Pharmacological Characterization of AZD5069, a Slowly Reversible CXCR2 Antagonist.


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Abstract

In normal physiological responses to injury and infection, inflammatory cells enter tissue and sites of inflammation through a chemotactic process regulated by several families of proteins including inflammatory chemokines, a family of small inducible cytokines. In neutrophils, chemokines CXCL1 and CXCL8 are potent chemoattractants and activate G protein-coupled receptors CXCR1 and CXCR2. Several small molecule antagonists of CXCR2 have been developed to inhibit the inflammatory responses mediated by this receptor. Here we present the data describing the pharmacology of AZD5069, a novel antagonist of CXCR2. AZD5069 was shown to inhibit binding of radiolabelled CXCL8 to human CXCR2 with a pIC<sub>50</sub> value of 9.1. Furthermore, AZD5069 inhibited neutrophil chemotaxis with a pA<sub>2</sub> of approximately 9.6 and adhesion molecule expression with a pA<sub>2</sub> of 6.9 in response to CXCL1. AZD5069 was a slowly reversible antagonist of CXCR2 with effects of time and temperature evident on the pharmacology and binding kinetics. With short incubation times AZD5069 appeared to have an antagonist profile with insurmountable antagonism of calcium response curves. This behavior was also observed in vivo in an acute lipopolysaccharide induced lung inflammation model. Altogether, the data presented here show that AZD5069 represents a novel, potent and selective CXCR2 antagonist with potential as therapeutic agent in inflammatory conditions.
Introduction

Overstimulation of the inflammatory response plays a role in the pathology of a variety of diseases. The process is propagated by circulating leucocytes entering into inflamed tissue in response to inflammatory mediators. The inflammatory cells entering the tissue are directed through a chemotactic process regulated by several families of proteins including inflammatory cytokines, adhesion molecules, matrix metalloproteases and chemokines.

The CXC chemokine receptor 2 (CXCR2) is a G-protein coupled receptor for a number of chemokines and is known to be elevated in several inflammatory diseases, including chronic obstructive pulmonary disease (COPD), severe asthma, acute respiratory distress syndrome, rheumatoid arthritis, psoriasis and inflammatory bowel disease. In addition, both CXCL8 and CXCL1 (endogenous ligands of CXCR2) have been shown to be increased in inflammation (Schulz et al., 1993; Kurdowska et al., 2002; Banks et al., 2003; Beeh et al., 2003). Recent advances indicate that chronic inflammation is a key risk factor for cancer and there are several associations between CXCR2 and cancer (Jamieson et al., 2012; Highfill et al., 2014; Katoh et al, 2014).

Chemokines are important in host defense mechanisms such as calcium mobilization, releasing granule contents and respiratory burst. Chemokine CXCL8 activates both CXCR1 and CXCR2. CXCR1 is stimulated by relatively few chemokines, CXCL6 and possibly CXCL7, while CXCR2 binds to all seven ELR+ CXC chemokines CXCL1-CXCL3 and CXCL5-CXCL8 (Bachelerie et al., 2014). These are potent chemo attractants for neutrophils and hence, CXCR1 and CXCR2 constitute the primary mechanism for recruitment of neutrophils to sites of inflammation and infection. CXCR1 and CXCR2 have also been detected on other cells associated with chronic inflammation, including macrophages, lymphocytes, mast cells, dendritic cells and endothelium (Murdoch and Finn, 2000; Mukaida et al., 2002; Traves et al., 2004; Reutershan et al., 2006).
CXCR1 and CXCR2 have similar signaling mechanisms (Stillie et al., 2009) and CXCL8 can potentiate several neutrophil functions triggered through both receptors, including phosphoinositide hydrolysis, intracellular Ca\(^{2+}\) mobilization and chemotaxis. However, CXCR1 has been specifically implicated in phospholipase D activation, respiratory burst activity and the bacterial-killing capacity of neutrophils (Jones et al., 1996) suggesting that CXCR1 and CXCR2 might have different physiological roles under inflammatory conditions.

COPD and severe asthma are both characterized by sustained neutrophilic inflammation of the airways (Bousquet et al., 1990; Linden et al., 1993; Barnes, 2007; Chapman et al., 2009; Kamath et al., 2005; Sabroe et al., 2002) and the number of neutrophils in sputum is negatively correlated with lung function as measured by forced expiratory volume in 1 second (FEV\(_1\)) (Perng et al., 2004; Little et al., 2002; Hogg et al., 2004; Singh et al., 2010). Neutrophils are recruited to the lung from the systemic circulation via the microvasculature mediated by a range of chemokines including CXCR1 and CXCR2 ligands (Quinton et al., 2004). Targeting CXCR2 is therefore expected to reduce neutrophilic inflammation, mucus production and neutrophil proteinase-mediated tissue destruction to the lung (Chapman et al., 2007).

