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Themed Section: Molecular Pharmacology of GPCRs

REVIEW

Pharmacological modulation of chemokine receptor function

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G protein-coupled chemokine receptors and their peptidergic ligands are interesting therapeutic targets due to their involvement in various immune-related diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, chronic obstructive pulmonary disease, HIV-1 infection and cancer. To tackle these diseases, a lot of effort has been focused on discovery and development of small-molecule chemokine receptor antagonists. This has been rewarded by the market approval of two novel chemokine receptor inhibitors, AMD3100 (CXCR4) and Maraviroc (CCR5) for stem cell mobilization and treatment of HIV-1 infection respectively. The recent GPCR crystal structures together with mutagenesis and pharmacological studies have aided in understanding how small-molecule ligands interact with chemokine receptors. Many of these ligands display behaviour deviating from simple competition and do not interact with the chemokine binding site, providing evidence for an allosteric mode of action. This review aims to give an overview of the evidence supporting modulation of this intriguing receptor family by a range of ligands, including small molecules, peptides and antibodies. Moreover, the computer-assisted modelling of chemokine receptor–ligand interactions is discussed in view of GPCR crystal structures. Finally, the implications of concepts such as functional selectivity and chemokine receptor dimerization are considered.

LINKED ARTICLES

This article is part of a themed section on the Molecular Pharmacology of G Protein-Coupled Receptors (GPCRs). To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-6. To view the 2010 themed section on the same topic visit http://onlinelibrary.wiley.com/doi/10.1111/bph.2010.159.issue-5/issuetoc

Abbreviations

7TM, seven-transmembrane; AA2AR, adenosine A_{2A} receptor; ADCC, antibody-dependent cellular cytotoxicity; ADRB, β-adrenoceptor; ATLL, adult T-cells leukaemia-lymphoma; bRho, bovine rhodopsin; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CRS, chemokine recognition site; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; DRD3, dopamine D_3 receptor; EL, extracellular loop; FRAP, fluorescence recovery after photobleaching; GRK, GPCR kinase; mAb, monoclonal antibody; PCA, protein complementation assay; RET, resonance energy transfer; TIRF, total internal reflection fluorescence; TM, transmembrane; TMS, transmembrane site; VHH, variable domain of heavy-chain antibody

Chemokines and their receptors

Chemokines and their receptors are key players in the immune defence by directing and controlling the migration, activation, differentiation and survival of the billion of leukocytes in our body (Viola and Luster, 2008). Some chemokine peptides are constitutively secreted in lymphoid tissues and involved in leukocyte homing during immune surveillance. The vast majority of chemokines, however, are secreted in response to inflammatory mediators or trauma, and function as paracrine chemoattractants to recruit leukocytes to sites of inflammation. To date, at least 45 chemokine subtypes have been identified in human, which are categorized into four classes (i.e. C, CC, CXC, CX_3C) on the basis of the number and spacing of conserved cysteine residues in their N-termini (Figure 1) (Alexander *et al.*, 2011). All chemokines share a similar tertiary protein fold that is stabilized by disulfide bonds between the four conserved cysteine residues (or two in the case of C-chemokines). The flexible N-terminus is followed by the C, CC, CXC or CX3C motif, and connected via an exposed N-loop to a highly structured core domain, which consists of a single-turn 3_{10} helix, three antiparallel β-strands and a C-terminal α-helix (Figure 2). Soluble chemokines bind via their C-terminal α-helix to glycosaminoglycans on the surface of endothelial cells to form an immobilized chemotactic gradient, which guides passing immune cells towards the source of chemokine secretion (Proudfoot *et al.*, 2003; Lau *et al.*, 2004). On the other hand, CXCL16 and CX3CL1 are initially expressed as membrane-bound chemokines to serve as adhesion molecules for cells that express CXCR6 or CX3CR1 respectively, but can be cleaved by ADAM enzymes to become soluble chemokines (Schulte *et al.*, 2007). Chemokines can form dimers as well as oligomers, which is essential for their *in vivo* but not their *in vitro* activity (Laurence *et al.*, 2000; Proudfoot *et al.*, 2003; Jin *et al.*, 2007).

Chemokine receptors are seven-transmembrane (7TM) receptors belonging to the superfamily of GPCRs (see below). The majority of chemokine receptors can bind a panel of chemokines, whereas some are highly specific (Figure 1). Chemokine receptors have been classified according to which chemokine subclass they bind, with one C, ten CC, seven CXC and one CX3C chemokine-binding receptors (Figure 1) (nomenclature follows Alexander *et al.*, 2011). With the exception of CXCR7, which is exclusively biased towards β-arrestin-mediated signalling (Rajagopal *et al.*, 2010), all C, CC, CXC and CX3C chemokine receptors signal at least through heterotrimeric G proteins (O'Hayre *et al.*, 2008). Three non-G protein-signalling (so-called decoy) chemokine receptors (i.e. D6, DARC and CCX CKR) are thought to be primarily involved in scavenging a (wide) variety of inflammatory chemokines from the extracellular microenvironment, thereby limiting the recruitment of leukocytes (Mantovani *et al.*, 2006). Differential expression of chemokine receptors on selective leukocyte (sub)populations allows these cells to sense and respond to local gradients of corresponding chemokines.

Inappropriate or prolonged expression of chemokines and/or chemokine receptors results in an excessive infiltration of (certain) leukocytes into (inflamed) source tissue or

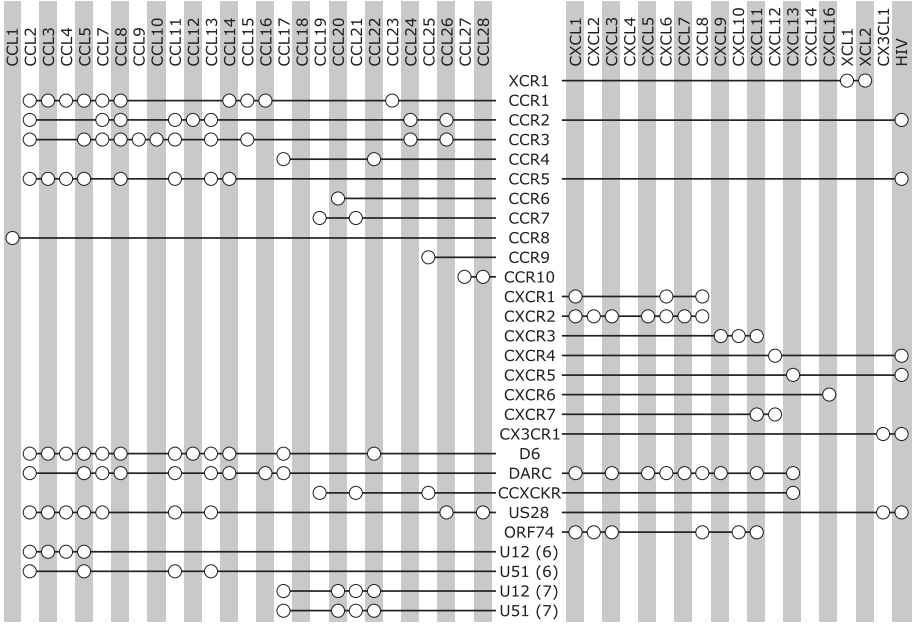


Figure 1

Overview of chemokine receptors (vertical) and their ligands (horizontal). An open circle indicates the specific ligand binds to the receptor connected to it by a black line. From top to bottom: human chemokine receptors (XCR1 to CX3CR1), chemokine decoy receptors (D6 to CCX CKR) and viral chemokine receptors (US28 to U51). For reasons of clarity, this figure does not show differences in affinity or activity between ligands for the same receptor. For more detailed information on ligand properties, visit the website of The International Union of Basic and Clinical Pharmacology (IUPHAR) (Murphy *et al.*, 2011).

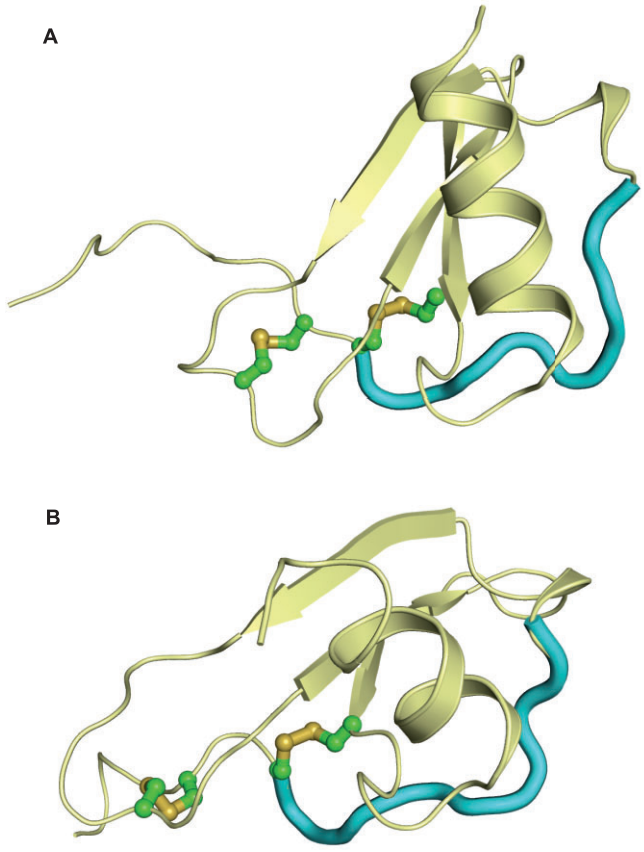


Figure 2

Overall tertiary structure of chemokines. (A) Structure of a CC-chemokine (CCL5). (B) Structure of a CXC-family chemokine (CXCL12). The disulfide bonds are indicated in green and yellow. The N-loop is shown in cyan. See text for more details.

confers chemokine sensitivity to cells that are normally not responsive to chemokines, respectively, resulting in chronic inflammation, autoimmune diseases, tumour growth, survival and metastasis (Figure 3) (Balkwill, 2004; O'Hayre *et al.*, 2008; Kraneveld *et al.*, 2011). In addition, the CCR5 and CXCR4 function as co-receptors for HIV entry into $CD4^+$ cells (Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996).

Many humans are latently infected by one or more herpesvirus species. Many of these herpesviruses encode GPCRs that are constitutively active but also responsive to human chemokines. These virally encoded chemokine receptors are thought to contribute to immune evasion and viral dissemination, but some of them are also involved in the development and/or progression of herpesvirus-associated inflammatory diseases and cancer (Figures 1 and 3) (Mausang *et al.*, 2009; Bongers *et al.*, 2010; Slinger *et al.*, 2010).

Considering the key role of (viral) chemokine receptors in the pathogenesis of autoimmune and inflammatory diseases, cancer and HIV infection, it is not surprising that these receptors gained increasing attention the last decade by both academia and pharmaceutical industry in their quest to develop drugs to treat such diseases.

Redundancy or (functional) selectivity?

The chemokine system is often 'accused' of showing significant redundancy, due to the fact that a single receptor binds multiple ligands, and conversely, a single ligand can bind several chemokine receptors (Figure 1) (Allen *et al.*, 2007). However, differential spatio-temporal expression patterns for different chemokines and receptors in our body indicate that they probably have distinct roles *in vivo* (Mantovani, 1999) (Figure 3). Furthermore, heteromerization of chemokine receptors may enable selective fine-tuning of chemokine receptor signalling (see section on 'cross-modulation within chemokine receptor oligomers'). Moreover, activation of a single receptor by different agonists might lead to differential signalling or *functional selectivity*, as observed now for different chemokine receptors, including CCR1, CCR2, CCR5, CCR7 and CXCR3 (Oppermann *et al.*, 1999; Kohout *et al.*, 2004; Tian *et al.*, 2004; Kouroumalis *et al.*, 2005; Leach *et al.*, 2007; Berchiche *et al.*, 2010). For example, both CCR7 chemokines CCL19 and CCL21 promote [^{35}S]-GTPγS binding and calcium signalling with similar potencies. However, only CCL19 induced efficient phosphorylation, β-arrestin recruitment and receptor internalization (Kohout *et al.*, 2004). A more recent study showed that CCR7 engages different GPCR kinases (GRKs) in response to CCL19 or CCL21, explaining the observed differences in receptor regulation (Zidar *et al.*, 2009). As a consequence, the signalling outcome does not depend on the receptor and ligand alone, but also on the expression levels of different signalling proteins in a specific cell, giving texture to the responses mediated by ligands. Along these lines, growing evidence supports differential binding of chemokines to a single receptor, and a single chemokine to different receptors, suggesting that these ligands have distinct roles (Ahuja *et al.*, 1996; Pakianathan *et al.*, 1997; Cox *et al.*, 2001; Blanpain *et al.*, 2003; Casarosa *et al.*, 2005; Jensen *et al.*, 2008). Overall, the evidence seems to refute the notion of redundancy in the chemokine receptor system. It rather indicates that the interplay between the timing and location of expression of ligands/receptors in the body, in combination with functional selectivity, is a mechanism for selectivity within the chemokine receptor family.

