CXCR3 antagonist VUF10085 binds to an intrahelical site distinct from that of the broad spectrum antagonist TAK-779

Belinda Nedjai1†, Jonathan M Viney1†, Hubert Li2, Caroline Hull1, Caroline A Anderson1, Tomoki Horie1,3, Richard Horuk4, Nagarajan Vaidehi2 and James E Pease1

1Leukocyte Biology Section, NHLI Division, Faculty of Medicine, Imperial College, London, UK,
2Department of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA USA,
3Tokyo Medical and Dental University, Tokyo, Japan, and 4Department of Pharmacology, University of California, Davis, CA, USA

BACKGROUND AND PURPOSE
The chemokine receptor CXCR3 is implicated in a variety of clinically important diseases, notably rheumatoid arthritis and atherosclerosis. Consequently, antagonists of CXCR3 are of therapeutic interest. In this study, we set out to characterize binding sites of the specific low MW CXCR3 antagonist VUF10085 and the broad spectrum antagonist TAK-779 which blocks CXCR3 along with CCR2 and CCR5.

EXPERIMENTAL APPROACH
Molecular modelling of CXCR3, followed by virtual ligand docking, highlighted several CXCR3 residues likely to contact either antagonist, notably a conserved aspartate in helix 2 (Asp-112:63), which was postulated to interact with the quaternary nitrogen of TAK-779. Validation of modelling was carried out by site-directed mutagenesis of CXCR3, followed by assays of cell surface expression, ligand binding and receptor activation.

KEY RESULTS
Mutation of Asn-132:33, Phe-207 and Tyr-271:61 within CXCR3 severely impaired both ligand binding and chemotactic responses, suggesting that these residues are critical for maintenance of a functional CXCR3 conformation. Contrary to our hypothesis, mutation of Asp-112:63 had no observable effects on TAK-779 activity, but clearly decreased the antagonist potency of VUF 10085. Likewise, mutations of Phe-131:32, Ile-279:59 and Tyr-308:43 were well tolerated and were critical for the antagonist activity of VUF 10085 but not for that of TAK-779.

CONCLUSIONS AND IMPLICATIONS
This more detailed definition of a binding pocket within CXCR3 for low MW antagonists should facilitate the rational design of newer CXCR3 antagonists, with obvious clinical potential.

Abbreviations
ECL, extracellular loop; HA, haemagglutinin, TM transmembrane; WT, wild type

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Correspondence
James E Pease, Leukocyte Biology Section, MRC and Asthma UK Centre in Allergic Mechanisms, National Heart & Lung Institute, South Kensington Campus, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, UK. E-mail: j.pease@imperial.ac.uk

†These two authors contributed equally to this study.

Received
22 July 2013

Revised
16 November 2014

Accepted
20 November 2014
Introduction

The recruitment of leukocytes from the circulation to the tissues is coordinated to a large extent by chemokines, a family of around 40 proteins in humans (Zlotnik and Yoshie, 2012). Chemokines bind to specific chemokine receptors located on the leukocyte surface and drive chemotaxis, the directional migration of the cell along the chemokine concentration gradient. Ordinarily, this is a desirable process, populating tissues with leukocytes to provide protection against invading microorganisms. However, in several clinically important diseases, the inappropriate or excessive production of chemokines is associated with increased leukocyte recruitment and tissue damage. Consequently, the notion of blocking chemokine receptors with small molecule antagonists has gained momentum in the field of medicinal chemistry, with several candidate molecules being developed and entering clinical trials (see Pease and Horuk, 2012).

