for translating of efficacy to clinical studies (Nicholson et al., 2007; Lazaar et al., 2011). Using elubrixin (SB-656933), the identical whole blood assay was used in phase 1 for dose selection for an ozone challenge study (Lazaar et al., 2011). Danirixin shifts the CXCL1 response curves to the right ex vivo in rat at doses consistent with inhibition of neutrophil migration to the lung. In human blood, the pic50 of 6.4 in the CD11b assay compared with the more potent values seen in binding or functional assays in purified systems is consistent with the moderately high protein binding observed with danirixin (98% in human plasma). This value is also consistent with the pic50 of 6.8 (69 ng/ml) determined by pharmacokinetic/pharmacodynamic modeling in phase 1 (Miller et al., 2015).

Inhibition of pulmonary neutrophilia in challenge models by danirixin is consistent with observations of a variety of other anti-inflammatory mechanisms in these rat models, as well as inhibition of LPS challenge in human by navirixin, and of ozone challenge by the related compound elubrixin (Holz et al., 2010; Lazaar et al., 2011; Leaker et al., 2013).

The previously reported receptor characterization of other CXCR2 antagonists navarixin and AZD5069 suggest more slowly reversible kinetics compared with danirixin (Nicholls et al., 2015). This observation can potentially be rationalized in terms of slightly different binding modes. By using mutagenesis, a group from Astra-Zeneca (Nicholls et al., 2008) identified the intracellular lysine residue K320 as a key interaction for diaryl urea and AZ5069-type antagonists. These results demonstrated that different types of small-molecule antagonists bind to the same allosteric binding site located on the intracellular side of the receptor. This hypothesis was confirmed in additional studies (Salchow et al., 2010); however, these studies also revealed differences in the interactions of the lipophilic aryl moieties with the receptor. In particular, navarixin and an analog of AZ5069 showed large reductions in affinity when the alanine residue A249 was mutated to a leucine (280- and 14-fold, respectively), whereas the affinity of the danirixin analog SB-265610 was essentially unaffected. This could suggest that navarixin and AZ5069 induces a conformational change that involves A249 and this in turn is responsible for the slower off-rate of these compounds. A conformation change would also explain the apparent noncompetitive, or insurmountable, binding kinetics observed in several assays (Gonsiorek et al., 2007) for navarixin. Although navarixin is reversible, as is danirixin, the different kinetics at the receptor in functional assays may result in distinct downstream signaling that translates into differences in pharmacology.

The role of CXCR2 antagonists in altering blood neutrophil levels has been investigated since the first reports of neutropenia in human subjects treated with navirixin (Rennard et al., 2015) or AZD5069 (Kirsten et al., 2015). Neutropenia was not observed either preclinically as described here for danirixin or

**Fig. 7.** Inhibition by orally administered danirixin (B form) of ozone-induced neutrophilia in the rat. Left: means and S.E.M. from groups of five rats; right: means with individual values. **P < 0.01

**Fig. 8.** Dose-related effects of danirixin on ex vivo shift in CXCL2-induced CD11b upregulation and inhibition of LPS-induced neutrophilia in the rat.
clinically with either danirixin (Miller et al., 2015) or the
related compound elubriskin (Lazarak et al., 2011). The dif-
f erences in the behavior of these compounds at the CXCR2
receptor, as well as selectivity with respect to CXCR1, may
explain the distinct clinical observations.

It has been shown by a variety of methods that CXCR2
is required for release of neutrophils from the bone
marrow to be maintained, and that CXCR2 and CXCR4
interact to maintain homeostasis of plasma levels (Eash
et al., 2010). CXCR2 is expressed at high levels on mature
neutrophils in the blood and bone marrow, and engagement
of CXCR2 plays a critical role in the recruitment of neutrophils
into tissues in many models of inflammation (Kobayashi,
2006). As neutrophils age, CXCR4 levels increase and CXCR2
levels decrease, leading to either a return to the bone marrow
or a decrease in migration to CXCR2 ligands (Rankin, 2010).
Mice engineered to have a deletion in CXCR2 showed impaired
neutrophil migration (Liu et al., 2013), and hyperfunctional
CXCR4 in severe combined immunodeficiency mice have accumulation
of neutrophils in the bone marrow (Rankin, 2010). In the CXCR2-deficient mice, CXCL1 was not efficiently scavenged in mice with greater than 90% deletion, but in mice with a less pronounced deletion, there was no defect in scavenging (Liu et al., 2013).

A possible mechanism for the neutropenia observed would be a sufficient decrease in CXCR2 signaling to limit the
neutrophils recruited from the bone marrow as CXCR4 continues
to signal for return to the bone marrow. Because danirixin,
naxirixin, and AZD-5029 all have differing receptor potencies and
kines, each will have differing functional blockade of
CXCR2 signaling in terms of percentage of blockade, duration
of blockade, and perhaps different effects on the downstream
signaling.

A detailed clinical study with naxirixin showed a decrease in absolute neutrophil count but no changes in bone marrow cell
mortality or symptoms of reduced neutrophils (Hastrup et al.,
2015), which is consistent with the hypothesis that changes in
peripheral neutrophil counts are related to trafficking and not
intracellular changes in the neutrophils. Although administration
of danirixin in clinical trials does not result in neutropenia or a
reduction in peripheral blood neutrophils at pharmacologi-
cally effective doses up to 150 mg, reduction in peripheral blood neutrophils was observed at higher doses (Miller et al.,
2015), suggesting a different but subtle effect on signaling or
receptor kinetics. Additional work comparing the receptor interactions and downstream effects of the various antago-
nists are required to understand the molecular basis of the differences in neutropenia. Danirixin has been shown to be a
selective CXCR2 antagonist that may offer benefit in diseases
of excess neutrophilia, such as COPD, without concomitant
neutropenia in the blood.

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Authorship Contributions

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