The two-step model of chemokine-receptor activation

The binding interactions of endogenous ligands in some class A GPCRs, such as the aminergic receptors, are relatively well known, especially with the recent successful crystallization of the β_1- and β_2-adrenoceptor (ADRB1/2), adenosine A_{2A}, and the dopamine D_3 receptor (DRD3) (Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008; Chien *et al.*, 2010). In contrast, binding modes of peptide ligands, such as chemokines, are less well characterized, due to their relatively large size and associated challenges in obtaining structural information. However, several studies have highlighted important regions in both chemokines and receptors that are involved in binding and function. The interaction of chemokines with their receptors is generally considered to be a two-step process (Allen *et al.*,

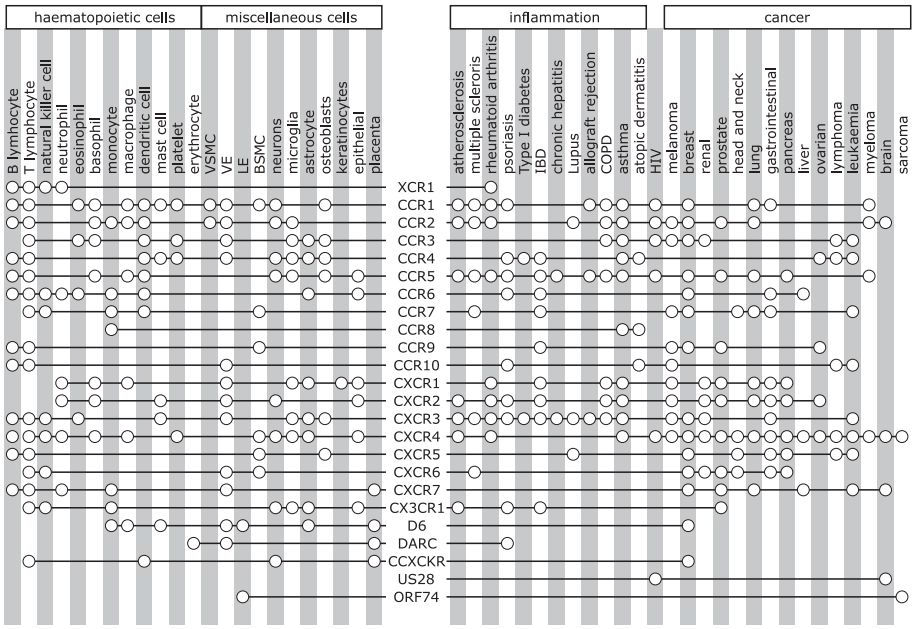


Figure 3

Chemokine receptors, their cellular expression profile and association with disease. The cellular expression profiles of the chemokine receptors are shown by open circles on the left-hand side. The cells are divided in haematopoietic cells and miscellaneous cells. Meaning of abbreviations: VSMC, vascular smooth muscle cells; VE, vascular endothelial cells; LE, lymphatic endothelial cells; BSMC, bronchial smooth muscle cells. The association of a particular receptor with disease is shown on the right-hand side of the figure. The diseases are categorized into inflammatory diseases and cancer. Abbreviations: IBD, inflammatory bowel disease; COPD, chronic obstructive pulmonary disease.

2007). First, the chemokine binds with its core region, including the N-loop (binding domain), to the N-terminus and extracellular loops (ELs) of the receptor (Figure 4B). We propose to use the term *chemokine recognition site 1* (CRS1), instead of *site I* often used in the literature, to avoid confusion with binding sites in the transmembrane (TM) pockets for small molecules. The binding to CRS1 is dominated by ionic interactions between positively charged residues in the chemokine and negatively charged amino acids at the N-terminus and extracellular surface of the receptor, including sulfonated tyrosines (Fernandez and Lolis, 2002; Colvin *et al.*, 2006; Allen *et al.*, 2007; Veldkamp *et al.*, 2008). In the second step, the flexible N-terminus (triggering domain) of the chemokine is positioned in such a way that it interacts with a second site (CRS2), formed by parts of the ELs and/or TM domains, resulting in receptor activation (Figure 4C) (Monteclaro and Charo, 1996; Wu *et al.*, 1996; Pease *et al.*, 1998; Gupta *et al.*, 2001; Blanpain *et al.*, 2003; Xanthou *et al.*, 2003; Ott *et al.*, 2004; Rajagopalan and Rajarathnam, 2004). This is supported by truncations or mutations in the N-termini of chemokines, generally leading to a loss in agonist activity, while often retaining high receptor binding affinity (Gong and Clark-Lewis, 1995; Proudfoot *et al.*, 1996; Clark-Lewis *et al.*, 2003; Richter *et al.*, 2009). In the case of CCR5, several reports indicate that a TXP motif in TM2 and surrounding aromatic residues in TM2 and 3 are involved in chemokine-mediated activation of CCR5, but not in high-affinity binding, suggesting that in step two the N-terminus interacts with residues in this TM region (Govaerts *et al.*, 2001; 2003; Blanpain *et al.*, 2003). As this motif is conserved among chemokine receptors, it is hypothesized that the TM2–TM3 interface in these receptors takes part in a common mechanism of ligand-induced conformational rearrangements leading to movements of helices, notably TM2 and TM3, and thereby chemokine receptor activation. For CXCR4, different studies have demonstrated that the core region of its ligand CXCL12 binds to the extracellular regions of CXCR4, while the N-terminus has additional interactions with TM residues, including $D97^{2.63}$ and $E288^{7.39}$ in TM2 and TM7 respectively (Kofuku *et al.*, 2009). Ballesteros–Weinstein numbering is used in superscript throughout the text to enable the comparison of residue positions between receptors (Ballesteros and Weinstein, 1995) (Table 1). Another recently described numbering scheme is used for residues in EL2 (de Graaf *et al.*, 2008).

TM2 has also been suggested to regulate functional selectivity through an extended allosteric interface by an hydrogen bonding network (Nygaard *et al.*, 2010; Rosenkilde *et al.*, 2010), as the mutation of a conserved proline in TM2 in the angiotensin II receptor (P2.60 in chemokine receptors) led to a loss in G_q coupling for the agonist angiotensin II, while functional selectivity for the biased agonist [Sar1-Ile4-Ile8]angiotensin II was lost at this same mutation (Reis *et al.*, 2007; Rosenkilde *et al.*, 2010). The triggering domain of chemokines is thought to interact with residues in this region as well (CRS2) (Table 1). Together with the finding that (modified) chemokines show functional selectivity at a single recep-

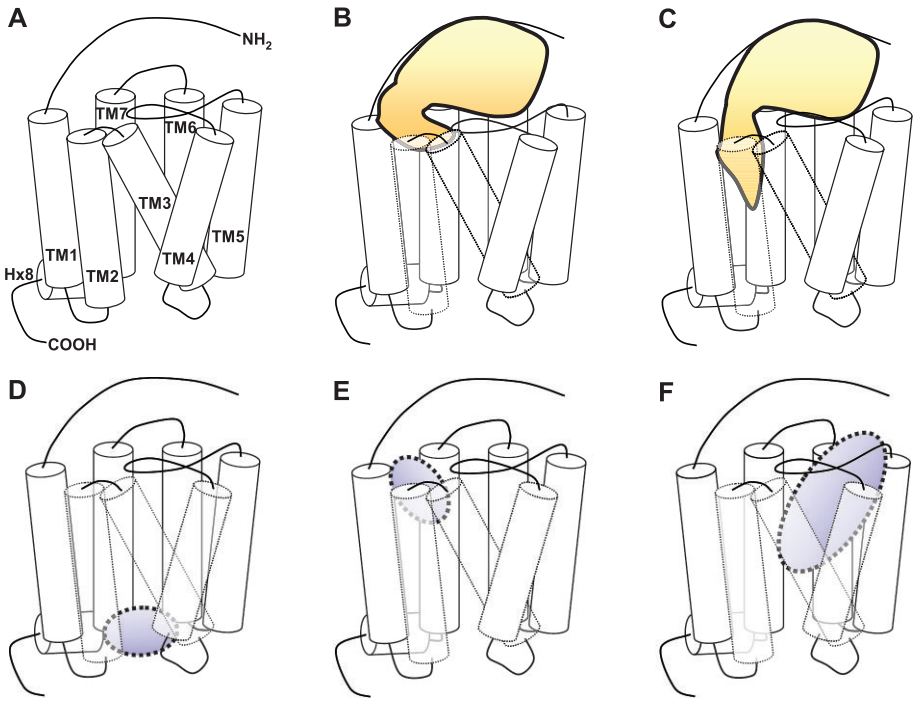


Figure 4

Schematic overview of GPCRs, their activation and ligand-binding sites. (A) The overall topology of a GPCR structure, with annotated TM helices, and N- and C-terminus. (B,C) Schematic representation of the two-step model of chemokine receptor activation. (B) The first step: chemokine binding to the extracellular surface of the chemokine receptor, including the N-terminus (CRS1). (C) The second step: the flexible N-terminus of the chemokine is positioned to interact with EL and TM residues (CRS2), mediating receptor activation. (D) Intracellular allosteric binding pocket used by CXCR2 and CCR4 ligands. Also the G protein binds in this region. (E) Binding pocket for small-molecule ligands in the chemokine receptor between TM1, 2, 3 and 7 (TMS1). (F) Binding pocket for small-molecule ligands in chemokine receptors between TM3, 4, 5, 6 and 7 (TMS2). Table 1 and Figure 5 give an overview of the interaction residues of small ligands binding to chemokine receptors.

tor, including CCR5 (Mack *et al.*, 1998), it can be hypothesized that this region is involved in functional selectivity of chemokines.

Despite the increasing evidence supporting the two-step model for chemokine receptor binding and activation, the exact regions of CRS1 and CRS2 used for these interactions seem to be different not only between receptors, but also for different ligands binding to the same receptor (Cox *et al.*, 2001; Blanpain *et al.*, 2003; Casarosa *et al.*, 2005; Jensen *et al.*, 2008).

Allosteric interactions in chemokine receptors and their consequences

Chemokines bind with high affinity to their receptors involving numerous interactions with the receptor extracellular surface (Allen *et al.*, 2007). Interestingly, low-molecular weight ligands (500–600 Da) are often able to disrupt binding or function of the roughly 100-fold larger chemokine ligands (or HIV gp120 envelope glycoprotein) with nanomolar potencies. From the size differences, it seems evident that these small ligands probably do not act via simple steric hindrance or competition, but rather operate in an allosteric manner. In general, allosteric ligands bind to sites that are topographically distinct from the orthosteric endogenous ligand-binding site. Only since the past decade we have begun to appreciate the different mode-of-action between allosteric and orthosteric ligands. The ability of allosteric ligands to change receptor conformations via distant, yet conformationally linked sites can positively or negatively impact the affinity as well as the efficacy of endogenous (orthosteric) ligands (Christopoulos and Kenakin, 2002).