Several small molecule chemokine receptor antagonists have been developed as a potential therapeutic approach for the treatment of inflammatory disease, for example, repertaxin, navarixin and danirixin (Chapman et al., 2009). These are in different stages of drug development and have been shown to have effect on neutrophil recruitment to the lung in clinical studies (Virtala et al., 2011; Holz et al., 2010; Lazaar et al., 2011, Pavord et al., 2013). In addition, the effect of inhibiting neutrophil recruitment has been shown for clinical biomarkers and endpoints indicative of disease efficacy as investigated in cystic fibrosis, severe asthma and COPD (Moss et al., 2013; Nair et al., 2012; Rennard et al., 2013). Despite a strong association of chemokine involvement in disease to date there are only two
marketed products targeting chemokine receptors; plerixafor, a small molecule antagonist of CXCR4, and maraviroc, an antagonist of CCR5 (Bachelerie et al., 2014).

AZD5069 was developed from a series of bicyclic CXCR2 antagonists (Walters et al., 2008; Allen et al., 2014). The studies presented here describe the pharmacological characterization of AZD5069 as a potent, reversible antagonist of CXCR2 and the effect in a rat in vivo LPS challenge model of lung inflammation. These data are discussed in the context of the properties of this compound determined in vitro. AZD5069 represents a novel small molecule antagonist for the potential treatment of inflammatory conditions arising from neutrophil infiltration.
Materials and Methods

Compounds

The compounds AZD5069, AZD8309 and AZ10397767 (Fig. 1) were synthesized by AstraZeneca Medicinal Chemistry Charnwood as described in Willis et al. (2001) and Cheshire et al. (2006).

Cell culture

HEK293 cells, expressing recombinant human CXCR2 or CXCR1, were grown to approximately 80% confluence in DMEM-Glutamax medium containing 10% (v/v) fetal calf serum and 0.5 mg/ml geneticin in a humidified incubator at 37°C, 5% CO₂. Cells were harvested from the flask using Accutase at 37°C for 3 to 5 min.

Cell membranes

Cells were resuspended on ice in hypotonic buffer and disrupted using a polytron tissue homogenizer at 22,000 rpm. The membrane preparation was purified by layering onto 41% (w/v) sucrose solution and then centrifuged at 140,000 g for 1 h at 4°C. The membrane fraction at the interface was harvested, diluted 4-fold with HEPES-buffered salt solution pH7.4 and centrifuged at 100,000 g for 20 min at 4°C. The membrane pellet was re-suspended in HEPES-buffered salt solution and subsequently stored in aliquots at -80°C. All buffers used for membrane preparation and storage were made in the presence of complete protease inhibitor cocktail tablets (Sigma), prepared according to the manufacturer’s instructions.

Polymorphonuclear (PMN) cell preparation

Peripheral venous human blood was collected in polypropylene tubes containing 10 Units/ml of heparin, 25 ml of blood were layered onto 17 ml of PolymorphPrep (Robbins Scientific)
and centrifuged at 450 g for 30 min at room temperature. The polymorphonuclear (PMN) layer was placed into a fresh polypropylene centrifuge tube and diluted in an equal volume of PBS containing 0.2% (w/v) glucose. The cells were centrifuged at 300 g for 5 min at room temperature. The supernatant was decanted and the cell pellet dispersed. Hypotonic lysis of red blood cells was performed by resuspending the pellet in 7.5 ml of 0.2% (w/v) NaCl for 1 min whilst inverting the tube and then adding an equal volume of 1.6% (w/v) NaCl. PBS (35 ml) containing 0.2% (w/v) glucose was added and the cells centrifuged at 300 g for 5 min. The procedure was repeated to ensure complete lysis of red blood cells. The PMN cell pellet was resuspended in 20 ml Tyrode’s/HEPES solution and the cell number determined.

**CXCL8 radioligand binding**

Radiolabelled [125I]-CXCL8 was used in the assay at a final concentration of 0.06 nM. The assay was performed in 0.1% DMSO and non-specific binding determined in the presence of 1 µM AZ10397767. The assay was initiated by adding membranes to give a total incubation volume of 100 µl per well in a 96-well multiscreen-filter plate, pre-wetted with assay buffer. The plates were sealed and incubated for 2 hr at 22°C. Plates containing the assay mixture were filtered then filter-washed with 200 µl cold HEPES-buffered salt solution using a Millipore vacuum manifold. The filtration plate was allowed to air dry, then the individual filters were punched out into polypropylene test tubes and the radioactivity measured by direct gamma counting using a Cobra II gamma counter (Packard BioScience) for 1 min per sample. Data were analyzed using 4-parameter logistic function in the Excel-based program ‘XL-fit’ (I.D. Business Solutions Ltd, Guildford, UK).

**AZD5069 radioligand binding**

Radiolabelled [3H]-AZD5069 was prepared at AstraZeneca R&D Charnwood. The affinity of AZD5069 was quantified by determining the specific binding of [3H]-AZD5069 to membranes of HEK293 cells expressing recombinant CXCR2 from various species. Non-specific binding
of [³H]-AZD5069 was determined in the presence of 10 μM AZ11705135, a concentration much higher than the IC₅₀ of this compound (2 nM) to displace radiolabelled CXCL8.