The CXC chemokine receptor CXCR3 is expressed on the surface of a variety of leukocytes, most notably T-cells and, like all other chemokine receptors, is a 7TM (7 transmembrane helix) receptor, binding the chemokines CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC) with affinities in the low nanomolar range (Cole et al., 1998). CXCL9, CXCL10 and CXCL11 are all induced by IFN-γ and therefore thought to promote Th1 immune responses (Luster and Ravetch, 1987; Farber, 1990; Cole et al., 1998), and play key roles in the inflammatory responses seen in rheumatoid arthritis (Ruschpler et al., 2003), atherosclerosis (Mach et al., 1999), psoriasis (Flier et al., 2001) and transplant rejection (Hancock et al., 2009). Accordingly, CXCR3 has attracted much attention as a therapeutic target for the treatment of these diseases, with several low MW antagonists of CXCR3 described by ourselves and others (Johnson et al., 2007; Hayes et al., 2008; Li et al., 2008; Verzijl et al., 2008; Liu et al., 2009; Chan et al., 2011; Wijtmans et al., 2011; Jenh et al., 2012). Although several studies of CXCR3 antagonists have shown their efficacy in in vivo models of disease, notably atherosclerosis (van Wunnik et al., 2008) and transplant rejection (Jenh et al., 2012), only one such antagonist has entered clinical trials in man, the compound AMG-487, originally identified by scientists at Chemocentryx and subsequently licensed to Amgen. Preclinical data showed AMG-487 to have excellent potency and efficacy in the inhibition of immune cell migration and efficacy in a bleomycin-induced model of lung inflammation in mice (Johnson et al., 2007). However, in phase II clinical trials for the treatment of psoriasis, AMG-487 failed to demonstrate efficacy leading to termination of the trial (Ribeiro and Horuk, 2005). This has led to the hypothesis that in certain inflammatory disorders where several chemokines are induced, it may be necessary to block more than one receptor to achieve efficacy (Pease and Horuk, 2010).

The low MW compound TAK-779 (Fig 1D) was originally developed by Takeda Pharmaceutical Company as a prototypic low MW antagonist of CCR5, and shown to be an inhibitor of HIV-1 entry in vitro (Baba et al., 1999), binding to an intrahelical site within the receptor (Dragic et al., 2000; Charo and Ransohoff, 2006; Hall et al., 2009). As CCR5 also shares 74% identity with the receptor CCR2, it is perhaps unsurprising that the molecule also has excellent potency and efficacy at the latter receptor and, via a programme of ab initio receptor modelling and site-directed mutagenesis, we have been able to compare the binding sites of this molecule in both CCR2 and CCR5 (Hall et al., 2009). What makes TAK-779 unique among current prototypic antagonists, is that it also has reasonable potency and efficacy at a relatively unrelated chemokine receptor of a different class, namely CXCR3 (Gao et al., 2003; Verzijl et al., 2008). Several in vivo studies have demonstrated the efficacy of TAK-779 in Th1 dominated diseases such as collagen-induced arthritis (Yang et al., 2002) colitis (Tokuyama et al., 2005), allograft rejection (Akashi et al., 2005) and ischaemia/reperfusion injury (Akahori et al., 2006), which is likely to be related to its broad spectrum activity in blocking CCR2, CCR5 and CXCR3 in both humans and rodents. More detailed knowledge of how a single compound interacts with three distinct chemokine receptors should facilitate the discovery of similar broad spectrum antagonists with therapeutic potential.

We describe here a programme of research in which ab initio modelling of CXCR3 coupled with site-directed mutagenesis and assays of receptor activation were used to characterize the binding sites of two known CXCR3 antagonists, the 3H-pyrido[2,3-d]pyrimidin-4-one derivative.
N-1R-[3-(4-ethoxy-phenyl)-4-oxo-3,4-dihydro-pyrido[2,3-d]pyrimidin-2-yl]-ethyl-N-pyridin-3-ylmethyl-2-(4-trifluoromethoxy-phenyl)-acetamide (VUF10085/AMG-487) and the broad spectrum quaternary ammonium anilide N,N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]-carbonyl]amino]benzyl] tetrahydro-2H-pyran-4-aminium chloride (TAK-779).

Methods

Generation of receptor mutants and their transient expression in the murine pre-B cell line L1.2
A previously described pcDNA3 plasmid containing human wild type (WT) CXCR3 cDNA with an HA epitope tag encoded at the N terminus (Meiser et al., 2008) was used as a template for the generation of point mutants by PCR using the QuikChange II site-directed mutagenesis kit (Stratagene, Amsterdam, the Netherlands). All constructs were verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany) before use. L1.2 cells were transiently transfected by electroporation with 1 μg of vector DNA/10^6 cells at 330V, 975 μF and incubated overnight in medium supplemented with 10 mM of sodium butyrate to enhance gene expression.

