Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
Molecular Pharmacology Fast Forward. Published on August 19, 2008 as doi:10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Identification of a Putative Intracellular Allosteric Antagonist Binding-Site in the CXC Chemokine Receptors 1 and 2.

David J. Nicholls, Nick P. Tomkinson, Katherine E. Wiley, Anne Brammall, Lorna Bowers, Caroline Grahames, Alasdair Gaw, Premji Meghani, Philip Shelton, Tracey J. Wright[1], Philip R. Mallinder.

Respiratory and Inflammation Research Area, AstraZeneca Research and Development, Loughborough, U.K.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Copyright 2008 by the American Society for Pharmacology and Experimental Therapeutics.

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running title: CXCL8 Receptor Intracellular Allosteric Site

b) Corresponding Author: Dr David J Nicholls

Address: Department of Discovery BioScience, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire LE11 5RH, UK.

Tel:+44 (0)1509 644000, Fax:+44 (0)1509 645506, email: david.nicholls@astrazeneca.com.

c)

Number of Text pages:	34
Number of Tables:	1
Number of Figures:	11
Number of References:	40
Words in Abstract:	240
Words in Introduction:	544
Words in Discussion:	1493

d) Non standard Abbreviations:

TM; trans-membrane. GPCR; G-protein coupled receptor. C-terminal; carboxyl–terminal. CXCR1-2-1; CXCR1 receptor with amino acids 168 to 218 (CXCR2 numbering system, equivalent to 159 to 209 in the CXCR1 sequence) substituted from CXCR2; CXCR1-2long; CXCR1 receptor with C-terminal 60 amino acids substituted from CXCR2, CXCR2-1long; CXCR2 receptor with C-terminal 59 amino acids substituted from CXCR1, CXCR1-2short; CXCR1 receptor with C-terminal 34 amino acids substituted from CXCR2, CXCR2-1short; CXCR2 receptor with C-terminal 33 amino acids substituted from CXCR1.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The chemokine receptors CXCR1 and CXCR2 are G-protein coupled receptors (GPCRs) implicated in mediating cellular functions associated with the inflammatory response. Potent CXCR2 receptor antagonists have been discovered, some of which have recently entered clinical development. The aim of this study was to identify key amino acid residue differences between CXCR1 and CXCR2 that influence the relative antagonism by two compounds which have markedly different chemical structures. By investigating the effects of domain switching and point mutations we found that the second extracellular loop, which contained significant amino acid sequence diversity, was not important for compound antagonism. Surprisingly, switching the intracellular C-terminal 60 amino acid domains of CXCR1 and CXCR2 caused an apparent reversal of antagonism at these two receptors. Further investigation showed that a single amino acid residue, lysine 320 in CXCR2 and asparagine 311 in CXCR1, plays a predominant role in describing the relative antagonism of the two compounds. Homology modeling studies based on the structure of bovine rhodopsin indicated a potential intracellular antagonist binding pocket involving lysine 320. We conclude that residue 320 in CXCR2 forms part of a potential allosteric binding pocket on the intracellular side of the receptor, a site that is distal to the orthosteric site commonly assumed to be the location of antagonist binding to GPCRs. The existence of a common intracellular allosteric binding site at GPCRs related to CXCR2 may be of value in the design of novel antagonists for therapeutic intervention.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Chemokines are small, secreted proteins of 8-14 kDa which regulate a broad spectrum of cellular functions and typically induce cell movement along a concentration gradient. There are three groups of chemokines exhibiting characteristic cysteine sequence motifs, the C-X-C, C-C and C-X3-C families (Horuk, 2001). The emergent role of chemokines in immune and inflammatory responses has identified chemokine receptors as attractive targets for therapeutic intervention in various diseases and disorders (D'Ambrosio et al., 2003). The two GPCRs CXCR1 and CXCR2 have been identified as important mediators of inflammation and display distinct ligand specificities. CXCL8 (IL-8) and CXCL6 (GCP-2) interact with both CXCR1 and CXCR2, however the chemokines CXCL5 (ENA-78), CXCL7 (NAP-2) and CXCL1 (GROα) are efficacious for CXCR2 only (Wolf et al., 1998). CXCR2 is expressed on a variety of cells including neutrophils, keratinocytes, mast cells, eosinophils, macrophages, endothelial and epithelial cells. In addition to chemotaxis, activation of CXCR2 is known to stimulate a variety of cellular responses including calcium mobilization, adhesion molecule up-regulation and angiogenesis. These pleiotropic effects have implicated CXCR2 in the pathology of various diseases with inflammatory components such as COPD, arthritis and psoriasis. The diverse nature of CXCR2 in biology has stimulated much interest in the pharmaceutical industry and the synthesis of several non-peptide antagonists have been described (White et al., 1998; Catusse et al., 2003; Matzer et al., 2004; Widdowson et al., 2004; Souza et al., 2004; Baxter et al., 2006; Gonsiorek et al., 2007). These non-peptide antagonists fall into various structural classes and display different receptor selectivity profiles (Busch-Petersen, 2006). Understanding the nature of antagonist

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

interactions with receptors and their selectivity is key for rational drug design. As CXCR1 is the closest homolog to CXCR2 and compounds have been shown to bind to both receptors, it is important to understand the origin of the observed selectivity. For GPCRs, predictions for ligand-binding interactions have until recently been largely based upon comparisons with rhodopsin. The retinal binding site is clearly defined in the exofacial core of rhodopsin and has been the focus of attention for mutagenesis and homology modeling studies to predict ligand-binding interactions (Klabunde and Hessler, 2002; Kristiansen, 2004; Schwartz et al., 2006). However, reports describing allosteric modulation of GPCRs suggest the existence of alternative interaction sites, which regulate receptor function and dimerization (Bertini *et al.*, 2004; Soudijn *et al.*, 2004; Birdsall and Lazareno, 2005; Gao and Jacobson, 2006). Although the term “allosteric” refers to a recognition domain on the receptor that is distinct from the primary (orthosteric) site (Neubig et al., 2003), the precise location of alternative interaction sites within GPCRs is not clearly understood. We describe here evidence to support the existence of a non-peptide antagonist-binding site near the intracellular C-terminal domain of CXCR2, which is distal to the classical retinal-binding site in rhodopsin. We have used domain swap experiments and site-directed mutagenesis in conjunction with homology modeling to identify amino acids within the intracellular region of CXCR1 and CXCR2 that are important for conferring receptor selectivity by structurally distinct non-peptide antagonists. In addition, we present evidence suggesting that access to the intracellular side of the receptor is required for inhibition by allosteric antagonists. The existence of a potential intracellular binding pocket in chemokine receptors and other GPCRs could influence the design of novel agents for therapeutic intervention.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Material and Methods

Reagents.

Oligonucleotide primers were synthesized by Eurogentec (Southampton, UK) using standard methods. Standard tissue culture and molecular biology reagents including restriction endonucleases, alkaline phosphatase, T4 DNA ligase and TOP 10 *E. coli* were supplied by Invitrogen (Paisley, UK). QuikChange XL site-directed-mutagenesis kits were purchased from Stratagene (Amsterdam, Netherlands). QIAquick Gel Extraction kits were supplied by Qiagen (Crawley, UK). CXCL8 and CXCL1 were purchased from BioSource (Nivelles, Belgium). ^{125}I-labeled CXCL8 (specific activity 74 TBq/mmol) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). CXCR2 antagonists (Fig. 1), compound A ((1*R*)-5-[[(3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-methylethyl]amino]thiazolo[4,5-*d*]pyrimidin-2(3*H*)-one (Walters et al., 2008) and compound B *N*-(3-(aminosulfonyl)-4-chloro-2-hydroxyphenyl)-*N*'-(2,3-dichloro-phenyl) urea (Podolin et al., 2002) were synthesized by the Department of Medicinal Chemistry, AstraZeneca (Charnwood, UK). Compound A is protected by European Patent No. 1222195, United States Patent No. 6790850 and corresponding patents and patent applications.

DNA constructs and site-directed mutagenesis.

The cDNAs encoding the human chemokine receptors CXCR1 and CXCR2 were cloned into pIRESneo2 using standard methods as described in Sambrook et al. (1989) and confirmed by sequencing. These plasmids were used as a template to produce the CXCR1 and CXCR2 chimeras. An alignment of CXCR1 and CXCR2 was generated (Fig. 2) and throughout this paper the amino acid numbering of all mutant and hybrid

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

proteins corresponds to that of CXCR2 in Figure 2. The amino acid residues at positions 320 and 325 in the CXCR2 sequence were equivalent to residues 311 and 316 in CXCR1 respectively. All oligonucleotide primers used for genetic manipulations are listed in the supplementary data. The first chimera was CXCR1-2-1 was generated by exchanging the cDNA sequence of CXCR1 encoding residues 159 to 209 with residues 168 to 218 of CXCR2 using an overlapping PCR reaction. Primers CXCR1 5' start and CXCR1 5' BS were used to amplify the N-terminal sequence of CXCR1. Primers CXCR2 F1 and CXCR1 R1 were used to amplify the middle CXCR2 region and primers CXCR1 3'TS and CXCR1stop were used to amplify the C-terminal region of CXCR1. The resulting PCR reaction was cloned into pIRESneo using NheI and NotI restriction sites on the PCR product. The next chimera constructs were generated by exchanging the cDNA encoding C-terminal 60 amino acids of CXCR2 with the C-terminal 59 amino acids of CXCR1 using an internal Xcm I site. These hybrid receptors were designated CXCR1-2long and CXCR2-1long. A second set of chimera constructs designated CXCR1-2short and CXCR2-1short were generated by first introducing an AflII restriction enzyme site in the cDNAs of both CXCR1 and CXCR2 by site directed mutagenesis, followed by exchanging the cDNA encoding the C-terminal 34 amino acids from CXCR2 and the C-terminal 33 residues from CXCR1. Receptor mutants CXCR1 N311K, CXCR1 F316L, CXCR2 K320N, CXCR1 N311K/F316L and CXCR2 K320N/L325F were generated using DNA primers with single or double base mismatches. Mutagenesis was performed using the QuikChange XL site-directed-mutagenesis kit. The correct sequence of all DNA constructs was confirmed by di-deoxy-terminator sequencing using standard methods.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Cell Culture.

HEK293 cells were grown in DMEM-Glutamax medium containing non-essential amino acids and 10% (v/v) foetal calf serum in a humidified incubator at 37°C with 5% CO_2:95% air. Cells were harvested at approximately 80% confluence from the flasks using 10x trypsin. The cells were transfected with plasmids for CXCR1 and CXCR2 receptor chimeras and mutants using the transfection reagent Fugene 6 (Roche, Burgess Hill, UK). Stable transfectants expressing CXCR1 and CXCR2 proteins were selected for and maintained by addition of Geneticin G418 at 1mg/ml (Invitrogen, Paisley, UK).

Functional calcium assays for CXCR2.