Membranes from HEK293 cells expressing recombinant CXCR2 were suspended in HEPES-buffered salt solution. The membranes were then bound to wheat germ agglutinin scintillation proximity assay (SPA) beads by mixing 37 μl SPA beads (100 mg/ml in water) with 10 ml of diluted membrane, and incubating at 37°C for 1 hr with gentle agitation. Membrane bound beads were then centrifuged for 5 min at 1000 rpm. The supernatant was drained off and replaced with fresh HEPES-buffered salt solution. [³H]-AZD5069 was added to each well of a white clear-bottomed 96-well plate in the presence and absence of 10 μM AZ11705135 to determine non-specific binding. The binding of [³H]-AZD5069 was initiated by addition of SPA bead-membrane preincubation mix to the wells in a total assay volume of 200 μl. The plate was sealed, mixed and incubated at 37°C for 2 hr. Radioactivity was determined in a Trilux 1450 Microbeta Scintillation counter (Wallac Ltd) with a 1 min read time. The non-specific binding control was subtracted then the data was fitted to a one site binding hyperbola using the GraphPad Prism™ Software package.

**HEK calcium flux assay**

HEK293 cells, expressing recombinant human CXCR2 and Gqi5, were grown and harvested as described above. The assay was performed in 96-well poly-D-lysine coated plates (Becton Dickinson). To each well, 25,000 cells were added in a final volume of 100 μl. Cells were allowed to adhere to the plates overnight at 37°C. Adhered cells were washed twice in buffer and test compound solution containing 1.5% (v/v) DMSO, was added to the appropriate wells at 3 x final concentration in a volume of 50 μl. The cells were incubated with compound at either 22°C for 30 min or 37°C for 3 hr. Prior to measurement of intracellular calcium transients, cells were pre-loaded with Fluo4-AM solution for 60 min. Calcium levels were monitored using a fluorescence imaging plate reader (FLIPR; Molecular
Devices) after 50 μl of solutions containing CXCL8 at 4x final concentration had been added. Calcium transients in response to various concentrations of CXCL8 were measured. Readings were taken every 2 s for a total run time of 6 min.

**Polymorphonuclear cell calcium flux assay**

100 μl of Fluo-3 (AM) solution diluted to 5 μM final concentration was added and the PMN cell suspension rolled for 90 min at room temperature. Fluo3-AM loaded cells were centrifuged at 300 g for 5 min at room temperature, the supernatant discarded then the cells re-suspended in Tyrode’s/HEPES solution at a concentration of 4 x 10^6/ml.

The assay was performed in 96-well poly-D-lysine coated plates (Becton Dickinson). To each well was added of 200,000 cells and test compound in 100 μl solution. Assay Buffer containing 1.5% (v/v) DMSO and compound at 3 x final concentration was added to the appropriate wells. The 96-well plates were centrifuged (200 g, 5 min) and then incubated at room temperature for 30 min in 0.75% DMSO. Calcium transients in response to various concentrations of CXCL1 were measured using a FLIPR, where 50 μl of solutions containing CXCL1 at 3 x final concentration were added and fluorescence readings were taken every 2 s for a total run time of 2 min. The CXCR2 selective ligand CXCL1 was used in PMN cells to investigate CXCR2-mediated effects in these cells as they express both CXCR2 and CXCR1 receptors.

**Reversibility of calcium flux inhibition in HEK cells**

HEK293 cells, expressing recombinant human CXCR2 and Gqi5, were grown and harvested as described above. Cells and plates were set up as described above. AZD5069 solution or PBS/HEPES solution, containing 1.5% (v/v) DMSO, was added to the appropriate wells in a volume of 50 μl to give 3nM final concentration. Cells were incubated with vehicle or compound at 37°C for two hr and then washed 3 times using 100 μL buffer and left in 100 μL
buffer for 4 hr before reading. A time-matched control was also conducted where cells were incubated in compound for 2 hr, 4 hr after the start of the experiment with no wash before reading. One hour before reading 50 μl of Fluo4-AM solution was added and the cells incubated for a further 60 min at 37°C. Calcium transients were then measured as described previously.

**Chemotaxis**

PMN cells were prepared as described above. A chemotactic gradient to CXCL1 in the presence and absence of test compound was performed in 96-well Neuroprobe chemotaxis plates (Receptor Technologies) with a 3 μm filter. An equal volume of compound or vehicle control was added to cells in Tyrode’s/HEPES solution. Cells in the presence or absence of compound were then incubated whilst rolling for 30 min at room temperature. Solutions of CXCL1 and compound were added to the lower well compartment of the chemotaxis plate. A 25 μl droplet of isolated PMN cells at 8 x 10⁶/ml was placed on top of each filter position. The filter-plate assembly was incubated at 37°C in 5% CO₂ for 3 hr to provide sufficient time for a slow acting compound to demonstrate an effect. The filter was removed and 5 μl Alamar Blue added to the cells in the lower well compartment. Cells were incubated at 37°C in 5% CO₂ for 45 min and the fluorescence determined at 560 nm using an fmax fluorimeter (Molecular Devices). Changes in fluorescence were expressed as number of cells by comparison with a calibration performed with Alamar Blue. The number of cells responding to the chemotactic gradient was expressed as percent relative to the maximum effect observed in the vehicle control.

**Whole blood CD11b expression**

Blood was collected in lithium heparin-coated (final concentration 10 IU/ml) tubes. Within 20 min of collection, 2.7 μl of DMSO vehicle control or various concentrations of compounds in
DMSO were added to individual tubes of blood to give a final DMSO concentration of 0.03%.

