Opportunities for novel therapeutic agents acting at chemokine receptors

John Saunders and Christine M. Tarby

Chemokines are proinflammatory mediators that primarily control leukocyte migration into selected tissues and upregulation of adhesion receptors. They also have a role in pathological conditions that require neovascularization and are implicated in the suppression of viral replication. By interaction with their respective G-protein-coupled receptor, chemokines have a profound influence over the selective recruitment of specific cell types in acute inflammatory disease and, hence, inhibition of their action should be of therapeutic benefit. Only now are small molecule inhibitors becoming available to validate this speculation. In this review, without seeking to be comprehensive, the authors provide an introduction to chemokines, their receptors and their role in certain disease processes. Also, recent disclosures claiming novel nonpeptide ligands for chemokine receptors are summarized.

Over the past five years there has been an explosion in the number of isolated low molecular weight proteins called chemokines (chemotactic cytokines)[1]. These proteins are involved in a variety of inflammatory responses via the chemoattraction and activation of leukocytes. In response to certain stimuli or insult to the immune system, chemokines are secreted by proinflammatory cells, leukocytes or endothelial cells to recruit new leukocytes from the circulation across the lumen and into the tissue (Fig. 1) and, as such, have earned the nickname 'crossing guards of the immune system'. The universal *in vitro* biological property of these molecules is the ability to effect the chemotaxis of specific cell types, but other chemokine functions have more recently been uncovered. These include inhibition or promotion of angiogenesis, inhibition or induction of cellular proliferation and induction of integrin receptors (Table 1).

Classification of chemokines

The chemokine proteins are segmented into four families (two major and two minor) based on the location of the first conserved Cys residues within the protein. Across and within the families, these proteins are 20–90% homologous with each other (see table of chemokine sequence homology and predicted secondary structure at the Chemokine Information Source: http://www.expasy.ch/cgi-bin/ChemokineTop.pl). The CXC family, in which IL-8 (see Box 1 for glossary of abbreviations) is the representative member, is characterized by the two conserved Cys residues (C), near the N-terminus, separated by an amino acid (X). Some of the CXC chemokines belong to the ELR subfamily (i.e. containing Glu-Leu-Arg motif), which are important in the recruitment and activation of neutrophils. All members of this subfamily contain the ELR triad of amino acids immediately before the first N-terminal Cys in the chemokine protein. This group of amino acids is extremely sensitive to modification. A point mutation in the ELR motif of IL-8 dramatically effects chemokine activity and is somewhat detrimental to receptor binding, with Arg

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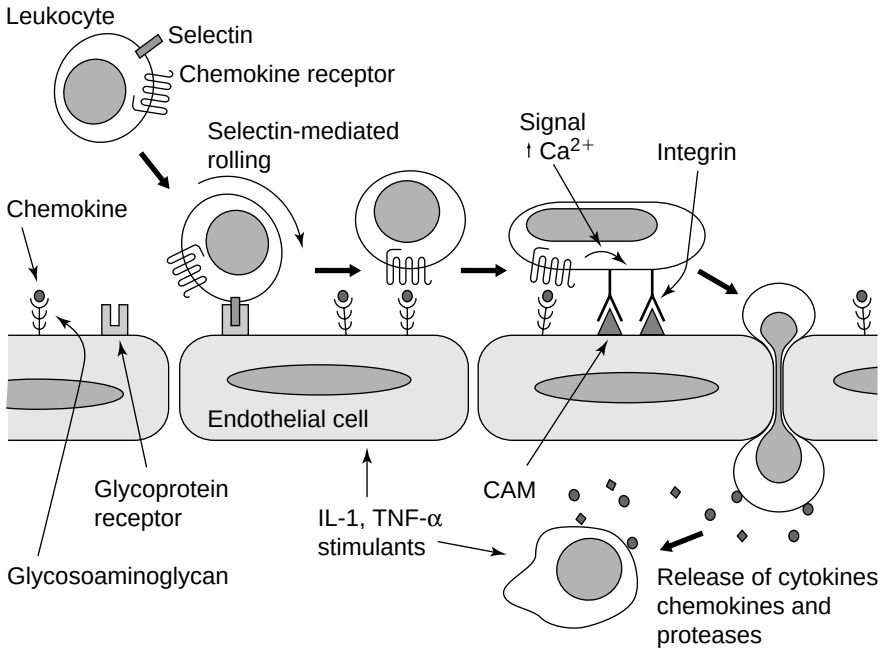