Flow cytometry
Cell surface expression of CXCR3 was assessed by flow cytometry after staining with an anti-HA antibody and FITC-conjugated secondary antibody as described previously (Vaidehi et al., 2009). Expression was analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View,
Chemotaxis assay
Assays of chemotactic responsiveness were carried out as previously described (Vaidehi et al., 2009) using 96-well ChemoTx® plates with 5 μm pores (Neuroprobe, Gaithersburg, MD, USA). Migrating cells were detected by the use of CellTiter Glo® Dye (Promega, Southampton, UK) and resulting luminescence measured using a TopCount scintillation counter (PerkinElmer, Waltham, MA, USA). Basal migration of cells to buffer alone was subtracted from the resulting data, with individual results expressed as a percentage of the total cells applied to the filter. In all experiments, each data point was assayed in duplicate. In every experiment, cells transiently expressing WT CXCR3 were used as a positive control.

Radiolabelled chemokine binding studies
Whole-cell binding assays on transiently transfected L1.2 cells were performed as described previously (Vaidehi et al., 2009) using 0.1 nM 125I-CXCL11 (Perkin Elmer) and increasing concentrations of unlabeled CXCL11 or antagonist. Cell-associated radioactivity was counted in a Canberra Packard Cobra 5010 gamma counter (Canberra Packard, Pange bourne, UK). Curve fitting and subsequent data analysis was carried out using the program PRISM (GraphPad Software, Inc., San Diego, CA, USA) and IC50 values were obtained by non-linear regression analysis. In all experiments, each data point was assayed in duplicate, with each individual experiment repeated three times. Background binding levels obtained in the presence of a 1000–3000 molar excess of unlabelled chemokine were subtracted from each data point and data are presented as the percentage of counts obtained in the absence of competing ligand. Kd values and the number of binding sites per cell were calculated from homologous binding curves prepared in Graph Pad Prism (La Jolla, CA, USA) as previously described (Nedjai et al., 2011).

Modelling the CXCR3 interaction with VUF 10085
The three-dimensional model of the seven helical TM bundle of human CXCR3 was predicted using the ab initio method MembStruk (Vaidehi et al., 2002; Hall et al., 2009). The extra and intracellular loops were added using the method, Modeller. VUF 10085 was built using the LigPrep module from the Schrodinger Glide suite (Schrodinger Inc.). Multiple ligand conformations were generated for the compound and docked using Glide XP (Schrodinger Inc., Portland, OR, USA). Subsequently, a short energy minimization was performed on each docked pose and the binding energy of this optimized pose was calculated. The binding energy was calculated as BE (binding energy) = PE (ligand in fixed protein) – PE (ligand in solution); where BE is the binding energy and PE is the potential energy. The compound poses were then sorted by binding energy and the top 20 conformations inspected visually to maximize the interactions with residues that are known to interact with ligands in chemokine receptors (Vaidehi et al., 2009). During the course of this work, the crystal structure of CXCR4 bound to a low MW antagonist was published (Wu et al., 2010). Therefore, we also generated a homology model of CXCR3 based on CXCR4 crystal structure as template (pdb ID:3ODU) using the program MODELLER (http://salilab.org/modeller/9v7/manual/node8.html). We selected the top 100 models from MODELLER and clustered these models by their root mean squared deviation in coordinates. The 100 models clustered into five clusters and the best energy structure from the cluster was chosen for docking. We then docked the VUF 10085 antagonist to this model using GOLD (http://www.ccdc.cam.ac.uk/SupportandResources/Support/pages/SupportSolution.aspx?supportsolutionid=110) flexible side chain docking to allow for protein flexibility. The side chains of the residues Y60, W109, D112, F131, F135, H202, Y271 and Y308 were treated as flexible using the built in rotamer library. A distance constraint was placed between D112 and the pyridine nitrogen of the VUF 10085 compound. The best docked pose was selected based on the experimental data in this paper. The final model was used in generating Figure 7A–C.

Data analysis
Data are expressed as the mean ± SEM of the number of experiments indicated in the Figure legends.