Each tube was rolled gently at room temperature for 60 min. Aliquots (80 μl) of the blood incubations were then added to deep well polypropylene 96-well plates containing FITC-labeled CD11b and PE-labeled CD16 antibodies or isotype controls (Caltag Medical Systems Ltd). Cells were stimulated by addition of various concentrations of agonist (10 μl) mixed for 5 s then incubated at room temperature in the dark for 40 min. The assay was stopped by placing cells on ice for 30 min. Plates were removed from the ice bath and fixed in Optilyse B by adding 0.1 m Optilyse B followed by vigorous shaking for 10 min at room temperature. Distilled water (1 ml) was added to each well and the plate mixed for at least 30 min at room temperature. Samples were then transferred to polypropylene LP4 tubes and stored in the dark at 4°C until ready to analyze by flow cytometry. CD11b expression was determined using a Coulter XL flow cytometer to detect the level of FITC fluorescence on the CD16 positive granular cells with high forward and side-scatter properties. Within the granulocyte region cells with high levels of CD16-PE fluorescence were gated as the “neutrophil region”. Neutrophil CD11b expression on the CD16-positive neutrophil population was measured by determining the median FITC fluorescence. Data describing concentration effect curves were fitted to a 4-parameter logistic function using ‘XL-fit’ and the response expressed percent relative to the maximum agonist effect observed in the vehicle control.

**AZD5069 radioligand binding kinetics**

The dissociation for AZD5069 was measured by displacement of [³H]AZD5069 from membranes of HEK293 cells expressing recombinant human CXCR2. Membranes from HEK cells expressing CXCR2 were bound to wheat germ haemagglutinin SPA beads as described above. To each well of a white clear-bottomed 96-well plate was added [³H]AZD5069 at various concentrations and either buffer or AZ10397767 (10 μM final concentration) to determine the non-specific binding. The binding of [³H] AZD5069 was
initiated by addition of 160 μl of membrane-coated SPA beads to the wells. The plate was sealed, mixed and incubated at either 37°C for 90 min or 22°C for 200 min. The dissociation of [3H] AZD5069 was initiated by addition of AZ10397767 to 10 μM in buffer containing 0.1% DMSO. The plate was mixed and counts per minute (CPM) sequentially determined at various time intervals in a Trilux 1450 Microbeta Scintillation counter (Wallac Ltd). For experiments performed at 22°C data points were collected in real time by incubation within the scintillation counter. For experiments incubated at 37°C plates were removed from the incubator briefly for scintillation counting for 30 s before being returned to the incubator. The non-specific binding control data was subtracted from the measured CPM and the data fitted to a single exponential decay model using Origin™ Software package.

**Rat airway LPS challenge model**

All animal studies were conducted under an approved UK Home Office project licence. The compound doses were selected based on a combination of data from *in vitro* CXCR2 potency, rat pharmacokinetics and rat protein binding. Male CRL:CD rats (weight range 315–427g) were allowed to acclimatize to housing conditions for at least 7 days after delivery. Compounds were prepared in with vehicle (1% HPMC/0.1% Tween 80/H2O).

Rats in treatment groups (8 per group) were dosed orally 1 hr before LPS challenge with AZD5069, AZD8309 or AZ10397767 or dexamethasone (5.8 μmol/kg). Rats in group 1 were challenged with 0.9% saline. Rats in groups challenged with 0.1 mg/ml LPS in saline (0.9%) or saline vehicle control. The rats were placed in perspex boxes and challenged with an aerosol generated with 2 jet nebulizers operated at an air-flow rate of 12 l/min for 30 min. At 4 hr following LPS challenge, the trachea was cannulated and the airway lavaged using 3 aliquots of 3.3 ml sterile PBS at room temperature. An aliquot of lavage fluid was removed for cell counting. Cytospin slides were prepared by adding a 100 μl aliquot of lavage fluid into cytospin funnels in a Shandon Cytospin3 operated at 700 rpm for 5 min. Slides were
stained with Wright-Giemsa stain on using a Hema-Tek-2000 automatic slide stainer and typically, 200 cells were counted under a microscope. Cells were classified as eosinophils, neutrophils and mononuclear cells. Mononuclear cells included monocytes, macrophages and lymphocytes. The number of neutrophils was quantified by expressing the cell number as a percentage of the total count. The number of neutrophils per animal was averaged across each treatment group and the result expressed as mean ± SEM. Results between treatment groups were compared using non-parametric statistics Mann-Whitney or Kruskal-Wallis methodology in Graphpad InStat. Rats were anaesthetized with isofluorane and terminal blood samples (0.5 ml and 2 ml) were taken from the abdominal vena cava of animals and collected. Animals were then terminated with 1.0 mL pentobarbitone sodium (Euthatal) intraperitoneally. One set of blood samples were assessed for differential cell numbers using an Advia Haematology System. The other set of blood samples (2 ml) were centrifuged at 2,800g for 10 min at 4°C. The plasma was removed and stored at -20°C for subsequent determination of compound concentration.
Results