Modulation by allosteric ligands is *saturable*, meaning that its maximum is attained with full occupancy of the allosteric sites on the receptor. In addition, this maximum effect further depends on the level of *cooperativity* between the two ligands. Furthermore, allosteric ligands exert effects that are generally *probe-dependent*, meaning that these effects are not the same towards all orthosteric agonists. This may be exemplified by allosteric CCR1 agonists, which enhance the binding of CCL3, while they inhibit the binding of CCL5 at the same receptor (Jensen *et al.*, 2008). In addition, an allosteric modulator can differentially affect receptor signalling mediated by orthosteric agonists, by selective potentiation of one signalling pathway while inhibiting a second, and leaving a third unaltered, reflecting the *permissive* nature of allosterism. Next to orthosteric ligand modulation, allosteric ligands can also exhibit agonistic activity in the absence of an orthosteric agonist, which is also referred to as *allosteric agonism* (Saita *et al.*, 2006; May *et al.*, 2007). This may include

Table 1

Evidence supporting the mechanism of action for small-molecule chemokine ligands

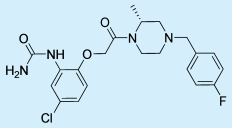
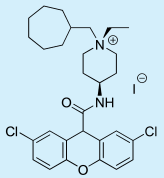
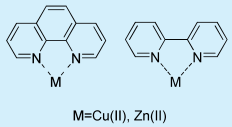
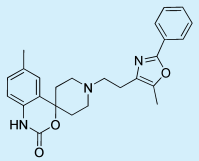
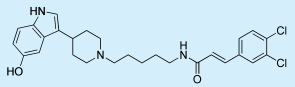
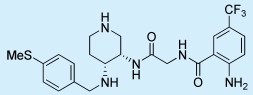
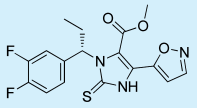
Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
CCR1						
BX-471 	Y1.39 Y7.43	Y3.32	Y3.33 I6.55	Radiolabelled analogue is not displaced by CCL3	Vaidehi *et al.*, 2006	S
UCB-35625 	Y1.39	Y3.32 **E7.39**	–	Incomplete displacement of ^{125}I-CCL3 binding CCL3-induced eosinophil shape change (↓, NC)	de Mendonça *et al.*, 2005 Sabroe *et al.*, 2000	
Metal chelators[1] 	–	**E7.39**	–	Agonist (IP turnover). CCL3 binding (↑) CCL5 binding (↓)	Jensen *et al.*, 2008	
CCR2						
RS-504393 	T7.40	**Y3.32** **E7.39**	H3.33	Antagonists	Berkhout *et al.*, 2003 Mirzadegan *et al.*, 2000	
SB282241 						
Piperidine 	–	**E7.39**	–	Antagonist	Cherney *et al.*, 2008	
JNJ-27141491 	–			CCL2-induced Ca^{2+} release (↓, NC)	Buntinx *et al.*, 2008	

Table 1

Continued

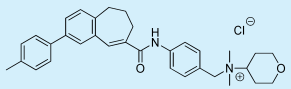
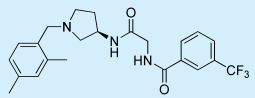
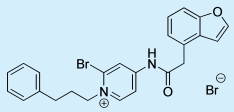
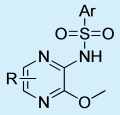
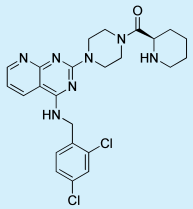
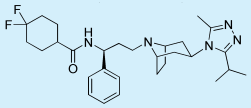
Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
TAK-779 	**W2.60** T7.40	**Y3.32** **E7.39**	–	Antagonist	Berkhout *et al.*, 2003 Hall *et al.*, 2009	
Teijin #1 	**W2.60**	H3.33 **E7.39** T7.40	I6.55	Antagonist	Berkhout *et al.*, 2003 Hall *et al.*, 2009	
CCR3						
UCB-35625 (see CCR1)	–	**Y3.32** **E7.39**	–	CCL1 binding (–). CCL11-induced chemotaxis (↓, C)	Wise *et al.*, 2007	
CH0076989[1] 	**Y1.39**	**Y3.32** **E7.39**	–	Agonist (chemotaxis, internalization, eosinophil shape change)	Wise *et al.*, 2007	
CCR4						
Pyrazinyl-sulfonamides 	C-tail (intracellular)			CCL22 dissociation (↑). Saturable antagonism of internalization of CCR4 by CCL22 (Schild analysis)	Andrews *et al.*, 2008	
BMS-397 	Not C-tail			Pyrazinyl-sulfonamides binding (–)	Andrews *et al.*, 2008	
CCR5						
Maraviroc (UK-427857) 	W2.60	**Y3.32** E7.39	I5.42 Y6.51	CCL3/CCL5 ^{35}S-GTPγS, Ca^{2+} (↓, NC). CCL3/CCL5 binding (↓, NC)	Watson *et al.*, 2005 Haworth *et al.*, 2007 Kondru *et al.*, 2008	A

Table 1

Continued

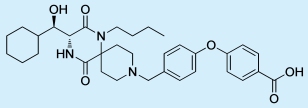
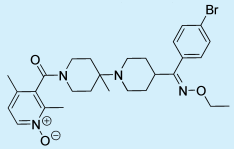
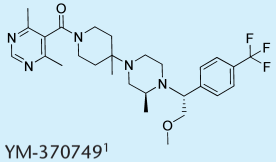
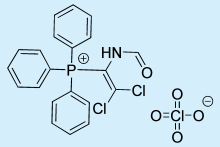
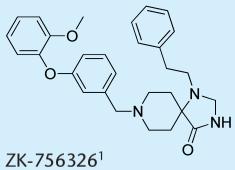
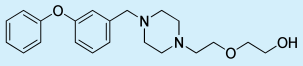
Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
Aplaviroc (AK602/873140) 	W2.60	**Y3.32** C45.50 **M7.35** E7.39	**F3.33** K5.35 T5.39 I5.42 W6.48 Y6.51	Incomplete displacement of ^{3}H-TAK-779, and ^{125}I-CCL5. Complete blockage of CCL5-induced Ca^{2+} response, complete displacement of CCL3. ^{3}H-Aplaviroc cannot be displaced by SCH-C and TAK-779	Maeda *et al.*, 2004 Watson *et al.*, 2005 Maeda *et al.*, 2006 Kondru *et al.*, 2008 Maeda *et al.*, 2008	S
SCH-351125 (SCH-C) 	–	**Y3.32** E7.39	I5.42	See also maraviroc Chemokine-induced internalization (↓, NC) CCL3L1 binding (–)	Maeda *et al.*, 2006 Muniz-Medina *et al.*, 2009	
TAK-779 (see CCR2)	L1.35 **Y1.39** W2.60	**Y3.32** E7.39 G7.42	I5.42 W6.48	Similar to SCH-C/maraviroc	Dragic *et al.*, 2000 Maeda *et al.*, 2006 Seibert *et al.*, 2006 Kondru *et al.*, 2008 Hall *et al.*, 2009	
Vicriviroc (SCH-D) 	W2.60	**Y3.32** E7.39	I5.42 Y6.51	Similar to SCH-C/maraviroc	Watson *et al.*, 2005 Kondru *et al.*, 2008	3
YM-370749[1] 	–	**Y3.32** E7.39	**F3.33** Y6.51 I5.42	Agonist (Ca^{2+}, internalization) No chemotaxis	Saita *et al.*, 2006	
CCR8						
LMD-009[1] 	Y1.39 F2.57 Q2.60	**S3.29** **Y3.32** E7.39	–	Full agonist (IP turnover, Ca^{2+}, chemotaxis) CCL1 binding (↓)	Jensen *et al.*, 2007	
ZK-756326[1] 	Does not seem to bind to the N-terminus of CCR8, in contrast to CCL1			Full agonist (Ca^{2+}, extracellular acidification). CCL1 binding (↓)	Haskell *et al.*, 2006	

Table 1

Continued

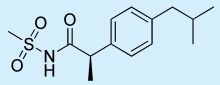
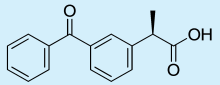
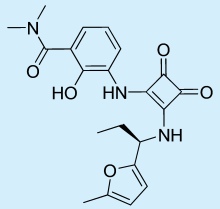
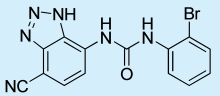
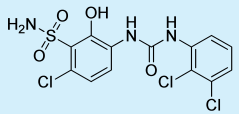
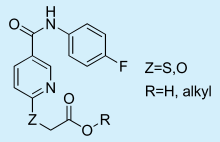
Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
CXCR1						
Repertaxin 	V1.35 Y1.39 K2.64	V3.28	–	CXCL8-induced neutrophil migration (↓, NC)	Moriconi *et al.*, 2007 Bertini *et al.*, 2004	2
(R)-Ketoprofen 	I1.36 Y1.39 K2.64	V3.28 I7.31 E7.39	–	CXCL8-induced neutrophil migration (↓). CXCL8 binding (–)	Allegretti *et al.*, 2005	
CXCR2						
SCH-527123 (SCH-N)[2] 	T2.39 D2.40 D3.49 A6.33 (IL3) Y7.53 K7.59 (Hx8)			Slow dissociation from CXCR2. CXCL8 does not displace ^{3}H-527123. Ca^{2+} and chemotaxis (↓, NC)	Gonsiorek *et al.*, 2007 Salchow *et al.*, 2010	2
Diarylureas[2] SB265610  SB332235 	T2.39 D2.40 D3.49 Y7.53 K7.59 (Hx8)			Chemokines do not compete for ^{3}H-SB225610 binding. CXCL8-mediated β-arrestin recruitment & CXCL8 binding (↓, NC)	Nicholls *et al.*, 2008 de Kruijf *et al.*, 2009 Salchow *et al.*, 2010	
Nicotinamide glycolates[2] 	Suggested intracellular binding mode, no structural data			Ester form is only active in whole cells, acid only in cell preparations	Maeda *et al.*, 2010	

Table 1

Continued

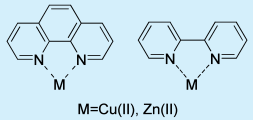
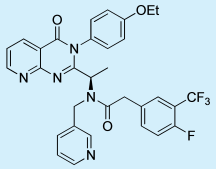
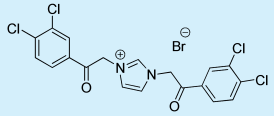
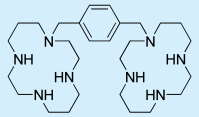
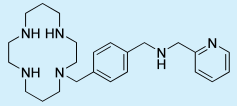
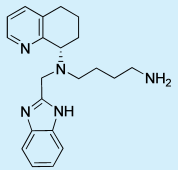
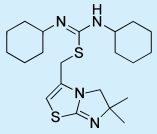
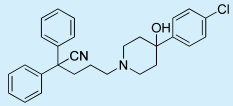
Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
CXCR3						
Metal chelator[1,4] 	–	F3.32 Y7.43 G3.29H	Y6.51	Agonist (IP turnover)	Rosenkilde *et al.*, 2007a	
NBI-74330 	–			CXCL10 and CXCL11-induced IP turnover (↓, NC)	Verzijl *et al.*, 2008	S
VUF10132 						
AMD3100[3] 	–	K7.35[3] S7.39[3]	–	Affinity increases 1000x for CXCR3	Rosenkilde *et al.*, 2007b Rosenkilde *et al.*, 2004 Hatse *et al.*, 2007	
CXCR4						
AMD3100 (see CXCR3)	Y1.39 W2.60	Y3.32 **E7.39**	D4.60 D6.58	Antagonist. 1000x more potent in antagonism of function than binding	Gerlach *et al.*, 2001 Wong *et al.*, 2008	A
AMD3465 	Y1.39 W2.60	Y3.32 H7.32 **E7.39**	D4.60 D6.58	Antagonist	Rosenkilde *et al.*, 2007b Wong *et al.*, 2008	
AMD11070 	Y1.39 W2.60 **D2.63**	**E7.39**	D4.60 D6.58	Antagonist	Wong *et al.*, 2008	2