Materials
Reagents were purchased from Invitrogen (Paisley, UK), unless stated otherwise. Recombinant human CXCL10 and CXCL11 were purchased from PeproTech EC, Ltd. (London, UK). The monoclonal mouse anti-haemagglutinin (HA) anti-HA.11 antibody was from Covance (Berkeley, CA, USA) and its corresponding IgG1 isotype control antibody from Sigma-Aldrich (Poole, UK). The anti-CXCR3 mAb (Clone 49801) was from R&D Systems (Abingdon, UK). The murine pre-B cell line L1.2 was maintained as described previously (Vaidehi et al., 2009). TAK-779 was obtained from the Programme EVA Centre for AIDS Reagents, NIBSC, UK, supported by the EC FP6/7 Europrise Network of Excellence, AVIP and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and was donated by Dr R. Gallo, University of Maryland School of Medicine. The synthesis of VUF 10085 has previously been described (Flier et al., 2001; Storelli et al., 2007).

Nomenclature
Ballesteros–Weinstein numbering (Ballesteros and Weinstein, 1995) is used in superscript throughout the text to denote the positioning of residues within the TM helices. In this nomenclature, a single most conserved residue among the class A GPCRs is designated x.50, where x is the TM helix number; all other residues on that helix are numbered relative to this conserved position.

Results
We have previously described the potency and efficacy of the specific low MW CXCR3 antagonist VUF10085 (Figure 1A and the broad spectrum antagonist TAK-779 (Figure 1D) at human CXCR3, with VUF 10085 the more potent of the two compounds in a variety of assays (Verzijl et al., 2008). In this study, we set out to elucidate the binding site of both compounds within the CXCR3 structure. Ourselves and others
Asp-112:63. We hypothesized that this would be a likely position, we turned our attention to an aspartate in helix II, Asp-112:63, acts as an anchor point for VUF 10085 to inhibit CXCL11 binding (Figure 1C), with a 13-fold increase in the relative IC50 values (WT = 2.34 μM and D112N = 0.70 μM). Notably, the F207A and Y271A mutants were expressed at levels barely above those of the isotype control antibody suggesting that they did not traffic to the cell surface. However, when the ability of the same panel of transfectants to bind [125I]-CXCL11 was examined, an imperfect correlation between detection of CXCR3 at the cell surface by flow cytometry. Expression of the CXCR3 mutants was on the whole robust, with only mutation of Asp-112:63 to asparagine (D112N = 0.56 μM). In contrast, little difference in the IC50 values for TAK-779 inhibition was observed (WT = 1.56 μM and D112N = 1.34 μM). Similarly, mutation of D112 decreased the ability of VUF 10085 to inhibit CXCL11 binding (Figure 1C), with a 13-fold increase in the relative IC50 values (WT = 169 nM, D112N = 2.34 μM). In contrast, IC50 values for TAK-779 inhibition were very similar (WT = 15.6 μM and D112N = 17.2 μM, Figure 1F). Thus, we conclude that an acidic residue in TM helix II, Asp-112:63, acts as an anchor point for VUF 10085 but not for TAK-779.

We subsequently explored further the binding site of the two CXCR3 antagonists by computational modelling using ab initio derived structures of CXCR3 (Vaidehi et al., 2009) docked with either VUF 10085 or TAK-779. The preliminary models thus obtained suggested an additional 10 residues likely to interact with the antagonists (Table 1), which were concentrated in five TM helices and the second extracellular loop (ECL2). Plasmids were generated in which the codon encoding each of these residues was singularly mutated to one encoding alanine and following transient transfection of cells, the effects of mutation upon CXCR3 cell surface expression and ligand binding were examined. As in previous studies, a conformationally insensitive N-terminal HA epitope was introduced into the constructs to aid detection at the cell surface by flow cytometry. Expression of the CXCR3 mutants was on the whole robust, with only mutation of Tyr-60:1.39, Asn-132:6.51, Phe-207 and Tyr-271:6.51 decreasing detection of CXCR3 at the cell surface (Figure 2A). Notably, the F207A and Y271A mutants were expressed at levels barely above those of the isotype control antibody suggesting that they did not traffic to the cell surface. However, when the ability of the same panel of transfectants to bind [125I]-CXCL11 was examined, an imperfect correlation between detection of the HA epitope and CXCL11 binding was observed, with the Y60A and F207A mutants binding levels of 125I-CXCL11 not dissimilar to those binding to WT CXCR3. This suggests that the accessibility of the anti-HA antibody to the N-terminal HA epitope of our CXCR3 construct may be restricted by the native conformational of CXCR3. This postulate was supported when the relative abilities of the anti-HA antibody and an anti-CXCR3 mAb to recognize WT CXCR3 were directly compared, with an approximate 75% reduction in CXCR3 detection levels observed between the two antibodies Figure 2C and D).