Intracellular calcium mobilization was determined in 96-well poly-D-Lysine coated plates. Cells (100000 per well) were allowed to adhere to plates overnight then incubated with 10µM final concentration of Fluo3-AM for 60 min in media at 37°C. Plates were washed twice in 100 µl HEPES-buffered PBS solution (25 mM HEPES, 10 mM phosphate buffer pH 7.4 containing, 137 mM NaCl and 1.5 mM $CaCl_2$) then 50 µl buffer/well added to the cells. Compounds in HEPES-buffered PBS solution, containing 1.5% (v/v) DMSO, were added in a volume of 50 µl/well and incubated at room temperature for 30 min. Cells were primed by addition of 50 µl/well carbachol solution (1 mM final concentration). After 3 min, 50 µl/well of solutions containing CXCL8 at various concentrations were added. Calcium transients were measured using a FLIPR^{TM} (Molecular Devices, Wokingham, UK).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Membrane Preparation.

Cells were resuspended and then disrupted by homogenisation using a Polytron tissue homogenizer in hypotonic buffer at 4°C (3:1 mix of water and HEPES-buffered Tyrodes solution). The membrane preparation was purified by centrifugation at 140000g for 1 h at 4°C on 41% (w/v) sucrose. The membrane fraction at the interface was recovered, diluted and centrifuged at 100000g for 20 min at 4°C. The membrane pellet was re-suspended at $1x10^8$ cell equivalents/ml (typically 3 mg/ml protein) in HEPES-buffered Tyrodes solution (10 mM HEPES pH7.4 containing 2.7 mM KCl, 137 mM NaCl, 0.4 mM $KHPO_4$, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% (w/v) gelatin and 100 µg/ml bacitracin) and subsequently stored in aliquots at -80°C.

Radio-ligand binding assays.

Radio-ligand binding assays were performed in HEPES-buffered Tyrodes solution using 0.45 mm 96-well filter plates (Millipore, Watford, UK). Membranes (30 µg/ well) and ^{125}I-CXCL8 (60 pM) were incubated with compounds for 2 h at room temperature in the presence of 1% (v/v) DMSO. Membrane-bound ^{125}I-CXCL8 was separated from ^{125}I-CXCL8 in solution by washing with 200 µl of HEPES-buffered Tyrodes solution at 4°C. Individual filters were transferred to polypropylene tubes and the radioactivity measured by direct gamma counting using a Cobra II Gamma counter (PerkinElmer, Beaconsfield, UK). Non-specifically bound radioactivity was determined in the presence of 10 µM unlabeled CXCL8.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Receptor Modeling.

Recently, two crystal structures for the β_2 adrenergic receptor have been described (Cherezov et al., 2007; Rasmussen et al., 2007) and these structures were compared with the rhodopsin structure to evaluate their suitability for modeling CXCR2. A multiple sequence alignment of all class A human GPCR receptors suggests the presence of a single residue gap in the β_2-receptor sequence at the bottom of helix 7, within 3 or 4 residues of the CXCR2 K320N mutation described in this study. This gap is not present in rhodopsin or CXCR1 or CXCR2 and when the rhodopsin and β_2-receptor structures are structurally aligned, this adversely affects any model of the intracellular region based on the β_2-receptor structure. More fundamentally, the β_2-receptor structures were solved using techniques likely to introduce artefacts into the intracellular portion of the receptor. The Rasmussen structure was obtained at low resolution as a complex with an antibody Fab bound to the intracellular site, and the Cherezov structure as a chimera with T4-lysozyme. A structural alignment of the rhodopsin and the chimeric β_2-receptor structures shows a very strong correspondence between the intracellular portions of the two receptors. However, the greatest deviation is in helices 5 and 6 where the lysozyme molecule is fused in the chimeric β_2-receptor and it is likely that this is an artefact introduced by engineering the chimera. For these reasons, the bovine rhodopsin structure was used as a starting point for modeling the intracellular site of CXCR2.

A sequence alignment of the CXCR2 coding sequence with other class A GPCRs and bovine rhodopsin was generated using ClustalW (Chenna et al., 2003) and then modified to reflect known TM defining motifs. This alignment along with the structure of bovine rhodopsin (Okada et al., 2002) was used as input to Modeler version 5 (Sali and

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Blundell, 1993) run via the InsightII 2000 interface (Accelrys Software Inc., Cerius2 Modeling Environment, Release 4.10, San Diego: Accelrys Software Inc., 2005.). A total of 20 models were produced and the structure with the lowest penalty function used. Hydrogens and charges were subsequently added in Sybyl version 6.9 and siteID (Tripos Inc. S^t Louis, USA) was used to visualise the intracellular binding site. The resulting model was manually refined and subjected to a series of constrained minimisations using CHARMm Version 31.1 (Accelrys Software inc., San Diego, USA). Dockings were performed manually and subjected to constrained minimisation in the active site using the Tripos forcefield.

Data Analysis

Concentration-response data were fitted to a 4 parameter logistic function using the Excel-based program 'XL-fit' (I.D. Business Solutions Ltd, Guildford, UK). Estimates of pA_2 values for compounds were determined by analysing the dose ratio relative to the vehicle control curve. The IC_{50} was defined as molar concentration of compound required to give 50% inhibition of specifically bound ^{125}I-CXCL8.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Agonist potency and compound antagonism at CXCR1 and CXCR2.

As expected agonist potency differences at CXCR1 and CXCR2 were clearly evident in CXCL8 and CXCL1 mediated calcium responses (Fig 3A and D). Antagonism and affinity differences were evaluated by comparing the ability of compounds to inhibit both CXCL8 mediated calcium responses and the binding of radio-labeled CXCL8 to membranes from HEK293 cells expressing recombinant receptors. Consistent with findings from previous studies (Podolin et al., 2002; Walters et al., 2008), both compounds A and B were at least 100-times more potent antagonists of CXCR2 compared to CXCR1 (Table 1). Moreover, compound A is representative of a diverse chemical series that shows a consistent 100-fold decrease in apparent affinity against CXCR1 compared to CXCR2 (data not shown). The antagonism of CXCR2 mediated intracellular calcium mobilization by compound A produced parallel right-shifted concentration-response curves (Fig. 4). In this respect, compound A appeared to demonstrate an inhibition profile resembling that of a classical competitive antagonist with a pA_2 of 8.7±0.1 (n=6), similar to the pIC_{50} for inhibition of radio-labeled CXCL8 binding to membranes in a cell-free system (Table 1).

Dependence of lipophilicity

A large series of compounds of which A is a representative was generated in order to investigate the structure activity relationship with CXCR2. Therefore all the compounds were tested in the cellular CXCL8 mediated calcium response assay and the CXCL8 cell-free radio-ligand displacement assay. The partition coefficient (logD) between octanol and aqueous solution at pH7.4 of each of these compounds was also

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

determined as a measure of lipophilicity. Compounds with lower lipophilicity showed a weak but significant trend towards reduced antagonism in the cellular calcium mobilization assay when compared with the cell-free system (Fig. 5). As permeability of a compound by passive diffusion through a biological membrane is dependent upon lipophilicity, molecular size and hydrogen bonding capacity, these data suggest that compounds in this series may require penetration of the biological membrane in order to interact with the receptor in the calcium response assay. Compounds A and B demonstrate a comparable degree of antagonism in the cell and cell-free systems and they both have high lipophilicity, each with a logD of approximately 3.5 (Fig. 5).

CXCR1-2-1 chimera construct.

The antagonism of CXCR1 and CXCR2 by compounds A and B was clearly different as shown by right-shifted CXCL8 mediated calcium mobilization in the presence of antagonist (Fig. 6A and 6B). We wished to understand, at a molecular level, the relative differences in antagonism for compounds acting at CXCR1 and CXCR2. It is generally assumed that small molecule binding sites in GPCRs are located in the retinal-binding pocket, described in the rhodopsin structure, which has extra cellular access for agonists and antagonists. Therefore the chimeric construct CXCR1-2-1 was generated to investigate the effect of exchanging the second extracellular loop and sections of the transmembrane regions of CXCR1 and CXCR2. This region has a high degree of sequence diversity (Fig. 2), is equivalent to the retinal binding pocket and was considered likely to have amino acid residues involved in compound interactions. When expressed in HEK293 cells the chimeric protein retained functional activity after stimulation with the agonists CXCL8 or CXCL1 showing the CXCR1-2-1 construct

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

possesses a similar agonist profile to wild-type CXCR1 (compare Fig. 3A with 3C). The CXCR1-2-1 chimera is similar to the ABA chimera generated previously (Ahuja et al., 1996) which also maintained agonist potency differences between CXCL8 and CXCL-1. Surprisingly, when incubated with compounds A or B the CXCR1-2-1 construct gave a similar antagonism profile to wild type CXCR1 (compare Fig. 6A with 6C). Thus, exchanging the second extracellular loop and sections of the transmembrane region of CXCR1 and CXCR2, comprising the equivalent of the retinal binding pocket in rhodopsin had little effect on either agonist potency or antagonist activity.

Long C-terminal tail chimera constructs.

Two chimeric constructs were generated to investigate the effect of exchanging the C-terminal region of CXCR1 and CXCR2 (CXCR1-2long and CXCR2-1long). Exchange of these C-terminal domains had little effect on agonist potency for either CXCL8 or CXCL1 (Fig. 3) suggesting that key amino-acid residues describing differences in agonist effects between CXCR1 and CXCR2 were not located in the C-terminal domain. However, differences in compound antagonism were observed when the chimeras were compared with the wild type CXCR1 and CXCR2 receptors. CXCR1-2long showed enhanced compound antagonism (Fig. 7A), which was previously absent in the CXCR1 wild-type receptor. Conversely, the reciprocal mutation CXCR2-1long had a reduced compound antagonism when compared with the CXCR2 wild-type receptor.

Short C-terminal tail chimera constructs.

To further investigate key amino acid residues dictating the selectivity difference between CXCR1 and CXCR2, hybrid receptor constructs were created where the most

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

divergent region, the last 34 C-terminal amino acid residues, was exchanged (CXCR1-2short and CXCR2-1short). Surprisingly, there was no effect on the antagonist profile when this smaller domain was exchanged as both hybrid receptors (Fig. 7 C and D), were similar to wild-type CXCR1 and CXCR2 respectively.

Site-directed mutagenesis.

Taken together, switching of the short and long C-terminal domains demonstrated that key amino acid residues which influence compound antagonism were located in the intervening 25 amino acid region between residues 300 and 325 (as shown in Fig. 2). The most prominent difference between CXCR1 and CXCR2 in the amino acid sequence alignment between residues 300 and 325 occurs at position 320 (Fig. 2) where asparagine in CXCR1 is substituted for lysine in CXCR2. Other differences are relatively conservative changes with differences in leucine, isoleucine and phenylalanine residues. These residues are similar in terms of bulk and lipophilicity, whereas the physicochemical properties of the asparagine and lysine amino acid side chains have markedly different ionisation states at physiological pH.