Table 1

Continued

Receptor/ligand(s)	TMS1	Interface	TMS2	Pharmacology	Reference(s)	C
IT1t[5] 	E1.26 W2.60 **D2.63** A2.64 W2.68 V3.28	H3.29 Y3.32 R45.47 I45.49 C45.50 **D45.51** R45.52 **E7.39**	–	Antagonist	Wu *et al.*, 2010 Thoma *et al.*, 2008	
CVX15[5] (peptide)	–	P1.21 H3.29 Y3.32 C45.50 **D45.51** R45.52 E7.28 H7.32 E7.35 S7.36 **E7.39**	T3.33 D4.60 F45.53 Y45.53 P45.54 N45.55 D45.57 D5.35 V5.39 F5.42 Q5.43 Y6.51 D6.58 I6.61 L6.62	Antagonist	Wu *et al.*, 2010	
RSVM/ASLW[1] (peptides)	–			Not inhibited by AMD3100 RSVM is partial agonist, ASLW is a superagonist (less internalization)	Sachpatzidis *et al.*, 2003	
CXCR7						
AMD3100[1] (see CXCR3)	–			β-arrestin recruitment. CXCL12 binding (↑) CXCL12-induced arrestin recruitment (↑)	Kalatskaya *et al.*, 2009	
CCR1-2, CCR4-5, CXCR4						
Trichosanthin (protein)	–			Chemokine-mediated chemotaxis (↑) and [^{35}S]-GTPγS (↑)	Zhao *et al.*, 1999	
US28						
VUF2274 	–	–	E7.39	Inverse agonist (IP_x). CCL5 binding (↓)	Casarosa *et al.*, 2003	

Ligands are shown for which evidence exists on the interaction with the receptor. Indicated residues are in Ballesteros–Weinstein notation (Ballesteros and Weinstein, 1995), and significantly affect ligand affinity or antagonism when mutated. Bold residues indicate that mutation also influenced chemokine binding or function. The residues are categorized into TMS1 (left column), 'interface' (centre column) or TMS2 (right column), based on their location in the binding pockets. 'Interface' signifies residues that reside on the interface of TMS1 and TMS2. Explanation of used symbols: '–', there is no report on mutations of residues in either TMS1, interface or TMS2 that affects ligand binding; '↑', enhanced binding or function; '↓' inhibited binding or function; 'NC', non-competitive behaviour, 'C', competitive behaviour. The last column 'C' indicates the progress of a compound in clinical trials; 1, 2 or 3 refer to the clinical phase the compound currently resides in. If a ligand is approved or suspended it is indicated with an 'A' or 'S' respectively.

[1]This compound is an agonist for the indicated receptor.

[2]Residues important for ligand binding at this receptor are part of an intracellular binding pocket.

[3]The binding site of AMD3100 transferred from CXCR4 to CXCR3 by mutations S7.39E, and K7.35A in CXCR3.

[4]A G1283.29H mutation was introduced to facilitate binding of the metal chelator complex.

[5]Interacting residues (within 4Å from the ligand) in CXCR4 crystal structures (Wu *et al.*, 2010).

Table 2

Clinical trials with mAbs targeting chemokine receptors

Receptor/mAbs	Epitope	Isotype	Pathological condition(s)	Phase	Trial (PMID)	Status
CCR2						
MLN1202	N.a.	IgG1	Metastatic cancer	II	NCT01015560	S
			Atherosclerotic cardiovascular disease	II	NCT00715169	C
			Multiple sclerosis	II	NCT01199640	C
CCR4						
KW-0761	Nt	IgG1	Adult T-cell leukaemia-lymphoma and peripheral T-cell lymphoma	I + II	NCT00920790 NCT00355472 NCT00888927	O O O
KW-0761 + multidrug chemotherapy			Peripheral and cutaneous T-Cell lymphoma	II	NCT01226472	R
			Peripheral T/NK-cell lymphoma	II	NCT01192984	R
			Adult T-cell leukaemia-lymphoma	II	NCT01173887	R
CCR5						
HGS004/CCR5mAb004	EL2	IgG4	HIV infection	I	NCT00114699	C
PRO140	Nt & EL2	IgG1	HIV infection	I + II	NCT00110591 NCT00613379 NCT00642707	C C C
PRO140 + oral antiretroviral therapy			HIV infection	II	NCT01272258	R
CXCR4						
MDX-1338	N.a.	N.a.	Acute myeloid leukaemia	I	NCT01120457	R

Info obtained in January 2011 on http://www.clinicaltrials.gov. Epitope indicates the receptor region involved in mAb recognition: Nt, N-terminus; N.a., information not available. Status of clinical trials: C, completed; O, ongoing; R, recruiting; S, suspended.

selective or *biased* activation of signalling pathways, also referred to as *functional selectivity*, or *biased agonism* (Galandrin *et al.*, 2007; Kenakin, 2010a). Altogether, allosterism adds another layer of complexity to GPCR pharmacology, which has forced us to reconsider the approaches to identify and optimize ligands in drug discovery and development programmes.

Allosteric ligands have been identified for different chemokine receptors, including CC and CXC chemokine receptors (Sachpatzidis *et al.*, 2003; Watson *et al.*, 2005; Vaidehi *et al.*, 2006; Rosenkilde *et al.*, 2007a; Jensen *et al.*, 2008; Salchow *et al.*, 2010). These ligands include not only small molecules, but also metal chelators and peptides. In the next sections we will discuss some of the evidence supporting allosteric modulation and functional selectivity of chemokine receptors and the implications of receptor dimerization. In addition, development of biologicals for the treatment of chemokine-associated disease is discussed.

Allosteric small-molecule chemokine receptor antagonists

The growing evidence implicating chemokine receptors and their ligands in disease has boosted the discovery and development of related therapeutics in the past decade. About 10 years ago, Berlex Biosciences entered clinical trials with the first antagonist for a chemokine receptor, BX-471, a selective, potent and orally available CCR1 antagonist. However, the compound failed in a phase II trial with multiple sclerosis (MS) patients due to lack of efficacy. Nevertheless, it had set the stage for the clinical development of other chemokine receptor antagonists. Since then, several chemokine receptor antagonists have been developed but failed in clinical trials due to failure of reaching clinical endpoints (Proudfoot *et al.*, 2010). However, the recent approval of maraviroc (CCR5 antagonist) and AMD3100 (CXCR4 antagonist), together with numerous clinical trials currently being conducted with small molecules and biologicals, reflects the faith in the chemokine system as a tractable therapeutic target (Proudfoot *et al.*, 2010) (Tables 1 and 2).

Small molecules generally interact with residues in the TM helices of the receptor. Two binding pockets in these helices can be distinguished: the *minor* and the *major* binding pocket, formed by residues from TM1, 2, 3, 7, or TM3, 4, 5, 6 respectively (Figure 4E,F) (Surgand *et al.*, 2006). To avoid confusion with the chemokine recognition sites, we refer to these sites as TM site 1 (TMS1) and TMS2 respectively. Many ligands bind in both TMS1 and TMS2, but some seem to bind exclusively to TMS1 or TMS2 (Table 1). The mechanisms by which

small-molecule ligands modulate chemokine affinity and/or efficacy are largely unknown. Although some ligands have been demonstrated to act in an allosteric manner, there are also studies that show overlap in binding sites of chemokines and small molecules, supporting a competitive component in the mechanism of action. However, ligands with partially overlapping binding sites, such as the CXCR3 chemokines CXCL10 and CXCL11, also show non-competitive 'allosteric' behaviour (Cox *et al.*, 2001; Xanthou *et al.*, 2003). Blanpain and co-workers showed that mutations in TM2 and 3 of CCR5 affect the function but not the binding of CCL3, while CCL5 binding and function remains unchanged (Blanpain *et al.*, 2003). As many small-molecule ligands for CCR5 have also shown to bind in this region (e.g. $Y37^{1.39}$, $W86^{2.60}$, $Y108^{3.32}$ and $E283^{7.39}$) including maraviroc, aplaviroc, vicriviroc, SCH-C and TAK-779 (Maeda *et al.*, 2006; Kondru *et al.*, 2008; Hall *et al.*, 2009), it can be hypothesized that these compounds compete with the CCL3 N-terminus to bind in the TM region and, thereby block its ability to activate CCR5 (Figure 4B,E). However, a simple competition hypothesis is not sufficient to explain the fact that these CCR5 antagonists are able to inhibit the binding of both CCL3 and CCL5 in an insurmountable allosteric manner (Watson *et al.*, 2005). In addition, aplaviroc showed behaviour deviating from the other CCR5 antagonists, in that it only displaced 20% of CCL5 even at 10 μM ($K_i \pm 3$ nM), while the calcium response mediated by the chemokine was completely blocked by only 10 nM of the ligand. The latter suggests an allosteric mode of action for aplaviroc, illustrated by the saturability and probe dependence of the effects observed for this CCR5 antagonist (Watson *et al.*, 2005).

A study of the CCR1-specific compound BX-471 showed that mutation of residues in both TMS1 and TMS2 affect ligand binding, including residues $Y41^{1.39}$, $Y113^{3.32}$, $Y114^{3.33}$, $I259^{6.55}$ and $Y291^{7.43}$, which are not important for CCL3 binding and function (Vaidehi *et al.*, 2006). Nevertheless, the compound was still able to displace [^{125}I]-CCL3 from the receptor, indicating an allosteric mechanism of inhibition. In contrast, the chemokine did not affect the binding of a radiolabelled analogue of BX-471. UCB-35625 is another small-molecule CCR1 antagonist, for which residues $Y41^{1.39}$, $Y113^{3.32}$ and $E287^{7.39}$ (TMS1) were shown to be involved in ligand binding, sharing residues $Y41^{1.39}$ and $Y113^{3.32}$ with BX-471, indicating that these two small molecules bind to different yet overlapping sites (de Mendonça *et al.*, 2005). UCB-35625 has a potency in the picomolar range to block CCL3-induced eosinophil shape change, while it has a ~1000-fold lower potency in displacing the chemokine from the receptor (Sabroe *et al.*, 2000). Moreover, the displacement of CCL3 is incomplete, even at saturating concentrations of the compound. The latter suggests an allosteric inhibition of efficacy but not affinity. In contrast to CCL3, CCL5-induced activation of CCR1 is dependent on $E287^{7.39}$ (Jensen *et al.*, 2008), and although not investigated, it might be speculated that the observed effects of UCB-35625 on CCL3 binding and activity would have been different when CCL5 was used as a probe. Also CCR2 and CCR3 ligands, like RS-504393, TAK-779 and UCB-35625, interact with TM residues shown to be involved in chemokine-induced receptor activation (Mirzadegan *et al.*, 2000; Berkhout *et al.*, 2003; de Mendonça *et al.*, 2005; Wise *et al.*, 2007).

Evidence for the mode of action of antagonists is not only found for CC, but also for CXC chemokine receptors, including CXCR4. As for many other chemokine receptors, CXCL12 binding to CXCR4 follows the two-step activation model (Kofuku *et al.*, 2009). CXCR4 CRS2 contains residues from EL2 and TM domains, such as $D187^{45.51}$ (EL2), $D97^{2.63}$ and $E288^{7.39}$ that are involved in both chemokine binding and activation (Brelot *et al.*, 2000). In the recent crystal structures of CXCR4, the small-molecule antagonist IT1t is shown to bind to these same residues, and it was suggested that it competitively blocks the interactions of the CXCL12 N-terminus with CXCR4 (Wu *et al.*, 2010). Similarly, the binding site of the well-studied bicyclam antagonist AMD3100 is mainly lined by three acidic TM residues, $D171^{4.60}$, $D262^{6.58}$ and $E288^{7.39}$ (Gerlach *et al.*, 2001; Hatse *et al.*, 2003; Rosenkilde *et al.*, 2004; Wong *et al.*, 2008). Although the binding site of AMD3100 and IT1t do not seem to overlap completely, AMD3100 might bind partly to CRS2 where the N-terminus of CXCL12 interacts with the receptor (Kofuku *et al.*, 2009). Altogether these findings might, at least in part, suggest a competitive mechanism of action for these compounds, by preventing the binding of the CXCL12 to CRS2 on the receptor, leading to the observed antagonism of functional responses. Along with the data presented above for CC chemokine receptors, competition of small molecules with this triggering domain of the chemokine might pose a general mechanism of action for chemokine receptor antagonists that have effects on efficacy rather than affinity. Table 1 shows an overview of evidence for binding mechanisms of small ligands targeting chemokine receptors.