Inhibition of CXCL8 binding and specificity. Initial studies showed AZD5069 inhibited binding of radiolabelled CXCL8 to recombinant membranes expressing human CXCR2 and CXCR1. This system specifically measured binding to recombinant receptor as there was no significant binding to HEK293 cell membranes not expressing these receptors. AZD5069, AZD8309 and AZ10397767 all inhibited CXCL8 binding to CXCR2 to a similar degree with pIC\textsubscript{50} values ± SEM of 9.1±0.05, 9.0±0.04 and 8.8±0.06 respectively (Fig. 2). All three compounds also inhibited CXCL8 binding to CXCR1 to a similar degree with pIC\textsubscript{50} values of 6.9±0.08, 6.6±0.10 and 6.5±0.06 respectively. AZD5069 was investigated at 153 human receptor and enzyme targets at a concentration of 10 µM or higher. Significant activity was observed only at 2 of these targets CCR2 (pEC\textsubscript{50} = 5.2 and CCR5 pEC\textsubscript{50} = 4.9) (Supplemental Table 1 and 2).

Cross species activity of AZD5069. The specific binding of [\textsuperscript{3}H]-AZD5069 to HEK cell recombinant membranes was used to quantify the binding affinity of AZD5069 to CXCR2 across different species (Fig. 3). pK\textsubscript{D} values ± SEM for of [\textsuperscript{3}H]-AZD5069 binding to human, cynomolgus monkey, rat and dog were determined to be 9.4±0.11, 9.3±0.06, 9.2±0.05, and 9.0±0.17 respectively.

Calcium responses in recombinant HEK cell and PMNs. An increase in intracellular calcium was observed either when recombinant cells expressing CXCR2 were stimulated with CXCL8 or donor isolated PMN’s were stimulated with CXCL1 (Fig. 4A & D). AZD5069 produced a clear concentration-dependant inhibition of calcium responses in both cell types when AZD5069 was pre-incubated with cells for a relatively short time (30 min) and room temperature. In the presence of AZD5069 the pharmacological profile showed either little or no effect at low concentrations or marked inhibition and curve collapse at high concentrations of AZD5069. Due to the non-classical agonist responses in the presence of
AZD5069 an estimate of \( pA_2 \) could not be determined under these conditions. However, qualitatively it can be seen that AZD5069 more potently inhibited calcium responses in PMN cells than in HEK cells expressing recombinant CXCR2. In contrast, the profiles for agonist response curves in the presence of AZD8309 and AZ10397767 shifted in parallel with increasing concentrations of compound for both cell types (Fig. 4B, C, E & F). For AZD8309 and AZ10397767 \( pA_2 \pm SEM \) were determined to be 9.0 ± 0.11 and 8.7 ± 0.08 respectively for CXCL8 stimulated calcium responses from HEK cells with more potent inhibition observed for CXCL1 stimulated calcium responses from PMN cells (\( pA_2 \pm SEM \) determined to be 10.0±0.1 and 9.5±0.1 for HEK and PMN cells respectively).

**Effect of time and temperature on the pharmacology and reversibility of AZD5069.**

An assessment was made to determine whether the non-classical pharmacology of AZD5069 persisted under physiologically relevant conditions of longer exposure at 37°C. When AZD5069 was preincubated with HEK cells expressing recombinant CXCR2 for 3 hr at 37°C the pharmacological profile of the agonist curves demonstrated an apparent parallel shift in agonist curves with increasing concentrations of AZD5069. With the increased time and temperature of incubation there was also a more regular pattern to the response curves with an intermediate level of inhibition evident (Fig. 5A) as opposed to only low or high levels of inhibition observed during short preincubation at room temperature (Fig. 4A). Under conditions of 3 hr preincubation at 37°C a \( pA_2 \pm SEM \) was determined across 7 experiments to be 9.6 ± 0.11. However, for 8 other experiments, a \( pA_2 \) could not be determined due to excessive curve collapse. The data shown in Figure 5A is a composite from all 15 experiments. Prolonged incubation at 37°C could not be performed in PMN cells due to loss of function under these conditions. The reversibility of AZD5069 as an inhibitor of a human CXCR2-mediated response was determined *in vitro* by measuring inhibition of CXCL8-induced calcium flux in HEK293 cells expressing recombinant human CXCR2 and Gqi5. The reversibility of the inhibitory effect of AZD5069 was demonstrated by washing compound
from the cells followed by a 4-hr recovery period. AZD5069 (3 nM) produced a concentration-dependent inhibition of calcium flux induced by CXCL8 (Fig. 5B). The inhibition of functional responses to CXCL8 by AZD5069 was reversible after compound was removed by washing cells. Although the washing procedure employed to remove compound from the system was able to demonstrate that inhibition by AZD5069 was reversible, complete restoration of the CXCL8-mediated calcium response to control levels was not observed.