Mutant receptor constructs of CXCR1 and CXCR2 were generated to investigate the effects of the N311K mutation on compound antagonism. A mutation at F316L was also included as it is in close proximity to N311K and could be included in the same mutagenic oligonucleotide. Simultaneous introduction of the two mutations N311K and F316L into CXCR1 (Fig. 8A) resulted in a mutant receptor that displayed an antagonist profile similar to that observed with native CXCR2 and the CXCR1-2long hybrid receptor. The reciprocal mutation in CXCR2 resulted in diminished compound

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

antagonism at the concentrations tested (Fig. 8B). The single amino acid substitution of F316L into CXCR1 had no effect on receptor antagonism as this mutant receptor had an antagonist profile identical to wild type CXCR1 (Fig. 8E). Remarkably, the single amino acid substitution of N311K into CXCR1 (Fig. 8C) resulted in antagonism of the mutant receptor similar to the antagonist profile observed with wild-type CXCR2, CXCR1-2long and the CXCR1 N311K/F316L double mutation constructs. The reciprocal mutation K320N in CXCR2 reduced antagonism at the concentration tested such that the antagonist profile was similar to wild type CXCR1 (Fig. 8D). Taken together, these data suggest that the amino acid residue at position 320 (Fig. 2) has a profound effect on antagonism by both compounds A and B.

The contribution of lysine or asparagine at position 320 to compound potency in CXCR1 and CXCR2 was explored further by investigating the effect of mutations on inhibition of radio-labeled CXCL8 binding to membrane preparations expressing recombinant receptors (Table 1 and Fig. 9). The mutations N311K in CXCR1 and K320N in CXCR2 produced an increase and decrease in affinity respectively for both compounds tested. Mutation of the residue at position 320 does not result in a complete reciprocal change in affinity for compounds A and B between CXCR1 and CXCR2 (Table 1). The magnitude of the changes in affinity appeared to be larger for compound B than for compound A (Table 1).

Receptor Modeling.

A homology model of CXCR2 based on the rhodopsin structure (Okada et al., 2002) was generated (Fig. 10). The rhodopsin model clearly shows the presence of a hydrophobic

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

cavity in CXCR2 on the intracellular side of the TM bundle with apparent access from the cytosol. The surface of this cavity comprises residues of transmembrane helices 2, 3, 6 and 7 and the opening of the binding site is adjacent to lysine 320, which is highlighted in green in Figure 10. The cavity is able to accommodate compounds of the size of A and B, with minimal adjustment either of individual residues or helices. The binding mode was modelled by introducing compound A into the cavity by a process of manual docking, followed by minimization (Fig. 11). In this pose, the acidic nitrogen of the thiazole ring and the adjacent nitrogen of the pyrimidine ring interact directly with K320. The phenyl ring sits in a hydrophobic cavity within the transmembrane region, whilst the allaninol group is located in the interface between the membrane region and the cytosol, adjacent to the arginine and aspartate of the DRY motif on helix 3. A model of chemokine bound to receptor has recently been proposed where the N-terminus of the chemokine interacts with the receptor helical bundle and the core domain of the ligand interacts with the extracellular loops of the receptor (Allen *et al.*, 2007). It is possible that with a degree of flexibility in the receptor, the cavity containing the chemokine N-terminus could potentially extend through the receptor. However, the chemokine-binding model suggests that the N-terminus does not extend far enough down into the receptor to interact at the intracellular site proposed here.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The non-peptide compounds A and B inhibit CXCL8 binding to both CXCR1 and CXCR2 (Table 1), although the compounds have approximately 100 fold lower affinity at CXCR1. We investigated whether amino acid sequence variation between CXCR1 and CXCR2 could explain the differential antagonism of compounds A and B at these receptors. The second extracellular loop region between transmembrane domains IV and V was initially expected to be involved in compound binding, as this region (shaded red, Fig. 10) forms part of a potential pocket analogous to the retinal binding site in rhodopsin. However, it appears that residues in this region contribute little to agonist or compound interactions, since there were no apparent differences in either agonist potency or compound antagonism when the second extra cellular loop was substituted in CXCR1 with that of CXCR2 to generate the chimera CXCR1-2-1.

The observation of a loss in potency for compounds in the cell assay compared with the cell-free system related to decreasing lipophilicity (Fig. 5) is consistent with the cell membrane acting as a barrier to passive diffusion of the compounds; lipophilicity being a key determinant for permeability through a biological membrane. These data suggest that the compounds require intracellular access to the receptor antagonist binding site as described recently (Andrews et al., 2008) and may not be directly competing with CXCL8 at the extra cellular site. This would imply a binding site involving residues in the intracellular region; and the region of highest sequence dissimilarity between the two receptors, the C-terminus, was chosen as the next target for mutagenesis.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

When the long C-terminal domains of CXCR1 and CXCR2 were exchanged there was little effect on agonist signalling, consistent with previous reports where the agonist binding location was attributed to segments of the receptor other than the C-terminus (Ahuja et al., 1996; LaRosa et al., 1992; Gayle, III et al., 1993; Katancik et al., 2000; Rajagopalan and Rajarathnam, 2004; Andrews et al., 2008). In contrast, the chimera receptor constructs showed that exchanging the C-termini clearly affected the ability of small molecule antagonists to inhibit calcium responses. Whilst a decrease in potency can be attributed to trivial non-specific effects, the increased compound antagonism demonstrated at the CXCR1-2long hybrid was compelling evidence to support involvement of the C-terminal region in compound:receptor interactions as described for CCR4 and CCR5 (Andrews et al., 2008). Surprisingly, when the short C-terminal regions containing the greatest sequence diversity between CXCR1 and CXCR2 were exchanged, there was little change in compound antagonism of the chimeric receptor constructs (Fig. 7C and 7D). The striking difference in compound antagonism observed between the short and long amino acid C-terminal domains suggested that the intervening region (amino acid residues 303– 326) plays a prominent role in describing compound interactions. Amino acid sequence differences between CXCR2 and CXCR1 in this region were limited to positions 304, 312, 320 and 325 (Fig. 2). Compound antagonism profiles analogous to wild-type CXCR2 and CXCR1-2long were generated in CXCR1 by introduction of the double point mutations CXCR1 N311K/F316L or the single point mutation CXCR1 N311K (Fig. 8). In contrast, the reciprocal double and single mutations introduced into the CXCR2 receptor, CXCR2 K320N/L325F and K320N (Fig. 8), diminished compound antagonism, which was similar to the profile observed with wild-type CXCR1 and the CXCR2-1long receptor construct. In addition, the single

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

point mutation F316L was unable to confer compound antagonism in CXCR1. Taken together, these observations of mutually consistent reciprocal mutations suggest that the amino acid residue at position 320 (Fig. 2) plays a key role in describing compound antagonism differences between CXCR1 and CXCR2.

The contribution of the amino acid residue at position 320 was further investigated by determining the degree of inhibition of radio-labeled CXCL8 binding to membranes expressing recombinant receptors (Fig. 9). The mutation at position 320 did not produce a complete reciprocal switch in compound affinity between CXCR1 and CXCR2 (Table 1) suggesting that the residue at position 320 was not the sole factor in defining the relative compound affinity against these receptors. The increase in affinity afforded by the K311 mutation in CXCR1 was more pronounced for compound B (250-fold) than for compound A (60-fold) suggesting that residue 320 has a more profound effect on the relative affinity of compound B for CXCR1 and CXCR2. This may reflect differences in the structural and physicochemical nature of the acid isosteres.

The antagonist profile of compound A is consistent with competitive antagonism of the calcium response with a pA_2 similar to the pIC_{50} for inhibition of radio-labeled CXCL8 binding to membranes (Figure 4 and Table 1). In the absence of other information it might appear that compound A competes directly with CXCL8 at the orthosteric binding site. However, the weaker antagonism of hydrophilic compounds in cells coupled with the mutational data suggest a compound binding site in CXCR2 and CXCR1 that is intracellular and therefore most likely to be allosteric rather than orthosteric to the chemokine binding site. Classically, allostery induces a conformation of the receptor

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

that has either a very weak functional response or weak agonist binding. Antagonists that exert a strong negative allosteric effect can produce concentration dependent shifts in agonist dose response curves indistinguishable from competitive interactions, particularly at low concentrations (Ehlert, 1988). Thus, the observation that agonist concentration response curves shifted in a parallel fashion with different concentrations of compound A (Fig. 4) is consistent with either an orthosteric or allosteric mode of action (Avlani et al., 2004; Lazareno et al., 1998).

Whilst it is possible that the agonist and antagonist binding sites overlap, it is apparent that amino acid changes in the intracellular portion of the receptor can independently modify the activity of antagonists without affecting agonist activity suggesting that the chemokine does not extend as far as K320 into the receptor. This is consistent with the hypothesis presented here that the agonists and antagonists used exert their activity through independent sites. The hypothesis that an allosteric compound binding site at the C-terminus can induce receptor conformation changes which are propagated to the agonist binding site is supported by studies on a related receptor, ORF74 (Verzijl et al., 2006) where perturbations in the region of helix-8 resulted in a reduction or loss of agonist binding.

The presence of a hydrophobic cavity in the homology model leading from the cytosol into the central TM region is highly suggestive of a binding mode where the hydrophobic group of compound A binds within the lower half of the TM region and the acidic feature interacts directly with K320. The discovery here that the N311K mutant can confer CXCR2-like activity on CXCR1 and vice-versa, is supportive of this proposed

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

mechanism of antagonism. Nevertheless, the existence of many basic residues in the intracellular region (including, among others, arginine 144 of the DRY motif on helix 3) introduces uncertainty and the proposed binding mode is speculative in the absence of additional mutagenesis or structural data. This region of the protein has been shown previously to be critical in the function of GPCRs and alternative explanations for the mutagenesis data shown here may include indirect conformational effects as described previously (Li et al., 2007; Verzijl et al., 2006), however, the apparent requirement for access to the intracellular compartment alongside the mutagenesis data adds weight to the more straightforward explanation of the existence of a binding pocket in proximity to K320. Definitive location of the binding site by X-ray crystallography or NMR could provide the focus for future structural studies.

With muscarinic acetylcholine receptors, subtype selectivity has been achieved through ligand binding at an allosteric site (Lazareno et al., 1998). Binding to the intracellular face of the muscarinic M1 receptor has been proposed in a docking study of an allosteric modulator, KT5720 (Espinoza-Fonseca and Trujillo-Ferrara, 2006). Although no supporting experimental evidence was put forward, the study highlighted the same region proposed here as the binding site for compounds A and B.

The observation of a suitable binding site for a CXCR2 antagonist in a model based upon the dark state of rhodopsin suggests that any compound binding to this site and stabilizing an inactive state of the receptor should act as an antagonist or inverse agonist. As the surface of the proposed intracellular cavity comprises residues which are reasonably conserved throughout all GPCRs, it is tempting to speculate that other

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

GPCRs can be modulated in this way. Evidence has recently been put forward for a common intracellular antagonist binding site in CCR4 and CCR5 (Andrews et. al., 2008). It is also noteworthy that compound A used in the present study is an antagonist at the CCR2 receptor (Walters et al., 2008) which has a lysine residue at the equivalent position. The identification of this intracellular binding pocket should be of value in the design of new drugs and our data for CXCR1 and CXCR2 suggests that subtle amino acid changes in this region markedly modify compound activity. Thus, even in closely related GPCRs, selective antagonism can be obtained through binding to a common intracellular site.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

We would like to thank Iain Walters, Steve Hill, Fraser Hunt and Rhona Cox (Department of Medicinal Chemistry, AstraZeneca R&D) who synthesized the chemokine antagonists used in this study. Thanks also to Keith Wreggett and Glen Andrews for helpful discussions and critical review of the manuscript

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

References

Ahuja SK, Lee J C and Murphy P M (1996) CXC Chemokines Bind to Unique Sets of Selectivity Determinants That Can Function Independently and Are Broadly Distributed on Multiple Domains of Human Interleukin-8 Receptor B. Determinants of High Affinity Binding and Receptor Activation Are Distinct. *J Biol Chem* **271**:225-232.