The success story of chemokine receptor antagonists: CCR5 and CXCR4

The quest for therapeutics targeting chemokine receptors has been catalysed by their significant involvement in various human diseases. In the 1990s, it was shown that chemokine receptors were promising targets for treatment in HIV-1 infection (Deng *et al.*, 1996; Feng *et al.*, 1996). Genetic evidence was provided by the impact of the naturally occurring mutation CCR5-Δ32 that encodes a truncated, non-functional form of CCR5 with no apparent deleterious consequences. It was found that CCR5-Δ32 is significantly underrepresented in the HIV-1 infected groups, and individuals homozygous for the mutation are only rarely infected with HIV-1 (Gorry *et al.*, 2002) supporting the role of CCR5 in HIV-1 entry. In addition, CXCR4 was found as a second co-receptor for HIV-1. Namely, CCR5 and CXCR4 facilitate HIV-1 entry to macrophages and T-cells respectively (Berger *et al.*, 1999). In the first stages of infection the virus mainly uses CCR5 as a co-receptor (CCR5-tropic). These findings paved the way for discovery and development of small-molecule antagonists for CCR5. TAK-779 was the first to be discovered, showing inhibition of HIV-1 infection *in vitro* and *in vivo* (Baba *et al.*, 1999). Since then, several CCR5 antagonists have entered clinical trials, including aplaviroc, maraviroc and vicriviroc. Maraviroc (Celsentri™, developed by Pfizer) is the first CCR5 antagonist approved by the European Medicines Agency (EMA) for

use in treatment-experienced patients harbouring only CCR5-tropic viruses. It represents a novel class of antiretroviral drugs, as it is the first therapeutic targeting a cellular rather than a viral protein. Maraviroc is a potent inhibitor of CCL4 binding to the CCR5 receptor (IC_{50} = 2 nM) and a potent antiviral agent (EC_{90} = 1 nM) (Wood and Armour, 2005). In addition, Maraviroc has been demonstrated to behave as an allosteric antagonist. It binds to a site in the receptor that is topographically distinct to the site where the viral gp120 envelope protein binds (Watson *et al.*, 2005; Muniz-Medina *et al.*, 2009) and that involves key interactions with the TM domains of CCR5 (Kondru *et al.*, 2008) (Table 1). More recently, a second-generation maraviroc analogue has been described, PF-232798, which retains the attractive antiviral effect combined with improved absorption profiles in rat and dog (Stupple *et al.*, 2010) and is currently in phase II clinical trials. In addition, vicriviroc also showed long-term potent antiviral activity and is currently in phase III clinical trials (Klibanov, 2009).

During the course of disease, HIV-1 shifts its tropism from CCR5 to CXCR4, a hallmark of the symptomatic stage when the disease progresses to AIDS (Jekle *et al.*, 2003). Consequently, there has been an increased interest in the discovery and development of CXCR4 antagonists able to block the interaction of HIV-1 with CXCR4, preventing subsequent infection of cells. One of the early compounds showing anti-HIV activity was AMD3100 (De Clercq, 2003). However, despite its efficacy in clinical trials, AMD3100 treatments in HIV-1 patients were discontinued due to several events of cardiac toxicity. A serendipitous finding during these trials was that AMD3100 promoted mobilization of hematopoietic stem cells from the bone marrow to the periphery. Subsequently, AMD3100 (plerixafor, Mozobil™) has been successfully developed by Genzyme as an effective therapeutic for autologous bone marrow transplantations in patients suffering from non-Hodgkin's lymphoma and multiple myeloma (De Clercq, 2010). As can be seen from the blocking of CXCR4 with AMD3100, the CXCL12/CXCR4 axis is involved in multiple homeostatic processes. These include T cell trafficking and homing, stem cell localization and organ development (Tsibris and Kuritzkes, 2007). Since CXCR4 or CXCL12/SDF-1α knockout mice are not viable because of significant defects in B-cell lymphopoiesis and bone-marrow myelopoiesis (Nagasawa *et al.*, 1996), long-term CXCR4 antagonism might lead to severe adverse effects. Future *in vivo* studies are required to answer the question whether CXCR4 can actually be targeted safely for the (long-term) treatment of CXCR4-tropic HIV-1 infection.

Allosteric agonists for chemokine receptors and functional selectivity

Despite the therapeutic focus on chemokine antagonists, the process of screening for and optimization of chemokine receptor antagonists has led to the discovery of several small-molecule agonists for different chemokine receptors, such as CCR1, CCR3, CCR5, CCR8, CXCR3 and CXCR4 (Sachpatzidis *et al.*, 2003; Saita *et al.*, 2006; Stroke *et al.*, 2006; Jensen *et al.*, 2007; Rosenkilde *et al.*, 2007a; Wise *et al.*, 2007). Despite their relatively small size, these ligands are generally able to fully activate receptor signalling. Similarly to small-molecule antagonists, residues involved in receptor binding have been shown to reside in TMS1 and TMS2 of the receptors (Table 1). For example, CH0076989, a small-molecule agonist for CCR3, activates multiple signalling pathways including chemotaxis and receptor internalization by interacting with residues in TMS1 (i.e. $Y41^{1.39}$, $Y113^{3.32}$ and $E287^{7.39}$) (Wise *et al.*, 2007). Since these residues are also important for CCL11-induced receptor activation, this suggests that CH0076989 activates the receptor in a similar manner as the chemokine, probably by interacting with the TM2–TM3 interface (Blanpain *et al.*, 2003; Govaerts *et al.*, 2003). As pointed out earlier, this receptor region might be involved in ligand-biased signalling (Rosenkilde *et al.*, 2010). Indeed, CCR3 agonist CH0076989 seems to bind in that region (TMS1) (Table 1). Interestingly, while equal receptor internalization was observed when stimulating CCR3 with either CH0076989 or CCL11, the efficacy of the small agonist to induce chemotaxis was significantly lower than for the chemokine, suggesting *functional selectivity*. YM-370749, a small-molecule agonist for the CCR5 receptor, also exhibited functional selectivity, while it binds to TMS2 and not TMS1. This compound promoted calcium mobilization and receptor internalization, but was unable to induce chemotaxis (Saita *et al.*, 2006). Importantly, YM-370749 inhibited HIV-1 replication. The use of functionally selective agonists that down-regulate the receptor without concomitant undesired side effects, such as chemotaxis, might pose a novel therapeutic avenue for the treatment of diseases like HIV-1 infection. As chemokine receptors can initiate more signalling pathways than described here, including Janus kinase/signal transducers and activators of transcription (JAK/STAT) (Kehrl *et al.*, 2009), it would be interesting to see whether chemokine receptor agonists, for example CCR8 agonist LMD-009 (binds in TMS1), show selectivity in activation of these other signalling pathways. Moreover, there is accumulating evidence for GPCRs suggesting that selective activation of specific signalling routes, that is, G proteins versus β-arrestins, might be beneficial over non-biased agonists (Kenakin and Miller, 2010), again highlighting the therapeutic potential of such functionally selective ligands.

Intracellular binding sites in chemokine receptors

GPCR signalling is allosteric by nature, in which extracellular endogenous agonists act as positive allosteric modulators on the coupling of intracellular G proteins, and vice versa (Kenakin, 2010b). Indeed, high-affinity chemokine binding to a number of tested receptors is G protein dependent as revealed by experiments in which $G_{i/o}$ proteins are uncoupled using, e.g. GTPγS, Gpp(NH)p, or *Pertussis* toxin (Cox *et al.*, 2001; Staudinger *et al.*, 2001; Springael *et al.*, 2006; Bradley *et al.*, 2009; Nijmeijer *et al.*, 2010). Agonist-induced or constitutive coupling of a GPCR to G proteins can limit the availability of a shared G protein pool to interact with other receptors, which may subsequently hamper high-affinity agonist binding to the latter receptors (Nijmeijer *et al.*, 2010).

In addition, GPCRs can interact with multiple other interacting partners, such as receptor activity-modifying proteins (RAMPs), β-arrestins, GRKs and other GPCRs (Kenakin and Miller, 2010), via regions that do not overlap with the binding site of endogenous ligands. Experimental evidence for such binding sites was presented for CCR4 and CXCR2 where some small-molecule antagonists appeared to bind along the intracellular surface of the GPCRs instead of the TM domains (Andrews *et al.*, 2008; Nicholls *et al.*, 2008; Salchow *et al.*, 2010). Nicholls *et al.* (2008) studied two classes of CXCR2 antagonists that had a 1000-fold higher affinity for CXCR2 compared to CXCR1. By constructing different chimeras between CXCR1 and CXCR2, they found a reversal of antagonism when switching the intracellular C-terminal tails. Using a similar approach, evidence was presented for an intracellular binding site in CCR4 (Andrews *et al.*, 2008). In the case of CXCR2, the point mutant K320N$^{7.59}$ in Hx8 of CXCR2 led to a 10-fold decrease in affinity for the compounds, while mutation of N311K$^{7.59}$ at the same position in CXCR1 led to a 100-fold increase in affinity, providing additional evidence for an intracellular binding mode (Nicholls *et al.*, 2008). Moreover, other groups, including our own, have presented pharmacological evidence for an allosteric binding mode for these and other classes of CXCR2 ligands (Gonsiorek *et al.*, 2007; Bradley *et al.*, 2009; de Kruijf *et al.*, 2009). These include the inability of chemokines to displace a small-molecule antagonist with similar chemical structure, and insurmountable inhibition of CXCL8-promoted β-arrestin recruitment and [^{125}I]-CXCL8 binding. Site-directed mutagenesis of different intracellular residues was performed to further delineate the binding pocket for these CXCR2 ligands (Salchow *et al.*, 2010). Salchow and co-workers identified several key CXCR2 residues involved in interaction of CXCR2 antagonist SCH-527123, a ligand already suggested to bind in an allosteric manner (Gonsiorek *et al.*, 2007), and compounds similar to those used in the previous study (Nicholls *et al.*, 2008). The binding pocket seems to be lined by T83$^{2.39}$, D84$^{2.40}$, D143$^{3.49}$ (E/DRY motif), Y314$^{7.53}$ and K320$^{7.59}$ along the intracellular surface of the TM helices (see Table 1 and Figure 4D). Since studied mutations are in close proximity to the site of G protein coupling, or to a region that is involved in receptor signal transduction, this might in fact govern a mechanism of allosteric inhibition (Figure 4D).

Recently, pharmacological modulation through interactions with intracellular parts of CXCR4 has also been described by Tchernychev and colleagues who identified the pepducin ATI-2341 as a potent agonist of this receptor (Tchernychev *et al.*, 2010). Pepducins are synthetic molecules that are composed of a peptide derived from the amino acid sequence of an intracellular loop of the target GPCR coupled to a lipid tether. The peptide component of the pepducin confers receptor-modulating activity whilst the lipid counterpart facilitates cell penetration and access to the intracellular face of the target GPCR. The ATI-2341 is derived from IL1 of CXCR4 and activates CXCR4-mediated signalling pathways, induces receptor internalization, and promotes both *in vitro* and *in vivo* chemotaxis. Interestingly, ATI-2341 acts as functional antagonist *in vivo*, leading to a similar mobilization of hematopoietic stem cells from the bone marrow as is observed for the CXCR4 antagonist AMD3100 (Tchernychev *et al.*, 2010). The mechanism responsible for these seemingly contradictory effects requires further investigation. Although more evidence is needed regarding the molecular determinants of these ligand–receptor interactions, these studies indicate that targeting of allosteric regions other than the classical major and minor TM-binding pockets is feasible within the class of chemokine receptors.

Biological therapeutics targeting chemokine receptors

Monoclonal antibody (mAb)-derived therapeutics have been proven to be an excellent paradigm as high-affinity biopharmaceuticals in the diagnosis and treatment of cancer and inflammatory diseases, as exemplified by the acquisition of mAb technology companies by major drug companies (Nelson *et al.*, 2010). During the last 2–3 decades, 42 engineered mAbs that target growth factors and receptor tyrosine kinases have gained US Food and Drug Administration (FDA) approval (Walsh, 2010). Hitherto, no mAb-derived therapeutic against GPCRs has been approved for clinical use. The difficulty to develop such therapeutics may have been due to the intrinsic nature of GPCRs. Their limited availability as purified proteins as well as their low immunogenicity as membrane-embedded proteins render GPCRs difficult antigens for the generation of antibodies that recognize their targets with high specificity and affinity (Olson and Jacobson, 2009; Hutchings *et al.*, 2010). However, several attempts have been successful and clinical trials are currently evaluating the therapeutical potential of mAbs targeting chemokine receptors (Table 2).