We subsequently assessed the panel of CXCR3 mutants for their ability to bind and signal in response to CXCL11, using chemotaxis assays and competitive binding assays. WT CXCR3 behaved as previously reported (Xanthou et al., 2003) with a bell-shaped chemotaxis response reaching an optimum at a concentration of 30 nM CXCL11 (Figure 3A).

Table 1
Expression, chemotaxis and binding properties of CXCR3 mutants

<table>
<thead>
<tr>
<th>Construct</th>
<th>% of WT CXCR3 surface expression Mean ± SEM</th>
<th>Number of receptors per cell Mean ± SEM</th>
<th>Kd CXCL11 binding (nM) Mean ± SEM</th>
<th>% of WT chemotaxis to 30 nM CXCL11 Mean ± SEM</th>
<th>Number of experiments n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CXCR3</td>
<td>100 ± 1.6</td>
<td>51 843 ± 8678</td>
<td>2.52</td>
<td>100 ± 4.32</td>
<td>3</td>
</tr>
<tr>
<td>Y60A</td>
<td>49.8 ± 7.5</td>
<td>10 500 ± 3130</td>
<td>1.8</td>
<td>55.4 ± 16.4</td>
<td>3</td>
</tr>
<tr>
<td>F131A</td>
<td>77.6 ± 9.5</td>
<td>75 78 ± 1312</td>
<td>1.1</td>
<td>53.7 ± 5.6</td>
<td>3</td>
</tr>
<tr>
<td>N132A</td>
<td>18.4 ± 7.1</td>
<td>ND</td>
<td>ND</td>
<td>0.7 ± 0.5</td>
<td>3</td>
</tr>
<tr>
<td>H202A</td>
<td>95.3 ± 9.6</td>
<td>92 92 ± 5332</td>
<td>2.9</td>
<td>66.0 ± 34.8</td>
<td>3</td>
</tr>
<tr>
<td>Y205A</td>
<td>82.0 ± 13</td>
<td>13 124 ± 1398</td>
<td>10.4</td>
<td>35.5 ± 21.5</td>
<td>3</td>
</tr>
<tr>
<td>F207A</td>
<td>6.8 ± 2.3</td>
<td>ND</td>
<td>ND</td>
<td>0.8 ± 0.8</td>
<td>3</td>
</tr>
<tr>
<td>Y271A</td>
<td>6.8 ± 2.3</td>
<td>ND</td>
<td>ND</td>
<td>1.0 ± 0.6</td>
<td>3</td>
</tr>
<tr>
<td>V275A</td>
<td>66.3 ± 2.5</td>
<td>ND</td>
<td>ND</td>
<td>52.9 ± 8.2</td>
<td>3</td>
</tr>
<tr>
<td>I279A</td>
<td>70.2 ± 11.6</td>
<td>ND</td>
<td>ND</td>
<td>51.3 ± 6.2</td>
<td>3</td>
</tr>
</tbody>
</table>

Surface expression and chemotaxis are expressed as the percentage of the mean of the values obtained for transfectants expressing WT CXCR3. n, numbers of experiments. ND indicates not determined.
Of the panel of CXCR3 constructs assessed, three mutations were seen to severely affect the chemotactic response, namely the N132A3.33, F207A and Y271A6.51 constructs (Table 1). The remaining constructs all exhibited a bell-shaped response of similar potency, albeit with reduced efficacy. In competitive binding assays, WT CXCR3 bound CXCL11 with a $K_D$ of 7.7 nM (Figure 3B, Table 1) with several mutations appearing to decrease the apparent $K_D$. This was explained when non-linear regression analysis of the CXCL11 data was carried out to determine the number of receptors per cell. Decreases in the apparent $K_D$ of mutant receptors were found to correlate with reduced receptor expression (Table 1). In keeping with their lack of chemotactic activity, the maximum concentration of CXCL11 was unable to displace 50% of the $^{125}$I-CXCL11 at the N132A3.33, F207A and Y271A6.51 mutants (Figure 3B), suggesting that Asn-1323.33, Phe-207 and Tyr-2716.51 are required for the integrity of a functional CXCR3 conformation.