Allen SJ, Crown S E and Handel T M (2007) Chemokine: Receptor Structure, Interactions, and Antagonism. *Annu Rev Immunol* **25**:787-820.

Andrews G, Jones C and Wreggett K A (2008) An Intracellular Allosteric Site for a Specific Class of Antagonists of the CC Chemokine G Protein-Coupled Receptors CCR4 and CCR5. *Mol Pharmacol* **73**:855-867.

Avlani V, May L T, Sexton P M and Christopoulos A (2004) Application of a Kinetic Model to the Apparently Complex Behavior of Negative and Positive Allosteric Modulators of Muscarinic Acetylcholine Receptors. *J Pharmacol Exp Ther* **308**:1062-1072.

Baxter A, Cooper A, Kinchin E, Moakes K, Unitt J and Wallace A (2006) Hit-to-Lead Studies: the Discovery of Potent, Orally Bioavailable Thiazolopyrimidine CXCR2 Receptor Antagonists. *Bioorg Med Chem Lett* **16**:960-963.

Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, Zampella G, Cervellera M N, Di C, V, Cesta M C, Galliera E, Martinez F O, Di Bitondo R, Troiani G, Sabbatini V, D'Anniballe G, Anacardio R, Cutrin J C, Cavalieri B, Mainiero F, Strippoli R, Villa P, Di Girolamo M, Martin F, Gentile M, Santoni A, Corda D, Poli G, Mantovani A, Ghezzi P and Colotta F (2004) Noncompetitive Allosteric Inhibitors of the Inflammatory Chemokine Receptors CXCR1 and CXCR2: Prevention of Reperfusion Injury. *Proc Natl Acad Sci U S A* **101**:11791-11796.

Birdsall NJ and Lazareno S (2005) Allosterism at Muscarinic Receptors: Ligands and Mechanisms. *Mini Rev Med Chem* **5**:523-543.

Busch-Petersen J (2006) Small Molecule Antagonists of the CXCR2 and CXCR1 Chemokine Recep-Tors As Therapeutic Agents for the Treatment of Inflammatory Diseases. *Curr Top Med Chem* **6**:1345-1352.

Catusse J, Liotard A, Loillier B, Pruneau D and Paquet J L (2003) Characterization of the Molecular Interactions of Interleukin-8 (CXCL8), Growth Related Oncogen Alpha (CXCL1) and a Non-Peptide Antagonist (SB 225002) With the Human CXCR2. *Biochem Pharmacol* **65**:813-821.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson T J, Higgins D G and Thompson J D (2003) Multiple Sequence Alignment With the Clustal Series of Programs. *Nucleic Acids Res* **31**:3497-3500.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Cherezov V, Rosenbaum D M, Hanson M A, Rasmussen S G, Thian F S, Kobilka T S, Choi H J, Kuhn P, Weis W I, Kobilka B K and Stevens R C (2007) High-Resolution Crystal Structure of an Engineered Human Beta2-Adrenergic G Protein-Coupled Receptor. *Science* **318**:1258-1265.

D'Ambrosio D, Panina-Bordignon P and Sinigaglia F (2003) Chemokine Receptors in Inflammation: an Overview. *J Immunol Methods* **273**:3-13.

Ehlert FJ (1988) Estimation of the Affinities of Allosteric Ligands Using Radioligand Binding and Pharmacological Null Methods. *Mol Pharmacol* **33**:187-194.

Espinoza-Fonseca LM and Trujillo-Ferrara J G (2006) The Existence of a Second Allosteric Site on the M1 Muscarinic Acetylcholine Receptor and Its Implications for Drug Design. *Bioorg Med Chem Lett* **16**:1217-1220.

Gao ZG and Jacobson K A (2006) Keynote Review: Allosterism in Membrane Receptors. *Drug Discov Today* **11**:191-202.

Gayle RB, III, Sleath P R, Srinivason S, Birks C W, Weerawarna K S, Cerretti D P, Kozlosky C J, Nelson N, Vanden Bos T and Beckmann M P (1993) Importance of the Amino Terminus of the Interleukin-8 Receptor in Ligand Interactions. *J Biol Chem* **268**:7283-7289.

Gonsiorek W, Fan X, Hesk D, Fossetta J, Qiu H, Jakway J, Billah M, Dwyer M, Chao J, Deno G, Taveras A, Lundell D J and Hipkin R W (2007) Pharmacological Characterization of Sch527123, a Potent Allosteric CXCR1/CXCR2 Antagonist. *J Pharmacol Exp Ther* **322**:477-485.

Horuk R (2001) Chemokine Receptors. *Cytokine Growth Factor Rev* **12**:313-335.

Katancik JA, Sharma A and de Nardin E (2000) Interleukin 8, Neutrophil-Activating Peptide-2 and GRO-Alpha Bind to and Elicit Cell Activation Via Specific and Different Amino Acid Residues of CXCR2. *Cytokine* **12**:1480-1488.

Klabunde T and Hessler G (2002) Drug Design Strategies for Targeting G-Protein-Coupled Receptors. *Chembiochem* **3**:928-944.

Kristiansen K (2004) Molecular Mechanisms of Ligand Binding, Signaling, and Regulation Within the Superfamily of G-Protein-Coupled Receptors: Molecular Modeling and Mutagenesis Approaches to Receptor Structure and Function. *Pharmacol Ther* **103**:21-80.

LaRosa GJ, Thomas K M, Kaufmann M E, Mark R, White M, Taylor L, Gray G, Witt D and Navarro J (1992) Amino Terminus of the Interleukin-8 Receptor Is a Major Determinant of Receptor Subtype Specificity. *J Biol Chem* **267**:25402-25406.

Lazareno S, Gharagozloo P, Kuonen D, Popham A and Birdsall N J (1998) Subtype-Selective Positive Cooperative Interactions Between Brucine Analogues and

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Acetylcholine at Muscarinic Receptors: Radioligand Binding Studies. *Mol Pharmacol* **53**:573-589.

Li JH, Han S J, Hamdan F F, Kim S K, Jacobson K A, Bloodworth L M, Zhang X and Wess J (2007) Distinct Structural Changes in a G Protein-Coupled Receptor Caused by Different Classes of Agonist Ligands. *J Biol Chem* **282**:26284-26293.

Matzer SP, Zombou J, Sarau H M, Rollinghoff M and Beuscher H U (2004) A Synthetic, Non-Peptide CXCR2 Antagonist Blocks MIP-2-Induced Neutrophil Migration in Mice. *Immunobiology* **209**:225-233.

Neubig RR, Spedding M, Kenakin T and Christopoulos A (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on Terms and Symbols in Quantitative Pharmacology. *Pharmacol Rev* **55**:597-606.

Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau E M and Shichida Y (2002) Functional Role of Internal Water Molecules in Rhodopsin Revealed by X-Ray Crystallography. *Proc Natl Acad Sci U S A* **99**:5982-5987.

Podolin PL, Bolognese B J, Foley J J, Schmidt D B, Buckley P T, Widdowson K L, Jin Q, White J R, Lee J M, Goodman R B, Hagen T R, Kajikawa O, Marshall L A, Hay D W and Sarau H M (2002) A Potent and Selective Nonpeptide Antagonist of CXCR2 Inhibits Acute and Chronic Models of Arthritis in the Rabbit. *J Immunol* **169**:6435-6444.

Rajagopalan L and Rajarathnam K (2004) Ligand Selectivity and Affinity of Chemokine Receptor CXCR1. Role of N-Terminal Domain. *J Biol Chem* **279**:30000-30008.

Rasmussen SG, Choi H J, Rosenbaum D M, Kobilka T S, Thian F S, Edwards P C, Burghammer M, Ratnala V R, Sanishvili R, Fischetti R F, Schertler G F, Weis W I and Kobilka B K (2007) Crystal Structure of the Human Beta2 Adrenergic G-Protein-Coupled Receptor. *Nature* **450**:383-387.

Sali A and Blundell T L (1993) Comparative Protein Modelling by Satisfaction of Spatial Restraints. *J Mol Biol* **234**:779-815.

Sambrook J, Fritsch E and Maniatis T (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schwartz TW, Frimurer T M, Holst B, Rosenkilde M M and Elling C E (2006) Molecular Mechanism of 7TM Receptor Activation--a Global Toggle Switch Model. *Annu Rev Pharmacol Toxicol* **46**:481-519.

Soudijn W, Van W, I and IJzerman A P (2004) Allosteric Modulation of G Protein-Coupled Receptors: Perspectives and Recent Developments. *Drug Discov Today* **9**:752-758.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Souza DG, Bertini R, Vieira A T, Cunha F Q, Poole S, Allegretti M, Colotta F and Teixeira M M (2004) Repertaxin, a Novel Inhibitor of Rat CXCR2 Function, Inhibits Inflammatory Responses That Follow Intestinal Ischaemia and Reperfusion Injury. *Br J Pharmacol* **143**:132-142.

Verzijl D, Pardo L, van Dijk M, Gruijthuijsen Y K, Jongejan A, Timmerman H, Nicholas J, Schwarz M, Murphy P M, Leurs R and Smit M J (2006) Helix 8 of the Viral Chemokine Receptor ORF74 Directs Chemokine Binding. *J Biol Chem* **281**:35327-35335.

Walters I, Austin C, Austin R, Bonnert R, Cage P, Christie M, Ebden M, Gardiner S, Grahames C, Hill S, Hunt F, Jewell R, Lewis S, Martin I, David N and David R (2008) Evaluation of a Series of Bicyclic CXCR2 Antagonists. *Bioorg Med Chem Lett* **18**:798-803.

White JR, Lee J M, Young P R, Hertzberg R P, Jurewicz A J, Chaikin M A, Widdowson K, Foley J J, Martin L D, Griswold D E and Sarau H M (1998) Identification of a Potent, Selective Non-Peptide CXCR2 Antagonist That Inhibits Interleukin-8-Induced Neutrophil Migration. *J Biol Chem* **273**:10095-10098.

Widdowson KL, Elliott J D, Veber D F, Nie H, Rutledge M C, McCleland B W, Xiang J N, Jurewicz A J, Hertzberg R P, Foley J J, Griswold D E, Martin L, Lee J M, White J R and Sarau H M (2004) Evaluation of Potent and Selective Small-Molecule Antagonists for the CXCR2 Chemokine Receptor. *J Med Chem* **47**:1319-1321.