Therapeutic antibodies can act via two different mechanisms. First, mAbs can bind and block the target protein, directly interfering with its function (i.e. direct targeted therapy). Alternatively, the mAb triggers an indirect biological activity upon recognition of its antigen by recruiting cytotoxic monocytes/macrophages (i.e. antibody-dependent cell-mediated cytotoxicity) or by binding complement factors (i.e. complement-dependent toxicity). In addition, other proteins or drugs that are conjugated to such targeting mAbs can induce cellular responses (Satoh *et al.*, 2006; Schrama *et al.*, 2006).

MLN1202 is a genetically engineered human IgG1 mAb targeting CCR2 that has been developed by Millenium Pharmaceuticals, and optimized to reduce antibody- and complement-dependent cytotoxicity. MLN1202 inhibits chemokine-induced CCR2 signalling in transfected cells (Vergunst *et al.*, 2008). This mAb has been in clinical trials for the treatment of various inflammatory diseases involving CCR2-expressing monocytes/macrophages (Figure 2 and Table 2). Treatment of patients at risk for atherosclerotic diseases with MLN1202 significantly reduced median serum levels of C-reactive protein (Gilbert *et al.*, 2011), which is considered to be a predictive biomarker of inflammation associated with cardiovascular diseases (Ridker, 2007). In contrast, MLN1202 failed to block CCR2-mediated infiltration of macrophages into the inflamed synovium of rheumatoid arthritis patients or reduce the expression of synovial pro-inflammatory cytokines (Vergunst *et al.*, 2008). This failure may have been due to the incomplete receptor occupancy (86–94%) by MLN1202 or

the fact that CCR2 is not the correct/only therapeutical target for this pathological condition (Proudfoot, 2008; Vergunst *et al.*, 2008). Finally, clinical trials in multiple sclerosis patients have also been conducted with MLN1202 but no results are publicly available. Also, a phase 2 clinical trial for the treatment of bone metastasis by MLN1202 had been initiated but was recently suspended.

Two mAbs targeting CCR5 have been developed by Human Genome Science (HGS004) and Progenics Pharmaceuticals (PRO 140) and have been investigated in the context of CCR5-mediated HIV-1 infection (Weiss and Clapham, 1996). HGS004 binds to EL2 of CCR5 and inhibits chemokine-induced signalling as well as HIV co-receptor activity (Lalezari *et al.*, 2008). Phase 1 clinical studies demonstrated that HGS004 reduces plasma HIV-1 RNA levels ≥10-fold in 54% of treated patients (Lalezari *et al.*, 2008). However, the lack of a clear dose–response relationship indicated that the anti-HIV potency of HGS004 as a single agent might be suboptimal. Also, some patients treated with high doses of HGS004 showed a shift from CCR5- to CXCR4-tropic viruses or dual strains.

PRO 140 binds to the N-terminal region (residue D2) and EL2 ($R168^{45.40}$, $Y176^{45.48}$) of CCR5. Interestingly, PRO 140 is more potent in inhibiting HIV-1 co-receptor activity than antagonizing chemokine-induced signalling, giving the possibility to inhibit HIV-1 infection without affecting CCR5-mediated signalling, an example of permissive antagonism (Olson *et al.*, 1999). Phase 1 and 2 clinical studies demonstrated that a single intravenous injection of PRO 140 could reduce HIV-1 viral loads in 100% of treated patients (Jacobson *et al.*, 2008; 2010a). Importantly, some patients displayed a more than 100-fold decrease in viral load and patients treated with high doses of PRO 140 displayed no change in co-receptor tropism and no emergence of PRO 140-resistant viral strains (Jacobson *et al.*, 2010a). Such strong antiviral effects supported the development of subcutaneous formulations of PRO 140. Three weekly subcutaneous injections of PRO 140 led to an antiviral activity similar to that observed with one single intravenous injection (Jacobson *et al.*, 2010b). This phase 2 study provided proof-of-concept for the subcutaneous use of PRO 140 and the subcutaneous PRO 140 was selected for further development on the basis of its potential to be self-administered by patients (Jacobson *et al.*, 2010a,b).

mAbs against CCR4 have been optimized by Kyowa Hakko Kirin Ltd to block the receptor, but also induce antibody-dependent cellular cytotoxicity (ADCC) (Satoh *et al.*, 2006; Ishii *et al.*, 2010). To this end, these mAbs (i.e. KW-0761 and KM2760) have been modified to remove fucose groups from the Fc region of their heavy chains, thereby increasing their affinity for leukocyte receptors FcγRIIIa. Upon binding to CCR4-expressing cells, these mAbs recruit FcγRIIIa-expressing natural killer cells leading to the lysis of $CCR4^+$ tumour cells (Satoh *et al.*, 2006; Ishii *et al.*, 2010). Preclinical studies have shown that the antitumor activity of KW-0761 and KM2760 in adult T-cells leukaemia-lymphoma (ATLL) mouse models is mediated via ADCC (Niwa *et al.*, 2004; Ishii *et al.*, 2010). Furthermore, the clinical application of KW-0761 was demonstrated by its ability to induce ADCC-mediated cytotoxicity of primary ATLL cells *ex vivo* (Ishii *et al.*, 2010). Phase 1 and 2 clinical trials are currently ongoing to evaluate the therapeutic effect of KW-0761, alone or in combination with multidrug chemotherapy, in patients with T-cell and NK-cell lymphomas (Table 2).

A new class of antibody-based therapeutics has recently joined the family of GPCR-targeting biologicals. VHH antibody fragments, also defined as nanobodies by Ablynx, are immunoglobulin single variable domains of heavy-chain antibodies that occur naturally in the Camelidae family. Due to their small size of approximately 15 kDa, nanobodies can be easily expressed and produced in organisms such as yeasts or bacteria, and are also highly stable and soluble. Furthermore, complementarity-determining regions of nanobodies can penetrate into grooves and cavities of proteins, accessing a new range of non-planar epitopes, such as enzymatic clefts (Ghassabeh *et al.*, 2010). We recently described the first GPCR-specific antagonistic nanobodies against the chemokine receptor CXCR4 (Jähnichen *et al.*, 2010). 238D2 and 238D4 were generated and their epitopes were localized mainly in EL2 but also EL3 loop of CXCR4, with one amino acid common to both different epitopes. Both nanobodies showed high affinity towards CXCR4, with inhibition constants in [^{125}I]-CXCL12 displacement assays, signalling and chemotaxis assays in the nanomolar range. In addition, both nanobodies proved to behave as competitive antagonists. Interestingly, when both 238D2 and 238D4 monovalent nanobodies were linked together, the affinity of the resulting biparatopic nanobody increased almost 30 times compared to its monovalent counterparts, reaching a K_i value of 0.35 nM. The affinity of this biparatopic single domain antibody was 100-fold higher than the CXCR4 benchmark compound AMD3100. Preclinical characterization of the CXCR4 nanobodies also demonstrated their ability to fully inhibit entry of CXCR4-tropic HIV-1 strains *in vitro*. Finally, a single intravenous injection of biparatopic nanobody resulted in stem cell mobilization in cynomolgus monkeys, to a similar extent as AMD3100 (Jähnichen *et al.*, 2010). Currently, CXCR4 nanobodies are in phase 1 clinical studies for use in stem cell mobilisation (http://www.ablynx.com/wp-content/uploads/2010/11/7_ALX-06511.pdf). It will be interesting to see the potential of these molecules and their therapeutical benefit compared to the AMD3100/Pleraxifor drug currently used in the clinic.

A VHH antibody fragment against the atypical chemokine receptor Duffy antigen receptor for chemokines (DARC) has also been reported. Unlike the CXCR4 nanobody, the DARC VHH fragment has been generated solely against the N-terminal domain of the receptor (Smolarek *et al.*, 2010). The CA52 nanobody binds to DARC with a very high affinity of 0.2 nM. It displaces the endogenous DARC ligand CXCL8 and was able to prevent the infection of red blood cells by Plasmodium vivax. As such, this nanobody may serve as a basis for the development of therapeutics against malaria (Handel and Horuk, 2010; Smolarek *et al.*, 2010).

Crystal structures of CXCR4 and their impact on chemokine receptor-targeted drug design

The determination of the first GPCR crystal structure of bovine rhodopsin (bRho) in 2000 was a milestone in GPCR

B&W	1.35	1.36	1.39	2.39	2.40	2.56	2.57	2.58	2.60	2.63	2.64	3.28	3.29	3.32	3.33	3.49	4.60	45.50	45.51	5.35	5.39	5.42	6.33	6.48	6.51	6.55	6.58	7.31	7.32	7.35	7.39	7.40	7.42	7.43	7.53	7.59
CCR1	L	P	Y	T	S	T	L	P	W	Y	K	L	S	Y	Y	D	G	C	S	K	A	L	A	W	Y	I	S	L	D	V	E	V	A	Y	Y	R
CCR2	L	P	Y	T	D	T	L	P	W	S	A	F	T	Y	H	D	G	C	G	N	T	R	A	W	Y	I	N	L	D	T	E	T	G	M	Y	K
CCR3	V	P	Y	T	N	T	L	P	W	Y	V	L	S	Y	H	D	E	C	S	R	T	M	A	W	Y	I	S	L	D	M	E	V	A	Y	Y	R
CCR4	L	P	Y	T	D	S	L	P	W	Y	A	I	S	Y	L	D	G	C	K	K	S	I	A	W	Y	L	E	L	D	I	E	T	A	F	Y	K
CCR5	L	P	Y	T	D	T	V	P	W	Y	A	L	T	Y	F	D	G	C	S	K	T	I	A	W	Y	L	N	L	D	M	E	T	G	M	Y	K
CCR6	V	P	Y	T	D	T	L	P	W	S	H	L	K	Y	A	D	T	C	E	K	L	E	A	Q	H	L	T	I	G	K	E	V	A	F	Y	K
CCR7	L	P	Y	T	D	T	L	P	W	S	A	I	F	Y	K	D	E	C	S	F	Q	Q	A	Q	Y	V	Q	L	N	Y	Y	S	A	C	Y	K
CCR8	L	A	Y	T	D	S	F	P	Q	Y	L	V	S	Y	Y	D	L	C	Y	K	N	M	A	W	F	L	T	L	T	T	E	I	S	F	Y	K
CCR9	L	P	Y	T	D	T	L	P	W	A	A	V	N	Y	K	D	E	C	T	K	L	K	A	Q	Y	L	Q	I	D	F	Q	T	A	F	Y	R
CCR10	Q	P	S	T	S	T	L	P	A	G	A	I	S	Y	S	D	A	C	R	K	A	Q	A	Q	Y	L	D	K	D	L	S	G	A	L	Y	R
CXCR1	V	I	Y	T	D	T	L	P	W	S	K	V	S	K	E	D	F	C	Y	R	R	P	A	W	Y	L	D	I	G	L	E	I	G	F	Y	N
CXCR2	V	V	Y	T	D	T	L	P	W	S	K	V	S	K	E	D	V	C	Y	R	R	P	A	W	Y	L	D	I	D	L	E	I	G	I	Y	K
CXCR3	L	P	Y	T	D	T	L	P	W	D	A	A	G	F	N	D	D	C	Q	R	R	Q	A	W	Y	V	D	V	D	K	S	G	G	Y	Y	K
CXCR4	L	P	Y	T	D	T	L	P	W	D	A	V	H	Y	T	D	D	C	D	V	Q	H	A	W	Y	I	D	V	H	I	E	A	A	F	Y	K
CXCR5	V	P	Y	T	E	I	L	P	A	E	G	V	I	H	K	D	E	C	T	W	R	Y	A	W	Y	I	D	L	P	I	E	F	G	L	Y	K
CXCR6	L	P	Y	T	D	T	L	P	W	A	G	L	L	Y	T	D	Q	C	G	S	L	Q	S	Q	F	K	R	F	H	I	E	A	A	Y	Y	K
CXCR7	L	S	Y	T	H	T	I	P	W	S	L	T	H	F	S	D	D	C	R	L	E	S	S	W	Y	V	D	L	F	L	Q	C	S	L	Y	N
US28	T	L	Y	G	D	T	L	P	W	Y	L	L	T	F	Y	D	R	C	M	P	N	L	I	W	Y	L	D	L	K	L	E	S	A	F	Y	K