Inhibition of agonist mediated CD11b expression and chemotaxis in neutrophils and PMNs. AZD5069 produced a concentration-dependent inhibition of PMN chemotaxis induced by CXCL1 (Fig. 6A). Agonist response curves appeared to collapse in the presence of AZD5069. In contrast less curve collapse was evident at higher concentrations of the CXCL1 driven PMN chemotaxis response in the presence of AZD8309 or AZ10397767. Although response to a gradient of chemokine is not a simple equilibrium driven system, the pA2 for AZD5069 was estimated to be approximately 9.6 (range, 9.2-9.9). The integrin CD11b is up regulated on neutrophils in response to a variety of inflammatory mediators. AZD5069 produced clear inhibition of CXCL1 mediated CD11b expression with a rightward shift of the concentration effect curve to CXCL1 (Fig. 6B), with a pA2 of 6.9 ± 0.13 (mean ± SEM, n=8). CXCL1 agonist response curves also appeared to collapse in the presence of AZD5069 (Fig. 6B). AZD8309 and AZ10397767 demonstrated inhibition of CXCL1 mediated CD11b expression with a parallel shift in the agonist responses and no apparent decrease in maximum effect (Fig. 6B & C). This equated to pA2 values of 6.2 ± 0.10 (mean±SEM, n=6) and 5.8±0.09 (mean±SEM, n=15) for AZD8309 and AZ10397767, respectively. When several agonists inducing CD11b expression on neutrophils were investigated a high concentration of AZD5069 was found to specifically inhibit only the CXCL1 driven response with no significant inhibition observed for neutrophils stimulated by C5a, fMLP or LTB4 (Fig. 7).
Dissociation kinetics for AZD5069. Dissociation of $[^3]$H AZD5069 from CXCR2 in the presence of 10 μM AZ10397767 followed a single site exponential decay model consistent with a simple first-order dissociation process (Fig. 8). Temperature had a marked effect on the dissociation half-life. The dissociation of AZD5069 was shown to be slow at both 37 °C and 22°C but changed by more than 12-fold for the 15 °C decrease in temperature from a half-life of $28 \pm 2$ min (n=4) at 37 °C to $360 \pm 5$ min (n=3) at 22°C.

AZD5069 inhibition of LPS-induced neutrophilia. Rat LPS induced lung neutrophilia is a well validated model of non-allergic airway inflammation (Holmes et al., 2002). LPS inhalation in the rat initiates a cascade of events resulting in pulmonary inflammation characterized by neutrophil influx and associated pathology in the lung. At 4 hr after LPS challenge there is a robust, reproducible neutrophilia observed in the bronchoalveolar lavage fluid (BALF), which is significantly inhibited by standard anti-inflammatory compounds such as dexamethasone. A dose of 0.1 mg/ml LPS was chosen because it produces a robust and reproducible neutrophil influx that is sub-maximal and responsive to anti-inflammatory compounds.

LPS challenge significantly increased neutrophil levels in the BALF compared to saline challenged animals. Orally administered AZD5069 reduced the LPS-induced neutrophilia in a dose dependent manner (Fig. 9A). The effect of AZD5069 was statistically significant at 3 μmol/kg (p<0.05) and 10 μmol/kg (p<0.001), when the terminal plasma concentrations of AZD5069 were 60 and 310 ng/mL, respectively. AZD5069 also inhibited the LPS-induced blood neutrophilia in a dose dependent manner (Table 1). AZD5069 showed a steep dose response with no effect at 0.3 μmol/kg. In the present study, dexamethasone was used as a positive control and significantly reduced neutrophils in the BALF in all studies. However, in
contrast to the steep dose response relationship observed with AZD5069, both AZD8309 and AZ10397767 exhibited a more gradual dose response relationship (Fig. 9B and 9C).

**Discussion**

We profiled a series of compounds of different structure in the same cell systems to link the translational pathway between human *in vitro* pharmacology and animal physiology. AZD5069 is a potent, slowly reversible and selective antagonist of radiolabelled CXCL8 binding at human CXCR2. AZD5069 was developed with the aim to be selective for CXCR2. When measured in a similar system expressing recombinant CXCR1 it was apparent that all of the compounds in this study were around 100-fold more potent at CXCR2 than at CXCR1. This is comparable to known CXCR2 antagonists such as MK-7123 which is 80-fold less potent at CXCR1 than CXCR2 (Gonsiorek et al., 2007). Inhibition of CXCR1 function is associated with impaired bacterial killing and could, if specifically inhibited, potentially lead to risks such as increased susceptibility to bacterial infection in humans (Hartl et al, 2007).

Binding of radiolabelled AZD5069 to membranes expressing recombinant CXCR2 was similar for receptor orthologs across 4 different species; human, cynomolgus monkey, dog and rat. This is consistent with good cross-species activity demonstrated for other CXCR2 antagonists of different structural classes (Chapman et al., 2007). This cross-species activity of AZD5069 allows the use of AZD5069 as an effective tool compound to explore pharmacological consequences of CXCR2 antagonism in various animal models, as well as the toxicological consequences of both on-target and off-target effects.

AZD5069, AZD8309 and AZ10397767 were all antagonists of CXCL8 mediated increases in intracellular calcium in HEK cells expressing CXCR2. In this system the pharmacological profiles of AZD8309 and AZ10397767 were consistent with classical competitive antagonism i.e. parallel rightward shifts of the agonist curves in the presence of increasing
concentrations of antagonist, with no apparent, significant change in the maximum calcium response at high agonist concentrations (Fig. 4B & C). In contrast, AZD5069 showed unusual pharmacological behavior in respect of calcium flux antagonism. A small increase in concentration of AZD5069 from 32 nM to 100 nM produced a marked increase in antagonism with significant curve collapse (Fig. 4A).