Wolf M, Delgado M B, Jones S A, Dewald B, Clark-Lewis I and Baggiolini M (1998) Granulocyte Chemotactic Protein 2 Acts Via Both IL-8 Receptors, CXCR1 and CXCR2. *Eur J Immunol* **28**:164-170.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Address reprint requests to David J Nicholls, Department of Discovery BioScience, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire LE11 5RH, UK.
Email: david.nicholls@astrazeneca.com.

[1] Present address: GlaxoSmithKline, Clinical Immunology, Biopharm CEDD, Stevenage, UK.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1: Chemical structures of compound A, ((1*R*)-5-[[(3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-methylethyl]amino]thiazolo[4,5-*d*]pyrimidin-2(3*H*)-one. and compound B, *N*-(3-(aminosulfonyl)-4-chloro-2-hydroxyphenyl)-*N*'-(2,3-dichloro-phenyl) urea.

Figure 2: Amino-acid sequence alignment of CXCR1 and CXCR2. Amino acid positions are numbered according to the CXCR2 sequence. Text in bold denote region exchanged in CXCR1-2-1 chimera construct. Triangles (▲) denote the substitution positions for the long and short tail swap constructs. Brackets denote the predicted 7 TM spanning regions on the receptors.

Figure 3: Effect of receptor tail substitution on agonist induced calcium mobilization. Calcium mobilization in HEK293 cells expressing recombinant receptor constructs was determined in response to stimulation with CXCL8 (○) or CXCL-1 (▲). Receptor constructs are illustrated schematically where CXCR1 is represented in white fill and CXCR2 represented in grey fill. Panel A; CXCR1, Panel B; CXCR1-2long, Panel C; CXCR1-2-1, Panel D; CXCR2, Panel E; CXCR2-1long. Data are the mean of 3 - 8 individual experiments normalized to the maximum observed response determined in the absence of compound.

Figure 4: Antagonism of CXCL8 induced calcium mobilization by recombinant CXCR2. Calcium mobilization was determined in HEK293 cells expressing CXCR2 in the absence (○) and presence of 10nM (●), 32 nM (Δ) and 100 nM (▲) compound A. Data

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

are the mean of 6 individual experiments normalized to the maximum observed response determined in the absence of compound.

Figure 5: Relationship of lipophilicity to potency loss for inhibition of cellular calcium response. Potency loss was determined by subtracting pIC_{50} for inhibition of ^{125}I-CXCL8 to cell membranes expressing CXCR2 (cell-free assay) from pA_2 for antagonism of CXCL8 mediated calcium mobilization in HEK293 cells expressing CXCR2 (cell assay). Lipophilicity was determined by measuring logD, defined as the partition coefficient between aqueous solution and octanol at pH7.4. Compounds similar to those described by Walters et al. (2008). (○), compound A (●), compound B (▲).

Figure 6: Effect of second extracellular domain substitution on antagonism of CXCL8 induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated schematically where CXCR1 is represented in white fill and CXCR2 represented in grey fill. Panel A; CXCR1, Panel B; CXCR2, Panel C; CXCR1-2-1. Data are the mean of 3 - 4 individual experiments normalized to the maximum observed response determined in the absence of compound.

Figure 7: Effect of receptor C-terminal domain substitutions on antagonism of CXCL8 induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

schematically where CXCR1 is represented in white fill and CXCR2 represented in grey fill. Panel A; CXCR1-2long, Panel B; CXCR2-1long, Panel C; CXCR1-2short, Panel D; CXCR2-1short . Data are the mean of 3 - 4 individual experiments normalized to the maximum observed response determined in the absence of compound.

Figure 8: Effect of receptor point mutations on antagonism of CXCL8 induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated schematically where CXCR1 is represented in white fill and CXCR2 represented in grey fill. Panel A; CXCR1 N311K/F316L, Panel B; CXCR2 K320N/L325F, Panel C; CXCR1 N311K, Panel D; CXCR2 K320N, Panel E; CXCR1 F316L. Data are the mean of 3 - 4 individual experiments normalized to the maximum observed response determined in the absence of compound.

Figure 9: Effect of receptor point mutations on compound inhibition of ^{125}I-CXCL8 binding to recombinant membranes. Compound inhibition of ^{125}I-CXCL8 binding was determined in membranes prepared from HEK293 cells expressing recombinant CXCR1 (○), CXCR2 (●), CXCR1 N311K (Δ) or CXCR2 K320N (▲). Panel A; compound A, Panel B; compound B. Data are the mean of 3 - 7 individual experiments.

Figure 10: Ribbon representation of the homology model of CXCR2. The ribbon is colored to show the second extracellular domain substitution (Red) and the long C-

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

terminal substitution (Purple). The intracellular half of the inter-helical cavity is surfaced in grey. Lysine 320 is shown in a space-filling representation.

Figure 11: Intracellular view of CXCR2 with compound A (magenta) docked. The inter-helical cavity is surfaced in grey and helices – denoted H2,H3,H6,H7/H8 - are in red. The acidic heterocycle nitrogen of compound A is in contact with Lysine 320 (green).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Molecular Pharmacology Fast Forward. Published on August 1, 2008 as DOI: 10.1124/mol.107.044610
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

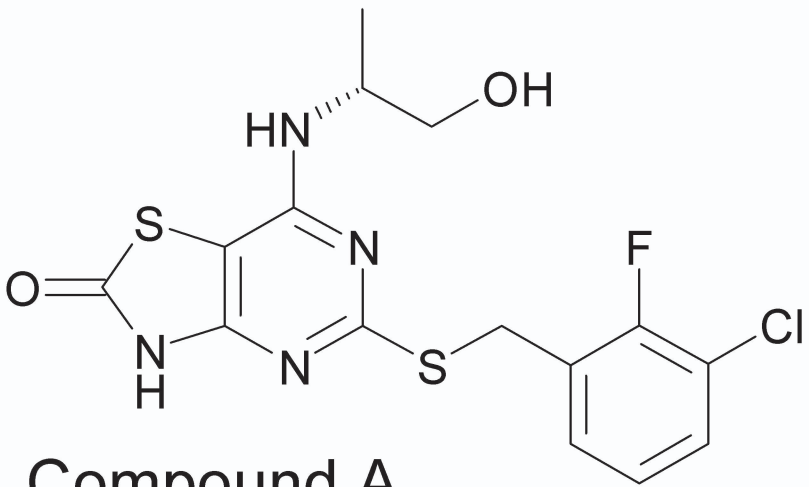
Table 1. Inhibition of CXCL8 binding

Compound inhibition at CXCR receptors and mutated receptor constructs was determined by measuring displacement of ^{125}I-CXCL8 binding to membranes. Compound, CXCL8 and membranes were incubated for 2 h at 22°C. Inhibition of specifically bound CXCL8 was determined and analyzed by non-linear regression. pIC_{50} values were derived from experiments presented in Fig. 9 and are mean ±SEM of 3 to 6 individual determinations.

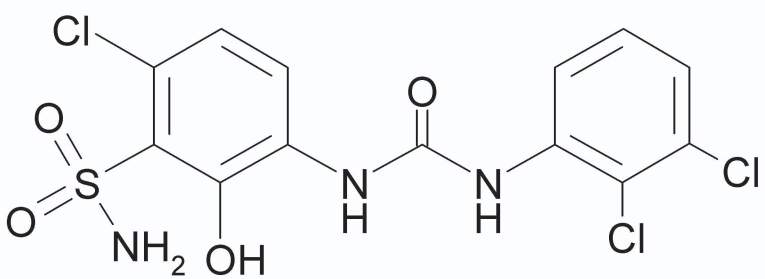
Construct	Compound A (pIC_{50})	Compound B (pIC_{50})
CXCR1	6.3±0.17	5.9±0.13
CXCR2	8.9±0.13	8.7±0.11
CXCR1 N311K	8.1±0.13	8.3±0.11
CXCR2 K320N	7.9±0.15	7.5±0.22