The functional CXCR3 constructs were subsequently assessed for their ability to be antagonized by either VUF 10085 or TAK-779 in chemotaxis assays, using the optimal 30 nM concentration of CXCL11 to drive cell migration (Figure 4A and B). In these assays, a construct showing a loss of sensitivity to either compound is interpreted as highlighting a CXCR3 residue contacting the antagonist. In the assessment of VUF 10085, three mutant constructs decreased the ability of VUF 10085 to inhibit chemotactic responses to CXCL11 (Figure 4A, Table 2). Notably, the Tyr-308A7.43 and Phe-131A3.32 mutations rendered VUF 10085 impotent, with calculation of an IC$_{50}$ value impossible. Similarly, mutation of...
Ile-279 resulted in a threefold increase in the IC50 value for VUF 10085. Mutation of Tyr-601 and His-202 increased the IC50 values for VUF 10085, but to a lesser degree. In contrast, the ability of TAK-779 to inhibit chemotaxis was hardly changed by mutation of the same five CXCR3 residues (Figure 4B, Table 2), with the H202A and Y308A mutations apparently increasing the IC50 values twofold when compared with WT CXCR3.

The same panel of CXCR3 mutants was then assessed in ligand binding assays, examining the ability of the antagonists to displace CXCL11 from the receptor (Figure 5A and B). In the assessment of VUF 10085, mutation of Ile-279 decreased the potency of VUF 10085 twofold while mutation of Y308 decreased the efficacy to such an extent that no IC50 value could be calculated (Table 2). In keeping with the chemotaxis data (Figure 4B), none of the five mutations examined appeared to impair the potency or efficacy of TAK-779 in binding assays (Figure 5B, Table 2), with two mutations, Y60A and Y308A resulting in an apparent twofold reduction in the IC50 values relative to WT CXCR3.

Taken together with our findings from the chemotaxis assays, we conclude that while VUF 10085 binds within an intrahelical site containing residues Phe-131, Ile-279, and Tyr-308 of CXCR3, the broad spectrum antagonist TAK-779 binds to a distinct site, which does not involve these residues.

We have previously used an alternative panel of six ‘loss of charge’ CXCR3 mutants to determine the counterion for a negatively charged small molecule CXCR3 agonist (Nedjai et al., 2011). Although these acidic residues lay outside our initially molecular modelling of the TAK-779:CXCR3 interaction, we tested the hypothesis that one or more of these acidic residues may contribute to TAK-779 binding. As we previously found, all six mutants were well expressed by L1.2 cells and responded robustly in chemotaxis assays to a fixed concentration of 30 nM CXCL11 (data not shown). Contrary to our hypothesis, the ability of TAK-779 to inhibit this migration was not reduced, suggesting that the TAK-779 binding site within CXCR3 does not involve these residues. (Figure 6).

Discussion and conclusions

We describe here details of the binding site within CXCR3 for the specific antagonist VUF 10085. Preliminary ab initio modelling suggested a series of CXCR3 side chains within the TM...
helices and ECL2, which were likely to interact with VUF 10085 and were necessary for its inhibitory activity. Mutation of these residues coupled with assays of receptor function was used to validate and refine the model. Mutation to alanine of the residues Asn-132, Phe-207 (ECL2) and Tyr-271 were noted for their deleterious effects upon CXCR3 expression and function, suggesting a role for these side chains in maintaining the correct conformation of the apo-protein. Hence, the possible contribution of these residues to antagonist binding could not be validated experimentally.