TMS1 (minor)
interface
TMS2 (major)
intracellular

Mutation of these residues significantly affect ligand binding
contact with any ligand (crystal)
X Specific contact with IT1t
X Specific contact with CVX15
X contact with both IT1t and CVX15

Figure 5

Alignment of important amino acid residues of TM domains and EL2. The TM residues are shown using the Ballesteros–Weinstein (B&W) numbering scheme (Ballesteros and Weinstein, 1995). An adapted version is also used for the EL2 residues (45.50 and 45.51) indicating residues in the loop between TM4 and TM5, using the conserved cysteine as reference: 45.50 (de Graaf *et al.*, 2008). Mutation of residues highlighted in gray significantly affected ligand binding. Also the TXP motif is shown (2.56–2.58), which is conserved among chemokine receptors (see text for details). The residues are coloured blue in case of TMS1 residues (minor pocket), orange for TMS2 residues (major pocket), yellow for interface residues between TMS1 and TMS2, and green for intracellular residues. For CXCR4, contacts from the crystal structures of the receptor with ligands are indicated with a thick black border. Magenta indicates contacts for the small-molecule IT1t, green for the peptide CVX15, and orange for both ligands. For more detailed information on the residues, ligands and associated literature references, see Table 1.

research (Palczewski *et al.*, 2000). The bRho structure served as template for *in silico* GPCR homology modelling, including chemokine receptors and structure-based drug design (de Graaf and Rognan, 2009). About 3 years ago the first structures of liganded GPCRs [i.e. ADRB1/2, and adenosine A_{2A} receptor (AA2AR)] were reported (Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008), which were subsequently used as new GPCR modelling templates. While the recently solved DRD3 crystal structure (Chien *et al.*, 2010) gives insights into bioaminergic receptor *subtype* specificity (by comparison with ADRB1/2), the recently solved CXCR4 chemokine receptor crystal structures (Wu *et al.*, 2010) open up new possibilities for structure-based drug design on the chemokine receptor family. One crystal structure has been elucidated with a large cyclic peptide CVX15 and several crystal structures have been elucidated with the small-molecule antagonist IT1t (Wu *et al.*, 2010).

The GPCR structure features seven TM helices and one intracellular helix (Hx8). Traditionally, the GPCR TM bundle is categorized in two subpockets in which ligands can reside. These are a minor pocket comprised of TMs 1, 2, 3 and 7 (TMS1) (Figure 4E), and a major pocket comprised of TMs 3, 4, 5, 6 and 7 (TMS2) (Surgand *et al.*, 2006) (Figure 4F). While the ligands in the bRho, ADRB1, ADRB2, AA2AR and DRD3 co-crystal structures all occupy TMS2, the CXCR4 crystal structures show for the first time ligand binding to not only TMS2 (CVX15) but also TMS1 (IT1t) (Figure 6A).

The cyclic peptide CVX15 resides in TMS2 and, due to its size, points out of the TM domain towards the extracellular side of the protein (Figure 6B). The peptide makes ionic interactions with D171$^{4.60}$ and D262$^{6.58}$ similar to other CXCR4 ligands that bind to TMS2 (Table 1, Figure 4F), and makes additional interactions with D187$^{45.51}$, D193$^{45.57}$ and E277$^{7.28}$ in the extracellular region (Figure 6B). The CXCR4 crystal structures with the antagonist IT1t are unique in the sense that they are the first to portray a ligand binding to TMS1 (Figures 4E and 6D). It forms ionic interactions with D97$^{2.63}$ and E288$^{7.39}$, the latter being a highly conserved binding partner in other chemokine receptors (Figure 5). The CXCR4 crystal structures as well as site-directed mutagenesis data of other chemokine receptors and their ligands (i.e. TAK-779 and AMD11070, Table 1) show that both pockets (TMS1 and TMS2) are interconnected. The existence of different ligand-binding sites makes the structure-based design of small-molecule ligands for chemokine receptors challenging.

Next to the novel ligand-binding modes, the CXCR4 crystal structures portray several other differences to those of previously resolved GPCRs. First, TM2 of the chemokine receptor family possesses a unique S/TXP motif (Figure 5: residues 2.56–2.58) which induces a unique helical kink to position the two ligand-binding residues W94$^{2.60}$ (Q in CCR8) and D97$^{2.63}$ (S or Y in other chemokine receptors) into TMS1 instead of towards the membrane layer as in the bRho, ADRB2, DRD3 and AA2AR crystal structures (Palczewski *et al.*,

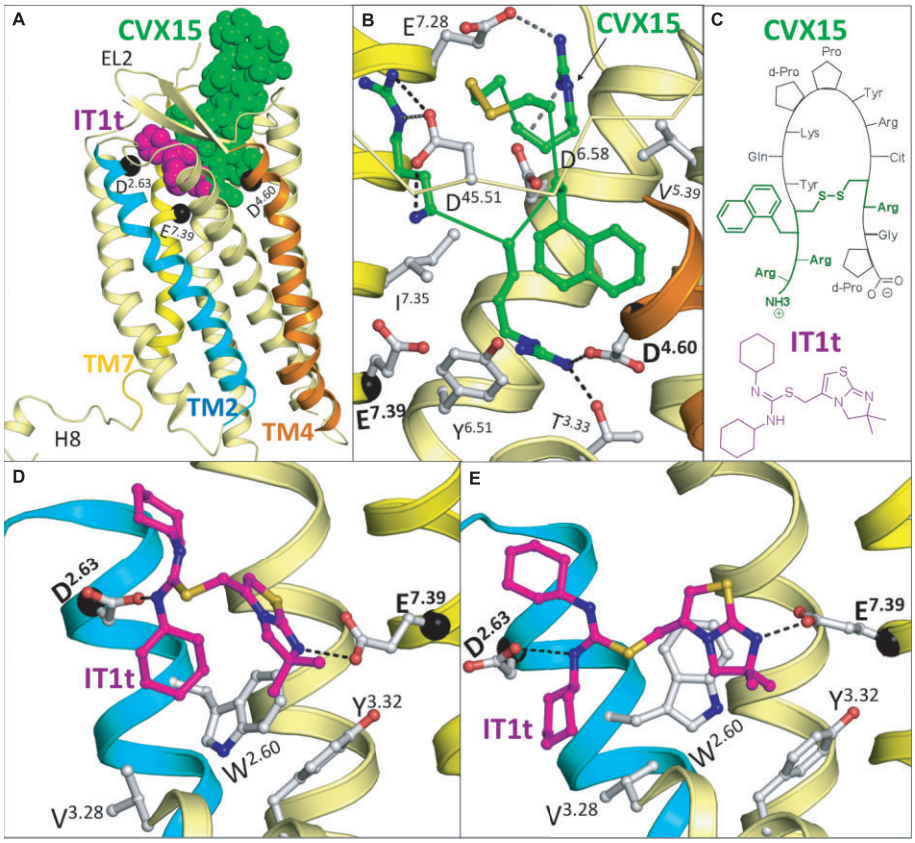


Figure 6

Crystal structures of CXCR4 receptors complexed with ligands. (A) Comparison of the ligand binding modes of the small-molecule antagonist IT1t (magenta, 3odu) and the peptide-like ligand CVX15 (green, 3oe0) in the (IT1t bound) CXCR4 crystal structure. While IT1t binds in the TMS1 (minor pocket) between TM helices 1, 2, 3 and 7, CVX15 binds primarily in TMS2 (major pocket) between TMs 3, 4, 5, 6 and 7. TMs 2, 4 and 7 are coloured cyan, yellow and orange respectively, and Cα atoms of residues D2.63 (TMS1), D4.60 (TMS2) and E7.39 (interface between TMS1 and TMS2) are indicated with black spheres (like in panels B, D and E). Furthermore, the beta sheet of EL2 and the disordered helix 8 are labelled. (B) Interactions between the residues 1–4 and 13–14 of CVX15 (green side chain in ball-and-stick, backbone as ribbon) and CXCR4 (3oe0). Important residues are displayed as ball-and-stick (grey carbon atoms), while CVX15-1T1t H-bond and ionic interactions are indicated with black and grey dashed lines respectively. Nitrogen, oxygen and sulphur atoms are coloured blue, red and yellow respectively. For reasons of clarity the top of TM3 is not shown, while only the first β strand of EL2 is displayed. (C) Chemical structures of IT1t and CVX15 (part of molecule displayed in panel B is coloured green). (D,E) Comparison of the interactions between IT1t (magenta carbon atoms) and CXCR4 in the experimentally determined X-ray crystal structure (3odu, panel D) and the best *in silico* CXCR4 model in the worldwide GPCR DOCK 2010 competition (panel E) correctly predicting the highest number of IT1t-CXCR4 contacts (prior to release of the CXCR4-IT1t crystal structure). Important residues are displayed as ball-and-stick (grey carbon atoms), while IT1t-CXCR4 H-bonds are indicated with black dashed lines. Colour coding of helices and heteroatoms are the same as defined in panels A and B. For reasons of clarity the top of TM3 is not shown.

2000; Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Chien *et al.*, 2010). This alternative kink of TM2 is supported by site-directed mutagenesis data probing the TM2–TM3 interface (Govaerts *et al.*, 2001) and receptor–ligand interactions (Table 1) and is in line with earlier predictions of TM flexibility (Deupi *et al.*, 2004).

Secondly, the crystal structures of ligand IT1t show a disordered Hx8 (Wu *et al.*, 2010). The impact of this distortion on future modelling attempts based on the CXCR4 crystal structures is that models for ligands that bind to a putative intracellular pocket (Figure 4D) will be difficult to construct since they contact TM7 (e.g. SCH-527123, SB265610 in CXCR2, see section on intracellular binding sites and Table 1).

Thirdly, EL2 of CXCR4 contains the same β strand in the crystal structure as observed in rhodopsin, but is oriented outward (Figure 6A), to accommodate the large peptide ligand or Hx8 of the CXCR4 monomer, demonstrating the plasticity of this loop to fold in an open or closed conformation.

Before the release of CXCR4-CVX15 and CXCR4-IT1t structures (Wu *et al.*, 2010), the scientific community was invited to submit a structural model of CXCR4 in complex with either CVX15 or IT1t in the open challenge GPCR-DOCK2010 (Kufareva *et al.*, 2011). Given the differences with previously released GPCR crystal structures and the lack of conclusive experimental data to guide the modelling procedure, correct prediction of the CXCR4 fold and CXCR4–ligand interactions was shown to be highly challenging. Our model of the CXCR4–IT1t complex (Figure 6E) was in fact the only model out of 103 models (from 25 different groups) which correctly captured both ionic interactions with D97$^{2.63}$

and E288$^{7.39}$, placing the dicyclohexylthiourea between TM2 (W94$^{2.60}$, ionic interaction with D97$^{2.63}$), TM3 (V112$^{3.28}$) and EL2 (I190$^{45.54}$), and the imidazothiazole ring between TM2 (W94$^{2.60}$) and TM3 (cation–π interaction with Y116$^{3.32}$), and TM7 (ionic interaction with E288$^{7.39}$). Although the overall position of the ligand was more shifted towards TM2 and TM3, the model was the only model that identified more than 20% of the ligand–receptor atomic contacts in the crystal structure (Figure 6D,E) (Kufareva *et al.*, 2011). The TM bundle of our CXCR4 model was constructed based on the ADRB2 crystal structure (Cherezov *et al.*, 2007) (next to ADRB1, the structural template with the highest resolution to CXCR4) and TM2 was modelled in an alternative kink, stabilized by the chemokine receptor specific TXP motif (Govaerts *et al.*, 2001; Kellenberger *et al.*, 2007; Shamovsky *et al.*, 2009) and orienting W94$^{2.60}$ and D97$^{2.63}$ towards the ligand-binding pocket. This modelling procedure was guided by site-directed mutagenesis data probing the TM2–TM3 interface (Govaerts *et al.*, 2001) and receptor–ligand interactions (Table 1), including the experimentally determined involvement of D97$^{2.63}$ in the binding of other CXCR4 ligands (Wong *et al.*, 2008). We docked IT1t into the CXCR4 TM bundle in line with ligand-binding mode hypotheses (de Graaf and Rognan, 2009) matching the essential positively ionizable thiorea and imidathiazole groups of IT1t (Thoma *et al.*, 2008) with the negatively charged carboxylate groups of D97$^{2.63}$, D171$^{4.60}$, D262$^{6.58}$ and E288$^{7.39}$ (Wong *et al.*, 2008). Furthermore, the steep structure–activity relationship around the dimethyl-imidathiazole group (Thoma *et al.*, 2008) supported the tight cation–π stacking interaction with Y116$^{3.32}$ at the bottom of TMS1 (Figure 6D,E). It should be noted that none of the CXCR4-CVX15 models predicted the important interactions in the crystal structure, indicating that accurate prediction of flexible peptide ligands in large receptor-binding pockets (without the availability of experimentally supported receptor–ligand interaction anchors) is currently beyond the reach of molecular modelling approaches. The low accuracy of the loop predictions in the GPCRdock challenge, compared to the reasonable accuracy of the predicted fold of the TM helices (Kufareva *et al.*, 2011), is furthermore in line with a previous evaluation of the implication of EL2 modelling on structure-based virtual screening (de Graaf *et al.*, 2008).