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 1

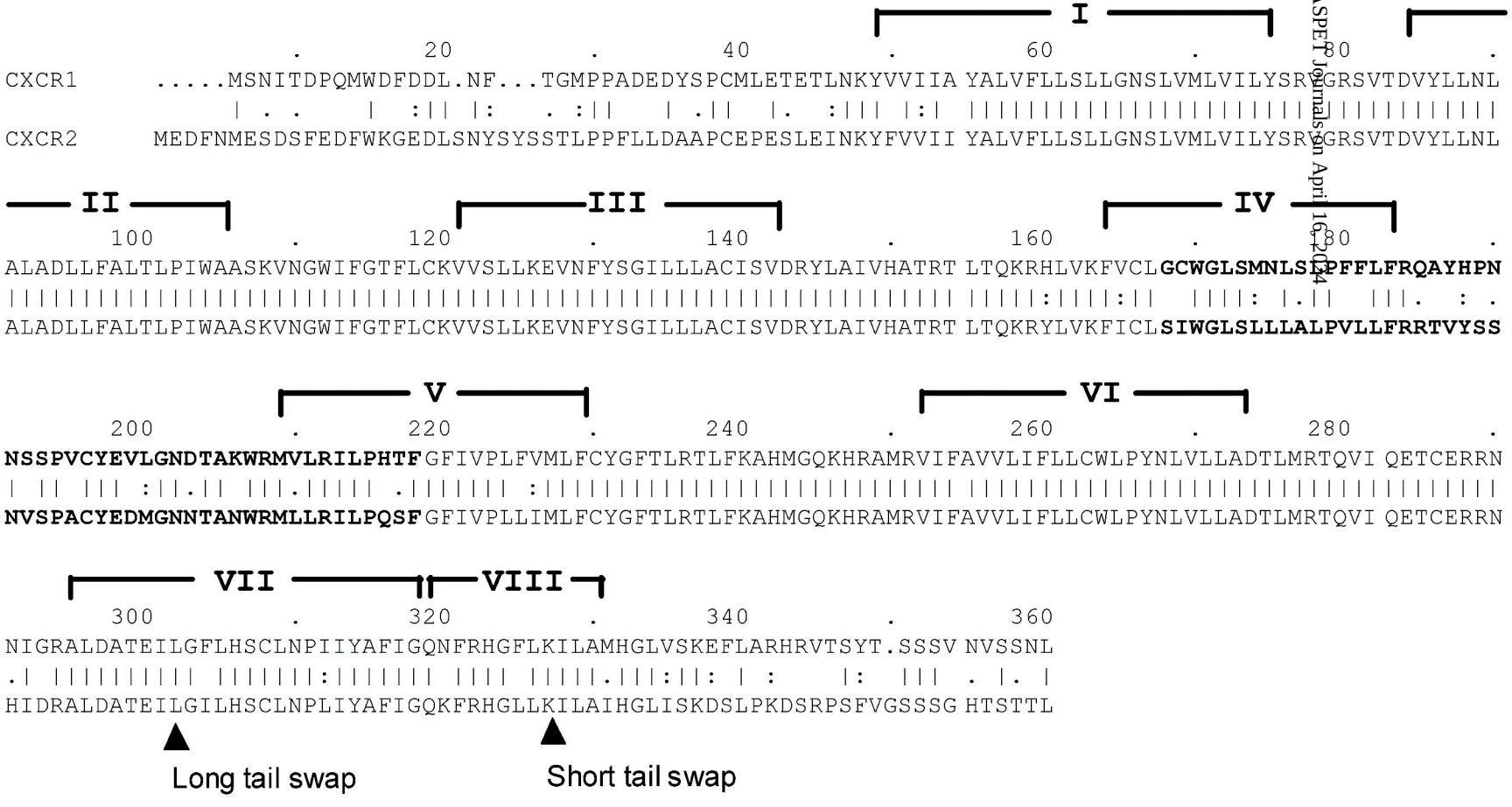


Compound A



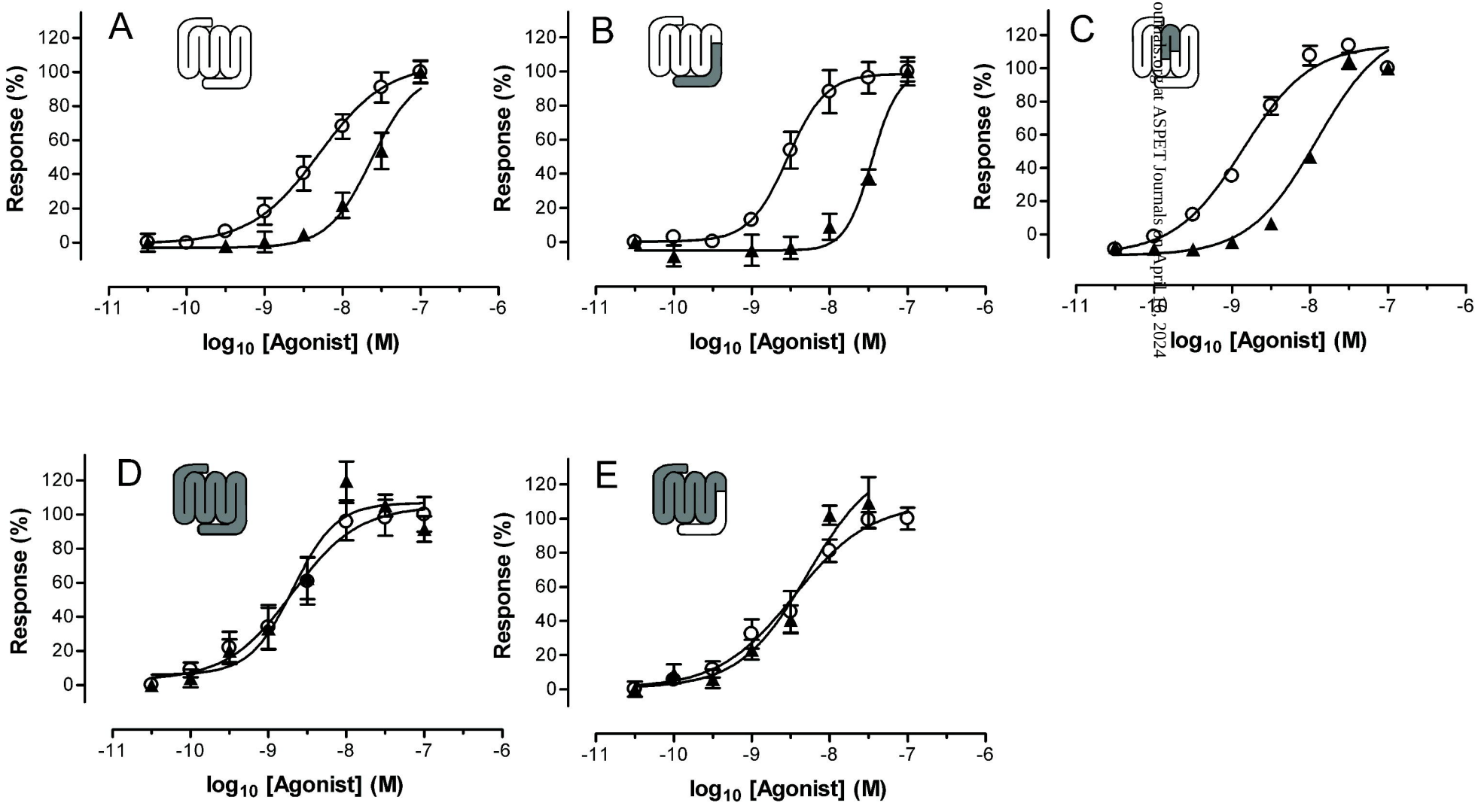
Compound B

Figure 2



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 3



om molpharm.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 4

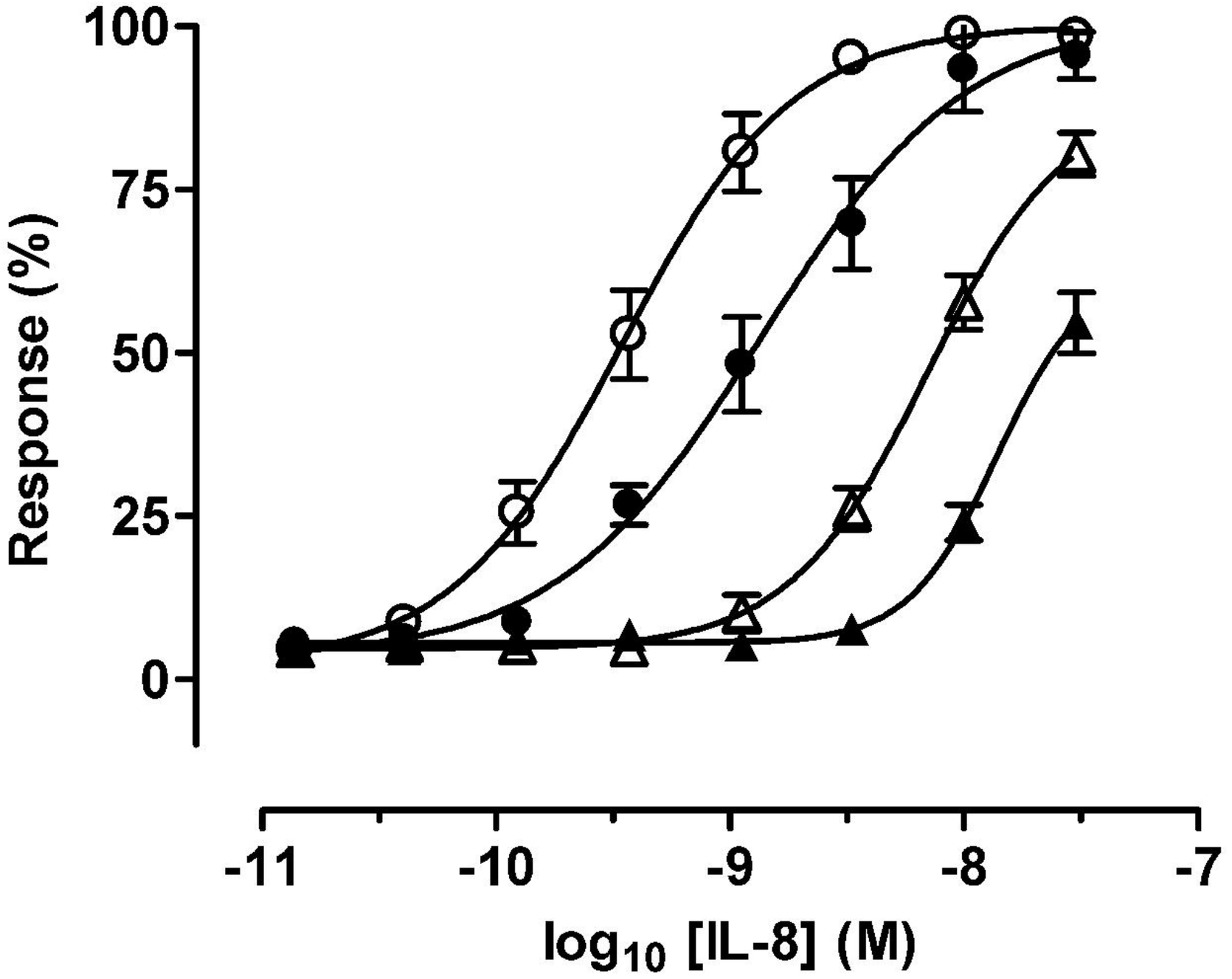


Figure 5

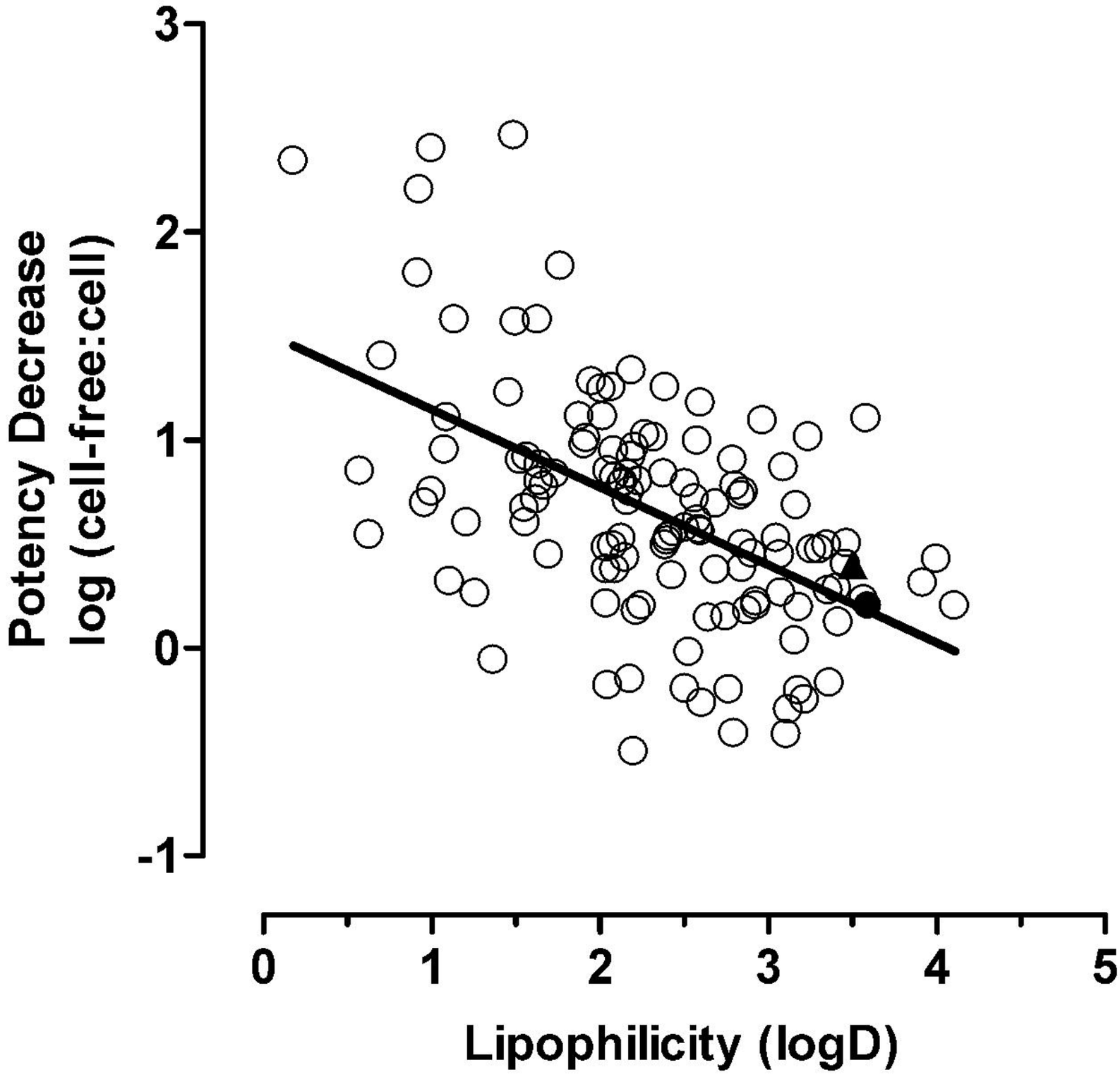

Potency Decrease
log (cell-free:cell)
3
2
1
0
-1
0
1
2
3
4
5
Lipophilicity (logD)