In keeping with studies of small molecule antagonists of other chemokine receptors, VUF 10085 binds in the minor pocket of CXCR3 as deduced by molecular modelling and experimental validation (Figure 7A-C). Asp-112 is postulated to form a close interaction with the nitrogen of the pyridine ring of VUF 10085 (Figure 7D). An earlier structure–activity relationship (SAR) study by Johnson and colleagues identified the pyridyl group of VUF 10085 as being important for activity in 125I-ligand displacement studies (Johnson et al., 2007). Tyr-308 is predicted to form a close interaction with the nitrogen atom of the quinazolinone ring of the compound. In the SAR study by Johnson and colleagues, this nitrogen atom was present in all of their derivatives and

### Table 2

<table>
<thead>
<tr>
<th>Construct</th>
<th>IC50 chemotaxis (mean ± SEM; μM)</th>
<th>IC50 ligand binding (mean ± SEM; μM)</th>
<th>IC50 chemotaxis (mean ± SEM; μM)</th>
<th>IC50 ligand binding (mean ± SEM; μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CXCR3</td>
<td>0.198 ± 0.035</td>
<td>0.241 ± 0.027</td>
<td>15.8 ± 3.10</td>
<td>31.3 ± 6.25</td>
</tr>
<tr>
<td>Y60A&lt;sup&gt;1.39&lt;/sup&gt;</td>
<td>0.292 ± 0.013</td>
<td>0.236 ± 0.054</td>
<td>10.2 ± 4.62</td>
<td>14.0 ± 7.07</td>
</tr>
<tr>
<td>F131A&lt;sup&gt;3.32&lt;/sup&gt;</td>
<td>ND</td>
<td>0.161 ± 0.040</td>
<td>15.0 ± 6.98</td>
<td>23.3 ± 6.57</td>
</tr>
<tr>
<td>H202A (ECL2)</td>
<td>0.373 ± 0.014</td>
<td>0.299 ± 0.120</td>
<td>32.7 ± 14.0</td>
<td>25.3 ± 10.2</td>
</tr>
<tr>
<td>I279A&lt;sup&gt;6.59&lt;/sup&gt;</td>
<td>0.676 ± 0.028</td>
<td>0.483 ± 0.210</td>
<td>10.5 ± 3.35</td>
<td>39.4 ± 15.0</td>
</tr>
<tr>
<td>Y308A&lt;sup&gt;7.43&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>41.4 ± 22.6</td>
<td>14.2 ± 3.0</td>
</tr>
</tbody>
</table>

Data are derived from three separate experiments. ND indicates not determined.
appeared to be important for activity at CXCR3 (Johnson et al., 2007). The aromatic sidechain of Phe-131 is believed to stabilize the pyridine ring of VUF 10085 via hydrophobic π–π stacking interactions. Our findings are in general agreement with a recent study published by Scholten and colleagues, which found that mutation of Asp-1122.63, F131A3.32 and Tyr-3087.43 impaired the ability of the structurally related compound NBI-74330 to displace CXCL11 binding to CXCR3 (Scholten et al., 2013). The overall interaction of CXCR3 with VUF 10085 is not dissimilar to that of another CXC receptor with an antagonist, as revealed by the crystal structure of the low MW antagonist IT1t bound to CXCR4 (Wu et al., 2010). The overall interaction of CXCR3 with VUF 10085 is not dissimilar to that of another CXC receptor with an antagonist, as revealed by the crystal structure of the low MW antagonist IT1t bound to CXCR4 (Wu et al., 2010). In this structure, the analogous residues of CXCR4, Asp-972.63 and Tyr-1163.32 form a salt bridge and hydrophobic contact respectively with IT1t. We postulate that Phe-1353.36 of CXCR3 contributes to the binding by forming π–π stacking interactions with VUF 10085, although this was untested by our mutagenesis study. Although a potent CXCR3 antagonist in vitro, the failure of VUF 10085/AMG-487 to achieve efficacy in the clinic was attributed in part to the production of a metabolite with inhibitory properties at cytochrome P-450 (CYP)-3A, and subsequent non-linear pharmacokinetics following multiple administrations (Henne et al., 2012). Occupation of the same minor binding pocket of CXCR3 with a compound with more favourable pharmacokinetics is perhaps worthy of further investigation.