This profile of antagonism, with curve collapse, was also observed following the inhibition of CXCL1 stimulated calcium release in PMN cells (Fig. 4D, E & F). As these data were collected following a 30 min incubation of compound at 22°C, it was considered that the unusual pharmacological profile of AZD5069 may be due to poor equilibration of AZD5069 with agonist in both the HEK and PMN calcium responses. When the calcium assay using recombinant HEK cells was performed at 37°C for 3 hr, AZD5069 showed increased antagonist potency and with increasing AZD5069 concentrations a more regular pattern of response curve depression was observed (Fig. 5A). Increasing the temperature and time of incubation would be expected to increase the rate of attainment of equilibrium conditions. Under these conditions, a more classical antagonist profile was observed but with insurmountable antagonism still evident in the calcium response curves. This is consistent with what would be expected for a slowly reversible antagonist where pre-formed antagonist-receptor complexes are reversible but dissociate so slowly that only part of the receptors can be liberated (Charlton and Vauquelin, 2010). These observations suggest that the non-classical calcium response curves obtained under conditions of 30 min incubation at 22°C were due to poor equilibration of the antagonist in the assay system. A prolonged incubation at 37°C was attempted in PMN cells in to investigate the effects of the compound under more physiologically relevant conditions; however, these cells did not survive long enough under these conditions for a stable measurement to be recorded.

All CXCR2 antagonists in the current study were found to be potent antagonists of CXCL1 mediated PMN chemotaxis. For CXCL1 mediated CD11b expression, AZD5069 displayed
rightward shifted curves but with some curve collapse evident at higher concentrations. This curve collapse can be explained by lack of equilibrium conditions similar to the pharmacology observed in the calcium flux experiments. This pattern of antagonism is similar to the insurmountable antagonism of calcium effects observed at the endothelin receptor by the slowly reversible antagonist Macitentan (Gatfield et al., 2012).

The kinetics of AZD5069 dissociation was measured using radio-labeled AZD5069. At room temperature the dissociation of AZD5069 was more that 12 fold slower than at 37°C (Fig. 8). This difference is larger than a temperature dependent change of about 2-4 fold which would be expected for a simple thermodynamically driven increase in collision frequency. The large effect of temperature suggests that dissociation of AZD5069 from the receptor is a more complex process possibly involving cooperativity or multiple steps. This process may involve protein isomerisation and/or interaction with membrane lipids. Both AZD5069 and AZ10397767 are similar in structure and would be expected to interact with CXCR2 at the same binding site, but it appears that the interaction is slower and more complex with AZD5069. However, as previously suggested for this class of compounds, the antagonist/receptor interaction occurs at a deeply buried internal binding site (Nicholls et al., 2008) and may add to the complexity and rate of the antagonist dissociation from the receptor.

AZD5069 was shown to specifically antagonize CXCL1 mediated adhesion molecule expression in PMN cells with out affecting the response to other inflammatory mediators. AZD5069 was therefore specific for the CXCR2 receptor and had no effect on the signal transduction mechanism for adhesion molecule expression. This specificity suggests that AZD5069 would be expected to preserve non-CXCR2-mediated innate immune responses to infection.

The anti-inflammatory effect of AZD5069 is anticipated to be produced by reducing neutrophil migration from the systemic circulation into sites of inflammation including lung
mucosa, with additional effect on neutrophil migration from the bone marrow (Kohler et al., 2011). The response by macrophages and epithelial cells to LPS administered to the rat lung results in the release of chemokines which attract inflammatory cells from the microvasculature (Quinton et al., 2004). AZD5069 was an antagonist of LPS driven lung neutrophilia in rats (Fig. 9A). The dose response profile for AZD5069 antagonism of the LPS driven lung neutrophilia was similar to that observed in the in vitro experiment where AZD5069 failed to equilibrate and showed a large change in antagonism for a relatively small increase in compound concentration. This suggests that under these conditions, AZD5069 may also equilibrate slowly in vivo in animal models. In contrast, both AZD8309 and AZ10397767 showed a more predictable dose response relationship in the LPS induced rat neutrophil assay. These data suggest that the potency of AZD5069 may be underestimated in acute animal models. In a repeated dosing study with sufficient time for equilibration between tissue and receptor, AZD5069 would be expected to perform according to the law of mass action and would be useful to investigate in chronic disease such as COPD, severe asthma, arthritis, inflammatory bowel disease and cancer.

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Authorship Contributions

Participated in research design: Nicholls, Gaw, MacIntosh.
Conducted experiments: Nicholls, Wiley, MacIntosh.
Performed data analysis: Nicholls, Wiley, MacIntosh.
Wrote or contributed to the writing of the manuscript: Nicholls, Wiley, Dainty, Phillips, MacIntosh, Gaw, Kärrman Mårdh.
References


Figure Legends

Figure 1. Structures of AZD5069, AZD8309 and AZ10397767.

Figure 2. Inhibition of $^{125}$I-CXCL8 binding HEK293 cell membranes expressing recombinant human CXCR2 (solid symbols) or CXCR1 (open symbols) receptors. Circles denote AZD5069, triangles denote AZD8309 and squares denote AZ10397767. Values are mean ± SEM (n=5-7). The radioligand concentration used was 0.06 nM and below the $K_D$ for CXCR2 and CXCR1 determined in to be 1.2 nM and 2 nM, respectively.