Figure 6

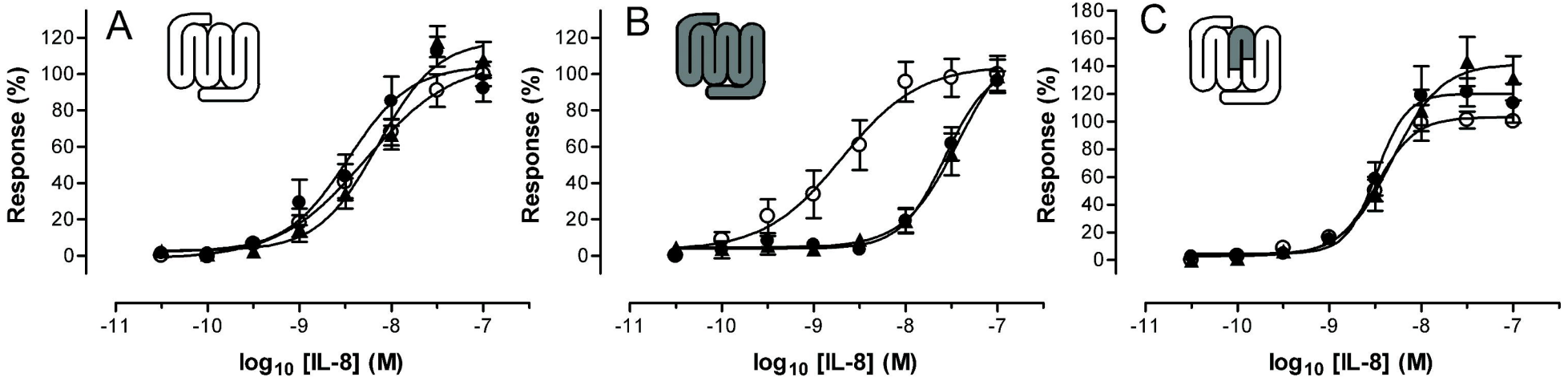
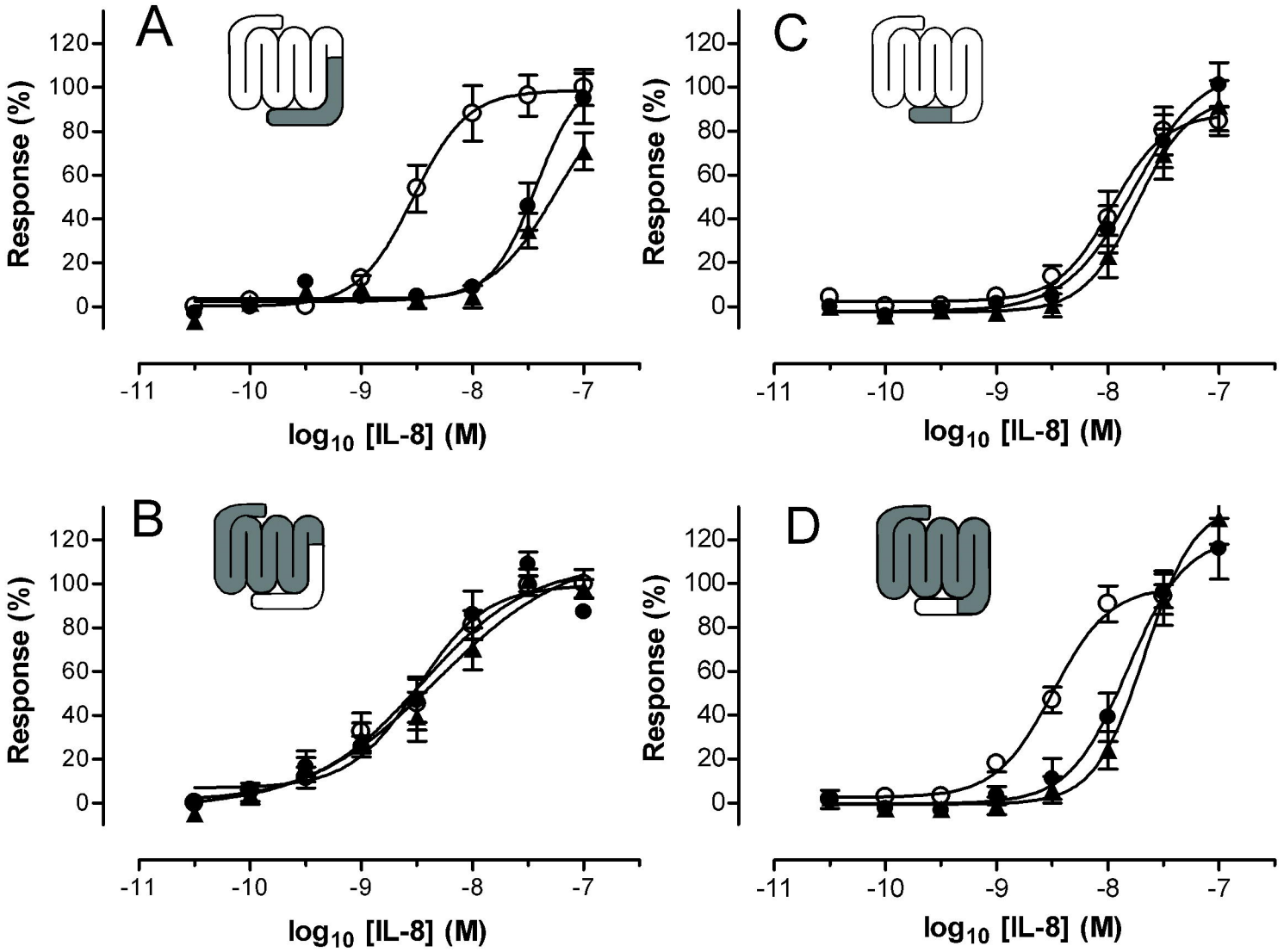
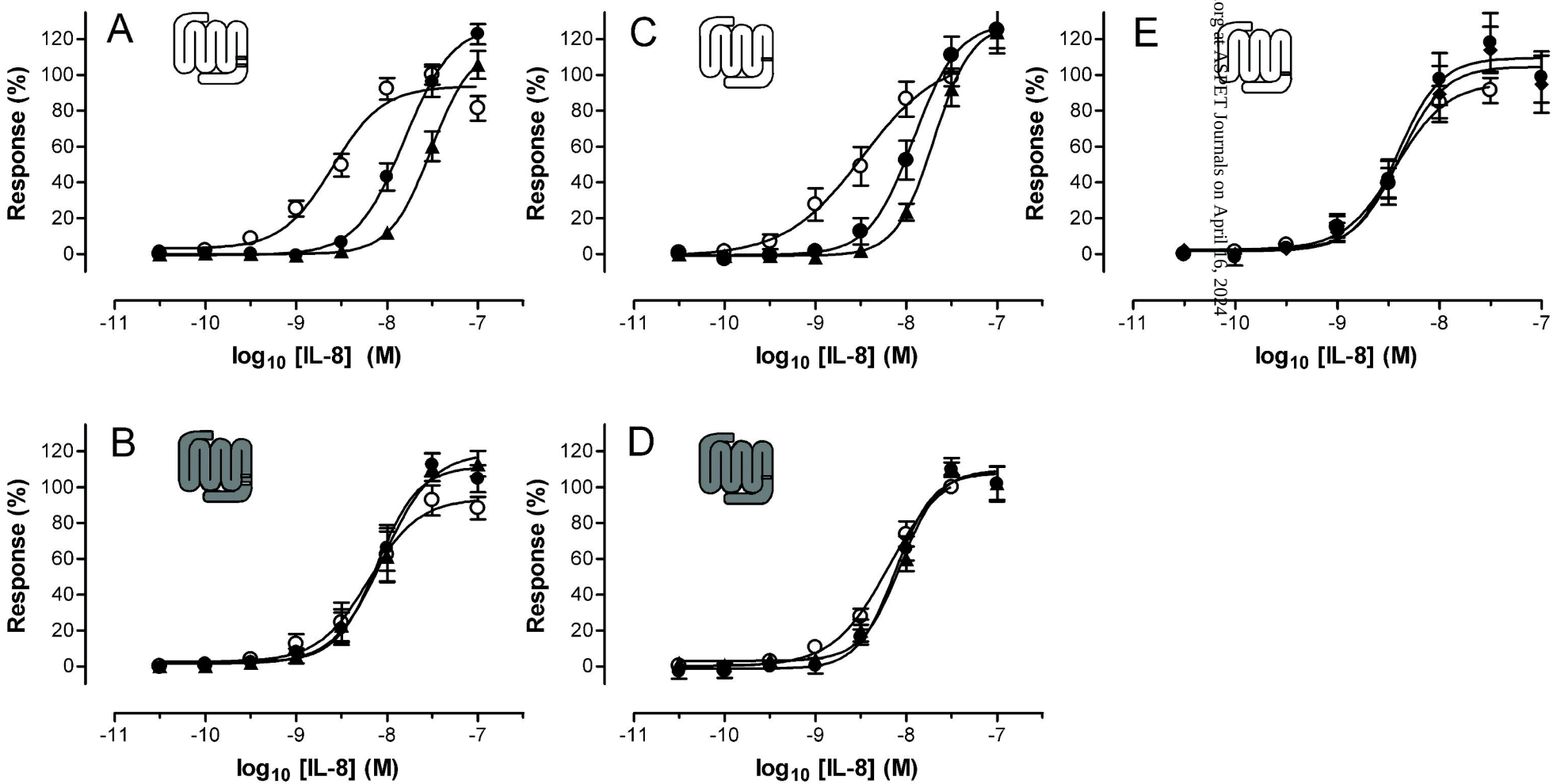

A
B
C
Response (%)
log10 [IL-8] (M)
-11
-10
-9
-8
-7
0
20
40
60
80
100
120
140
160
180