In contrast to our study of VUF 10085, mutation of the panel of intrahelical and ECL2 mutations that form the binding pocket of VUF 10085 had no discernable effect on the ability of TAK-779 to interact with CXCR3, suggesting that its binding site was distinct from that of VUF 10085. This is surprising, as we have previously mapped the binding site...
of TAK-779 to the minor pockets of both CCR2 and CCR5, where the conserved tyrosine residues at position 1.39 and 3.32 were critical for antagonist function. However, in this study, when the analogous Tyr-60\textsuperscript{1.39} and Phe-131\textsuperscript{3.32} of CXCR3 were mutated, no impairment of TAK-779 function was observed. This would appear to be against the trend of results from known CC chemokine receptor antagonists, as we previously found that tyrosine residues in the analogous 1.39 and 3.32 positions of the receptors CCR1 and CCR3 were critical for the function of the bi-specific antagonist UCB 35625 (de Mendonça et al., 2005; Wise et al., 2007). Thus we have concluded that TAK-779 binds outside the minor pocket of CXCR3.

It should be noted that the possibility for a type II error in our work exists, namely that due to insufficient experimentation, we have failed to show that some of the residues in CXCR3 do form a TAK-779 binding site. For example, these could include the residues N132\textsuperscript{3.32}, Y205 and F207 (ECL2) and Y271\textsuperscript{4.30}, which were devoid of signalling and therefore could not be subjected to the loss of sensitivity tests to TAK-779 that other mutants were subjected to. The use of additional assays of CXCR3 activation may have been informative in this instance, such as GTP\textgamma-S binding assays (Smit et al., 2003). It is also possible that some of the other residues such as Y308\textsuperscript{4.41}, which showed a trend towards decreased sensitivity to TAK-779 in chemotaxis assays (Figure 4B, Table 2), may make significant contributions to the TAK-779 binding pocket, with additional experimentation. However, the very modest effects that we observed from the three experiments suggest that any contribution of these residues to a binding site is minor at most, because in our experience, mutation of key ligand contact points results in a dramatic loss of activity (de Mendonça et al., 2005; Vaidehi et al., 2006; Wise et al., 2007; Hall et al., 2009; Nedjai et al., 2011). Moreover, we expected TAK-779 to be more sensitive to the effects of mutation within its binding site, as its potency is two orders of magnitude lower than that of VUF 10085 at WT CXCR3 (Figure 1 and Table 1). Based on our previous experiences, we therefore concluded that further assessment of this panel of residues was unlikely to be fruitful in the search for a TAK-779 binding site and was not a good use of resources.

We postulate that the quaternary nitrogen of TAK-779 is critical for the interaction of the compound with CXCR3, because the derived compound TAK-652, which has increased potency and oral bioavailability (Seto et al., 2006) is devoid of activity at CXCR3 (data not shown). Possible counterions for the quaternary nitrogen of TAK-779 exist in the TM domains, and Y271\textsuperscript{4.30}, which were devoid of signalling and therefore concluded that further assessment of this panel of CXCR3.

In summary, we describe the intrahelical binding site of a highly potent, specific CXCR3 antagonist. Knowledge of this binding site may pave the way for the rational design of novel antagonists of this clinically important chemokine receptor.

Acknowledgements

This work was supported by an Arthritis Research UK Project Grant (#18303) to J. E. P. We are grateful to Martine Smit, Rob Leurs, Chris de Graaf and Danny Scholten (Vrie University Amsterdam) for the supply of VUF 10085 and for their helpful discussions.

Conflict of interest

J. E. P. holds a consultancy with Boehringer Ingelheim.

References


et al.

time-dependent inhibition of the enzyme. Drug Metab Dispos 40:

metabolites that covalently modify CYP3A4 Cys239 and cause

novel CXCR3 antagonist, results in formation of quinone reactive

et al.

Subramanian R

Henne KR, Tran TB, VandenBrink BM, Rock DA, Aidasani DK,


antagonists of the chemokine receptor CXCR3. Bioorg Med Chem


et al.

1084–1097.

Optimization of a series of quinazolinone-derived antagonists of


Discovery and optimization of a series of quinazolinone-derived


Optimization of the heterocyclic core of the quinazolinone-derived


Optimization of a series of quinazolinone-derived antagonists of


Luster AD, Ravetch JV (1987). Biochemical characterization of a

gamma interferon-inducible cytokine (IP-10). J Exp Med 166:

1084–1097.


CXC chemokines by human atheroma-associated cells. J Clin Invest

104: 1041–1050.