Figure 3. Specific binding of $^3$H-AZD5069 to membranes prepared from HEK293 cells expressing recombinant human (solid circles), cynomolgus (open squares), rat (triangles) and dog (diamonds) CXCR2. Values are mean±SEM (n=4). To determine non-specific binding of $^3$H-AZD5069 the CXCR2 antagonist AZ11705135 was used at a concentration of 10 μM much higher than the IC$_{50}$ of 2 nM determined in separate $^{125}$I-CXCL8 inhibition assays.

Figure 4. Effect of compounds on intracellular calcium responses in HEK293 and PMN cells. at 22°C following preincubation with compounds for 30 min. Panels A-C, concentration response curves for CXCL8-induced calcium flux in HEK293 cells expressing human CXCR2 in the presence and absence of compounds. Panels D-F, concentration response curves for CXCL1-induced calcium flux in human PMN cells. Compound concentrations were 10 nM (solid circles), 32 nM (open triangles) and 100 nM (solid triangles) with the
control response in the absence of compound denoted by open circles. Values are mean+/SEM (n=6-8).

Figure 5. Panel A, concentration response curves for CXCL8 induced calcium flux in HEK293 cells expressing human CXCR2 in the presence and absence of AZD5069. Cells were incubated at 37°C for 2 hours, after one hour the Fluo4-AM solution was added and the cells incubated for a further 60 min at 37°C. Compound concentrations were 0.32 nM (solid squares), 1 nM (open diamonds) and 3.2 nM (solid diamonds) with the control response in the absence of compound denoted by open circles. Values are mean+/SEM (n=15). Panel B concentration response curves for CXCL8 induced calcium flux in HEK293 cells expressing human CXCR2 in the presence (triangles) and absence (circles) of AZD5069. Solid symbols denote initial control data, open symbols denote data collected 4 hr following a washing procedure to remove compound. Values are mean ± SEM (n=5).

Figure 6. Panel A, concentration response curves for CXCL1 stimulated CD11b expression on human neutrophils in blood in the presence and absence of AZD5069, values are mean+/SEM (n=7-13). Panel B, concentration response curves for CXCL1 stimulated chemotaxis of human PMN cells in presence and absence of AZD5069, values are mean+/SEM (n=5). Compound concentrations were 0.3 nM (solid squares), 1 nM (open diamonds), 3 nM (solid diamonds), 10 nM (solid circles) and 30 nM (open triangles) with the control response in the absence of compound denoted by open circles.

Figure 7. Concentration response curves for CXCL1 (panel A), C5a (panel B), fMLF (panel C) and LTB4 (panel D) stimulating CD11b expression on human neutrophils in blood in the
presence and absence of AZD5069. AZD5069 concentrations were 10 μM (solid circles) with the control response in the absence of compound denoted by open circles. Values are mean +/- SEM (n=4).

Figure 8. Time dependence for displacement of ^3H-AZD5069 (1 nM) with 10 μM AZ10397767 from HEK membranes expressing human CXCR2. Data is expressed as percent specific binding of AZD5069. Circles denote incubation at 37°C, triangles denote incubation at 22°C values are mean±SEM (n=2-4).

Figure 9. Dose response for AZD5069 (panel A), AZD8309 (panel B) and AZ10397767 (panel B) on neutrophils recovered from BALF 4 hours after LPS challenge. Dexamethasone dosed at 5.8 μg/kg was used as a control in both studies. Values shown are mean ± SEM for the number of neutrophils recovered per rat. The statistical significance of compound effects relative to the LPS challenge controls were determined by non-parametric ANOVA and are denoted as **, P<0.05 and ** P<0.001.
### Tables

**Table 1** The effect of AZD5069 on mean blood neutrophil numbers 4 hours after LPS challenge.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment/ challenge</th>
<th>Blood neutrophils (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle po/saline challenge</td>
<td>1.62 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle po/LPS challenge</td>
<td>3.16 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>AZD5069 (0.3 μmol/kg) / LPS challenge</td>
<td>2.78 ± 0.09</td>
</tr>
<tr>
<td>4</td>
<td>AZD5069 (1 μmol/kg) / LPS challenge</td>
<td>2.44 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>AZD5069 (3 μmol/kg) / LPS challenge</td>
<td>1.81 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>AZD5069 (10 μmol/kg) / LPS challenge</td>
<td>1.37 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>Dexamethasone (5.8 μmol/kg) / LPS challenge</td>
<td>1.96 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>AZ10397767 (120 μmol/kg) / LPS challenge</td>
<td>2.04 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>P<0.05, <sup>b</sup>P<0.001 when compared with the vehicle treated control group (group 2) using ANOVA followed by Bonferroni Multiple comparisons Test.**
Figures
Figure 1

AZD5069

AZD8309

AZ10397767
Figure 2
Figure 3

% Specific Binding

[AZD5069] log_{10} (M)

0 20 40 60 80 100

-10.5 -9.5 -8.5 -7.5
Figure 4
Figure 5

A

B

Calcium Response (%) vs. [CXCL8] log$_{10}$ (M)

Calcium response (%) vs. [CXCL8] log$_{10}$ (M)
Figure 9