Figure 7



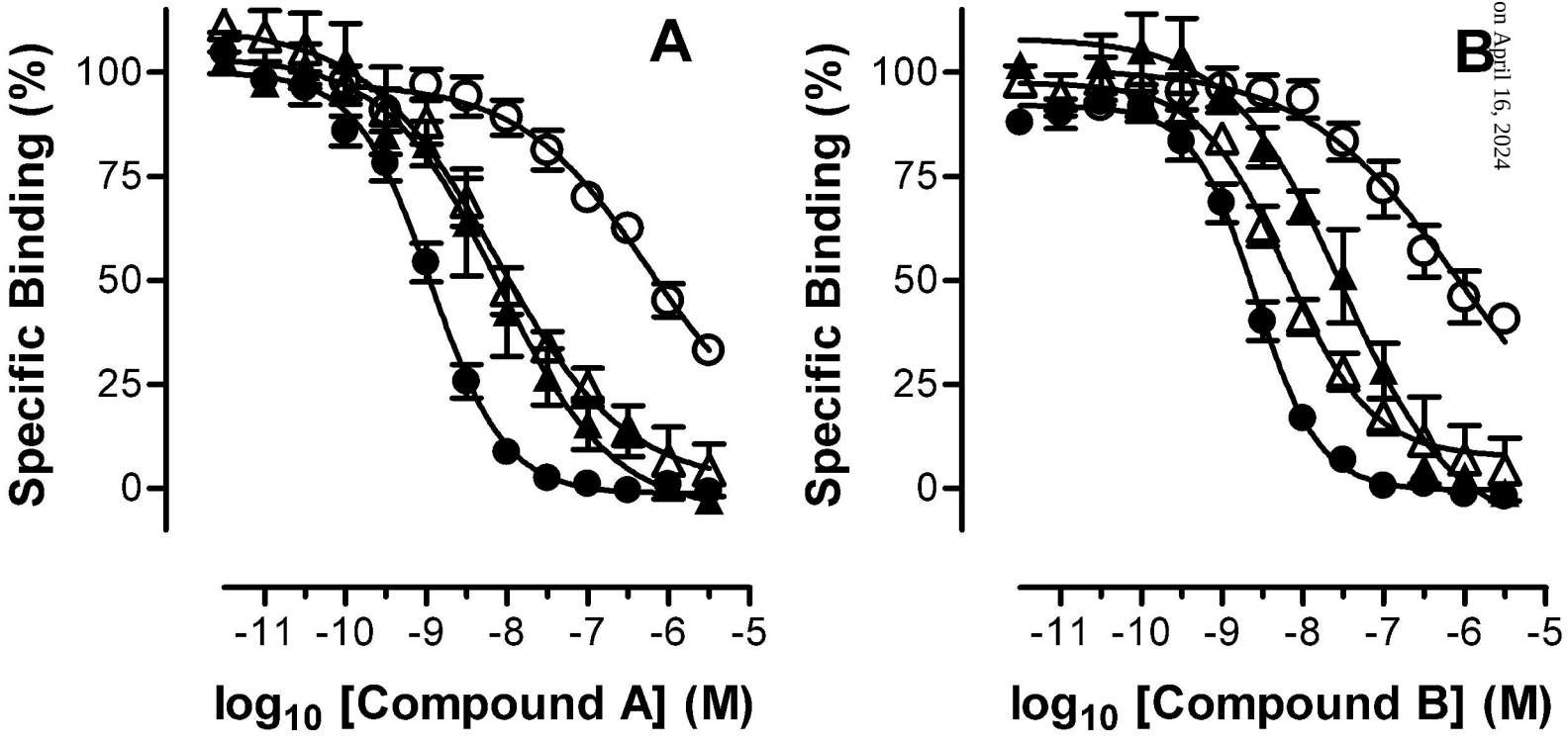
Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 8



molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 9



molpharm.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 10

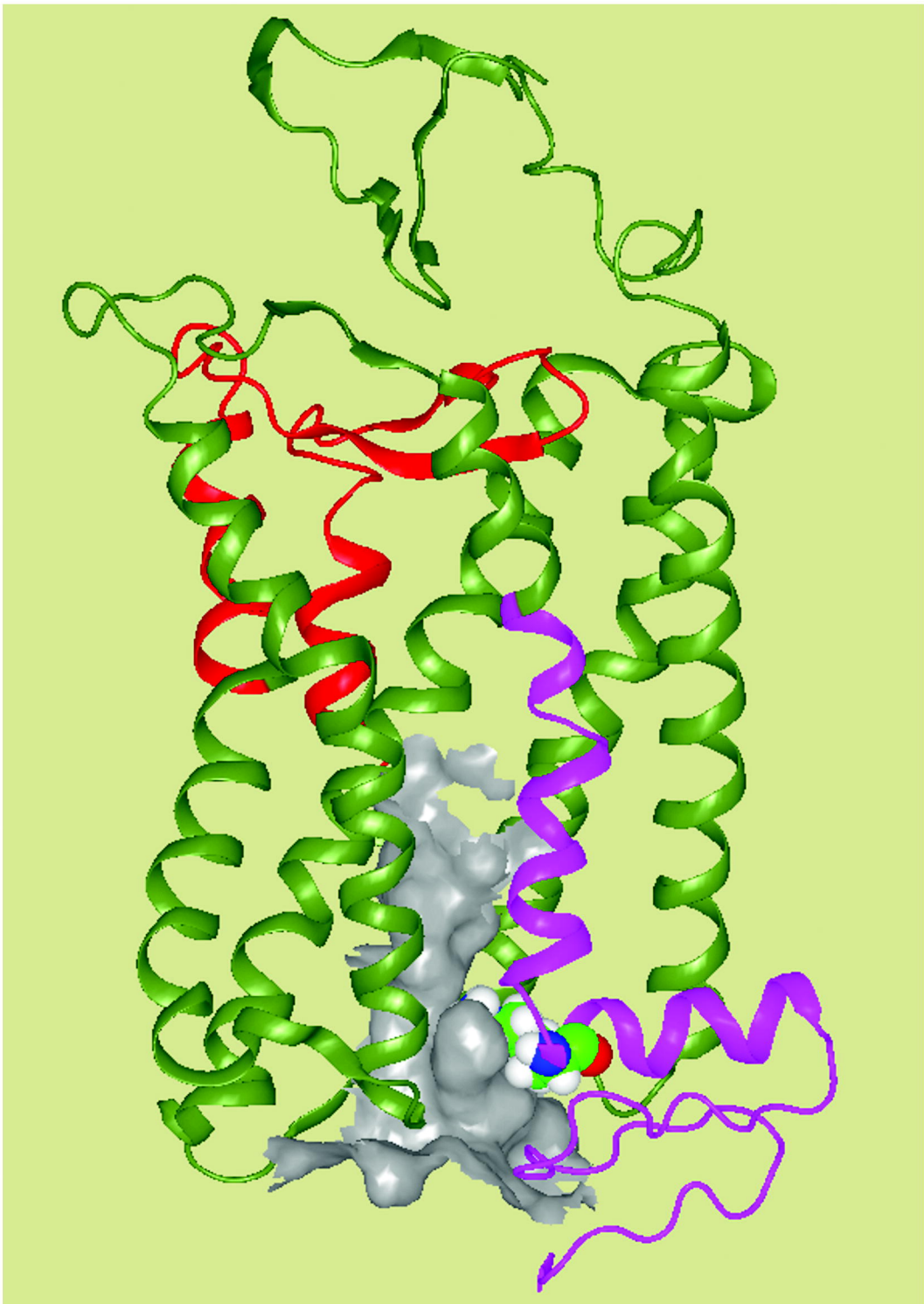
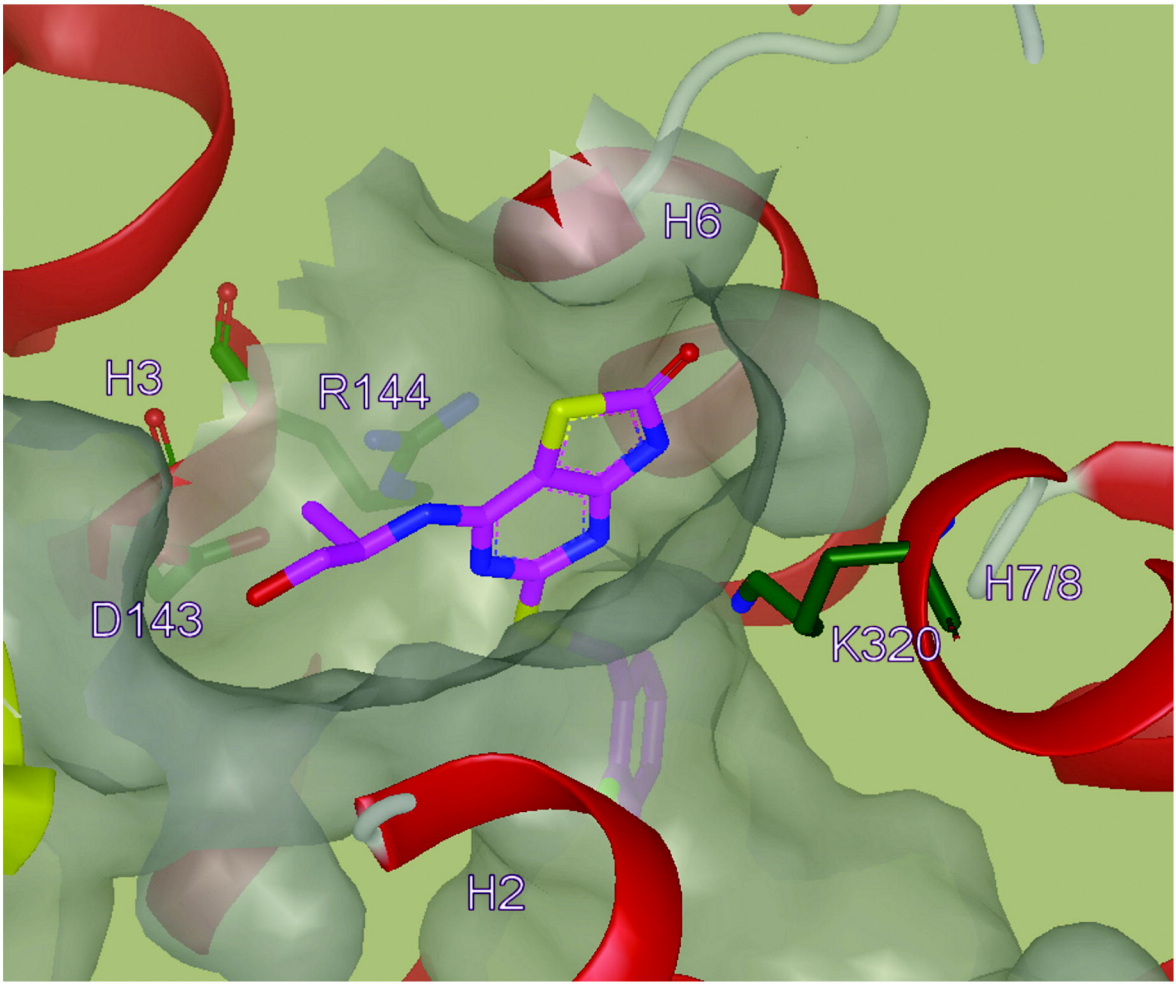



Figure 11