The binding modes of ligands in earlier ADRB1/2 homology models generally resemble the binding orientations of ligands in the respective crystal structures (Cherezov *et al.*, 2007; 2010; de Graaf and Rognan, 2009), and the recently solved DRD3-ligand co-crystal structure could be correctly predicted based on the closely related ADRB1/2 crystal structures (Kufareva *et al.*, 2011). The use of experimental anchors to guide the construction of a AA2AR-ligand crystal structure complex were, however, somewhat misleading (de Graaf and Rognan, 2009), while the spatial distribution of experimentally determined interaction partners in CXCR4 (negatively ionizable residues D97$^{2.63}$, D171$^{4.60}$, D262$^{6.58}$ and E288$^{7.39}$) (Wong *et al.*, 2008) and its ligand (two positively ionizable groups) allows the definition of several alternative binding modes. The success of our customized CXCR4 modelling approach demonstrates that a careful consideration of experimental data (site-directed mutagenesis data in combination with structure–activity relationships) is essential in predicting

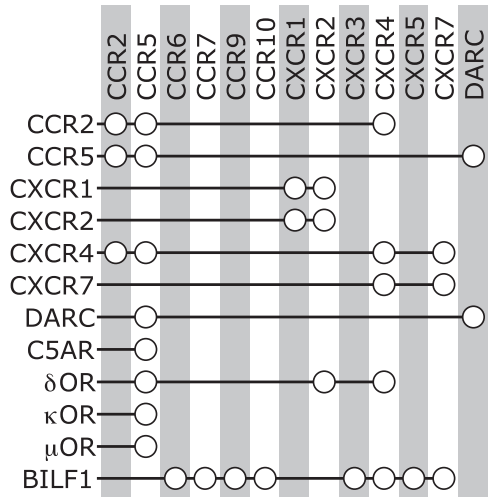


Figure 7

GPCR dimerization partners of chemokine receptors. The open circles connect the chemokine receptors with their suggested dimerization partners.

protein structure and protein–ligand interactions (de Graaf and Rognan, 2009). Finally it should be noted that customized chemokine receptor models based on bRho and ADRB2 crystal structures have already been successfully used to identify new ligands for CCR2 (Kim *et al.*, 2011), CCR3 (Becker *et al.*, 2004), CCR4 (Bayry *et al.*, 2008; Davies *et al.*, 2009), CCR5 (Kellenberger *et al.*, 2007; Liu *et al.*, 2008), CXCR4 (Perez-Nueno *et al.*, 2009) and CXCR7 (Jones *et al.*, 2006) in the past. Therefore, it is expected that the novel crystallographic data on CXCR4 will improve the resolution of *in silico* models and aid the structure-based development of future drugs for targets belonging to the chemokine receptor family.

Cross-modulation within chemokine receptor oligomers

Although GPCRs can function as monomeric signalling units by coupling of intracellular heterotrimeric G proteins or β-arrestins upon agonist binding to their extracellular surface in a 1:1:1 stoichiometry (Whorton *et al.*, 2007; Kuszak *et al.*, 2009; Arcemisbehere *et al.*, 2010; Tsukamoto *et al.*, 2010), accumulating evidence suggest that GPCRs are assembled in homo- and/or heteromeric complexes for at least part of their lifetime (Pin *et al.*, 2007; Milligan, 2009; Lohse, 2010). Several examples of homo- and heteromeric interactions between chemokine receptors, but also between chemokine receptors and other GPCR subtypes have been reported in the last decade (Figure 7).

Initial cross-linking experiments suggested that chemokines induce homo- and/or heteromerization of CCR2, CCR5 and CXCR4 (Rodriguez-Frade *et al.*, 1999; Vila-Coro *et al.*, 1999; 2000; Mellado *et al.*, 2001; Hernanz-Falcon *et al.*, 2004; Rodriguez-Frade *et al.*, 2004). However, more recent co-immunoprecipitation, resonance energy transfer (RET) and protein complementation assay (PCA)-based studies

revealed that all tested chemokine receptors oligomerize in a ligand-independent manner (Issafras *et al.*, 2002; Babcock *et al.*, 2003; El-Asmar *et al.*, 2005; Wilson *et al.*, 2005; Sohy *et al.*, 2009). The latter fits well with the current dogma that GPCR oligomers are formed during protein biosynthesis and maturation in the endoplasmic reticulum (ER) to facilitate appropriate protein folding and cell surface targeting (Milligan, 2010). On the other hand, fusion of an ER-retention motif to the C-tail of CXCR2 not only impaired its own trafficking to the cell surface, but also of co-expressed wild-type CXCR1 and CXCR2 through the formation of heteromeric complexes (Wilson *et al.*, 2005). Similar entrapment of wild-type CCR5 by the dominant negative CCR5Δ32 truncation mutant was proposed to explain the delayed progression of HIV-1 infection in heterozygous individuals (Benkirane *et al.*, 1997), although others raised scepticism on the dominant negative nature of this observation (Venkatesan *et al.*, 2002).

Recent studies using fluorescence recovery after photobleaching (FRAP) and total internal reflection fluorescence (TIRF) microscopy revealed that some GPCR subtypes are engaged in short-living transient homodimers that are formed and fall apart within seconds in a ligand-independent manner, whereas others are assembled in stable higher order oligomers at the cell surface (Dorsch *et al.*, 2009; Fonseca and Lambert, 2009; Hern *et al.*, 2010). Similar FRAP or TIRF microscopy evaluation of chemokine receptor oligomer stability remains to be performed. However, CXCR4 and δ-opioid receptors were proposed to exist in a ligand-dependent dynamic equilibrium between homo- and heteromers (Pello *et al.*, 2008). Stimulation with either CXCL12 or [D-Pen2, D-Pen5]-enkephalin shifted this equilibrium towards signalling homomers, whereas simultaneous addition of both agonists induced the formation of signalling-deficient heteromers.

Heteromerization of the non-G protein-signalling chemokine receptors CXCR7 and DARC with CXCR4 and CCR5, respectively, blunted chemokine-induced G protein signalling by the latter two receptors (Chakera *et al.*, 2008; Levoye *et al.*, 2009). On the other hand, heteromerization of CCR5 with CCR2 or CXCR4 shifted G protein coupling from G_i- towards G_q-mediated signalling pathways resulting in cell adhesion rather than chemotaxis (Mellado *et al.*, 2001; Molon *et al.*, 2005; Contento *et al.*, 2008). Recruitment of CCR5/CXCR4 heteromers to the immunological synapse stabilizes the interaction of T cell with antigen-presenting cells in response to chemokine secretion by the latter (Mellado *et al.*, 2001; Molon *et al.*, 2005; Contento *et al.*, 2008).

Chemokines can induce changes in basal RET and/or PCA signals, which are generally interpreted as conformational rearrangements within existing chemokine receptor oligomers, rather than de novo formation or dissociation of oligomers (Toth *et al.*, 2004; Percherancier *et al.*, 2005; Sohy *et al.*, 2007; Chakera *et al.*, 2008; Isik *et al.*, 2008; Levoye *et al.*, 2009; Luker *et al.*, 2009). These conformational rearrangements result in allosterism between individual chemokine receptors within oligomers. Negative chemokine binding cooperativity has been observed within CCR2, CCR5 and CXCR4 heteromers in equilibrium (competition) binding and 'infinite' radioligand dilution experiments (El-Asmar *et al.*, 2005; Springael *et al.*, 2006; Sohy *et al.*, 2007; 2009). Assuming that chemokine affinities for their cognate receptors are within the same order of magnitude (Murphy *et al.*, 2011), these heteromeric chemokine receptor configurations allow cells to respond to the highest chemokine (gradient) concentration at the expense of other chemokine subtypes by allosterically inhibiting their interaction to partnering receptors. Moreover, low-molecular-weight (allosteric) antagonists acting at one receptor can cross-inhibit *in vitro* and *in vivo* chemokine-induced immune cell recruitment mediated through the other chemokine receptor within the heteromer (Sohy *et al.*, 2007; 2009). In contrast, however, the CXCR2 antagonist SB225002 increased signalling of CXCR2/δOR heteromers in response to opioid agonists (Parenty *et al.*, 2008). Hence, this allosteric cross-modulation should be kept in mind when screening for ligands against a particular chemokine receptor to avoid side effects through heteromerized receptor partners. On the other hand, one can also take advantage of allosteric modulation by targeting a widely abundant receptor in a more cell type-specific manner through its less widely expressed heteromeric partner. Moreover, future development of chemokine receptor heteromer-selective (bivalent) ligands may also contribute to the specific targeting of a smaller subset of cells (Waldhoer *et al.*, 2005; Jähnichen *et al.*, 2010; Tanaka *et al.*, 2010; Zhang *et al.*, 2010).

Summary

In summary, the family of chemokines receptors is a perfect showcase for the GPCR family to illustrate the effectiveness of GPCR targeting with small-molecule allosteric modulators and/or biologicals. This is underscored by the recent FDA approval of two small molecules targeting CXCR4 and CCR5. Whereas most biologicals are considered to target extracellular parts of the receptor, the present data on binding modes of the small-molecule antagonists and chemokines suggests that competing with or blocking the entrance of the N-terminus of a chemokine to TM residues poses a mechanism of allosteric (or partially competitive) inhibition of chemokine-mediated receptor activation for many of the chemokine receptor antagonists binding to the classical TMS1- and TMS2-binding pockets (Table 1, Figure 4). As this region is generally not important for chemokine binding, using the term allosterism is, in our opinion, justified. Whether these interactions are purely allosteric or partially competitive largely depends on the used chemokine probe and its specific receptor interactions. There is no discussion needed on the allosteric nature of chemokine receptor antagonists suggested to bind to intracellular binding sites of CXCR2 or CCR4. It remains to be established whether other chemokine receptors or other members of the large GPCR family can be modulated in a similar manner, but the two examples are intriguing. Similarly intriguing is the possibility to specifically target chemokine receptor heterodimers. We would like to stress though, that the evidence for such a mechanism of action of small-molecule modulator still remains to be established.

Targeting chemokine receptors in a functionally selective manner, as suggested to be feasible for CCR5 and CXCR4 (Sachpatzidis *et al.*, 2003; Watson *et al.*, 2005; Saita *et al.*, 2006), is a further promise for future drug discovery. The association of specific signalling pathways with disease or

adverse drug effects is starting to emerge, and the overall challenge remains to identify what signalling pathway to target in a particular disease. On the other hand, insights in the structure activity relationships governing functional selectivity is needed, and *in vivo* studies will have to shed more light on the potential of functional selective ligands in the treatment of chemokine-related disease.

Finally, the recent breakthrough of the CXCR4 crystal structure will give a strong impetus to additional receptor crystallization, mutagenesis, modelling and pharmacological studies, which will be essential to delineate the mechanism of action of the various small-molecule allosteric modulators and/or biologicals.

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Conflicts of interest

None.

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