Figure 1. *Process of leukocyte migration. The secreted chemokine is putatively bound by the extracellular glycosoaminocglycan matrix of the endothelial cell for display to leukocytes rolling along the endothelial wall. During post selectin-mediated rolling of the leukocyte, chemokines bind to a 7-transmembrane receptor on its surface inducing firm adhesion to the endothelium via upregulation of integrin receptors. The adhered leukocyte transmigrates into the tissue where it can secrete inflammatory cytokines, chemokines and degradative proteases that act on the subendothelial matrix facilitating the migration of additional leukocytes to the site of injury. CAM, cellular adhesion molecule; GAG, glycosaminoglycan; TNF-α, tumor necrosis factor α.*

being the most critical residue of the three for receptor affinity (1000-fold loss upon mutation)[2]. The non-ELR-containing CXC chemokines are not chemotactic for neutrophils but, rather, act on lymphocytes. In angiogenesis, the presence or absence of the ELR motif modulates the chemokines functionality. ELR-containing CXC chemokines (such as IL-8 and GRO-α) promote angiogenesis. The non-ELR-containing IP-10 and PF4 are angiostatic even in the presence of angiogenic factors.

In the CC chemokine family, the first pair of conserved Cys residues are located next to each other, hence the CC designation. Some prominent members of this class include MCP-1, RANTES and the MIP-1 subfamily of proteins. Most of the CC chemokines genes have been localized to human chromosome 17, while those of the CXC chemokine can be found on chromosome 4. This chemokine family is primarily involved in the recruitment of monocytes, macrophages and B and T cells. However, MIP-1α and a newly discovered member, LKTN-1, have been shown to attract neutrophils *in vitro*[3].

More recently, two newer chemokine classes have been elucidated: the C chemokines – lacking two of the four Cys residues – represented by SCM-1/lymphotactin[4] and the CXXXC chemokines – where the N-terminal cysteines are separated by three amino acid residues – of which fractalkine/neurotactin is the sole representative[5,6]. Fractalkine is unique in that it is a membrane-bound chemokine. Furthermore, endothelial-bound fractalkine can induce firm adhesion of monocytes and T cells, independently of upregulation of the integrin receptors[7].

The conserved Cys residues in all chemokine classes are involved in disulphide-bond formation, stabilizing the solution structure of the protein. Although the CXC and CC chemokine families have approximately the same secondary structure – three β-sheets and one α-helix – and tertiary structure (Fig. 2), the quaternary structures as determined by solution-phase NMR analysis of the parent molecules for each family are quite different. This can be illustrated by examining the minimized composite NMR structures of the IL-8 and MCP-1 dimers (Fig. 2). Figure 2 depicts the overall secondary structure for a single chain in each of the dimers shown. In the dimer structures, the disulphide C→C bonds (for IL-8: C9→C50 and C7→C34; for MCP-1: C11→C36 and C12→C52) stabilizing the loop to the β-sheet structure are displayed. The important ELR motif for several CXC chemokines is also shown on the IL-8 structure, located in the N-terminal coil region. In the CXC chemokines, the overall quaternary structure is formed by a β-sheet dimer with the two α-helices sitting on top of the six antiparallel β-sheet. In the CC chemokines, the solution structure is characterized as an end-on-end dimer.

The solution structures of these proteins may not necessarily represent their true binding mode. Recall that the chemokines are putatively displayed for binding to the receptors on the glycosoaminoglycan (GAG) surface of the endothelium, and the manner in which the chemokine is

Table 1. *In vitro* properties of the chemokine ligands

Type	Chemokine[a]	Receptor	Target[b]	Expression
CXC	IL-8 (I)	CXCR1, CXCR2	Neutrophils, T cells, basophils[c], eosinophils, keratinocytes[d], HUVECs, endothelial cells[e], NK cells	Monocytes, macrophages, lymphocytes, epithelial cells, T cells, epidermal keratinocytes, some tumor cells
CXC	GRO-α (I)	CXCR2	Neutrophils, lymphocytes, monocytes, epidermal melanocytes[h], endothelial cells[e], erythrocytes[f]	Monocytes, lung, macrophages, neutrophils, endothelial cells, epidermal keratinocytes, some tumor cells
CXC	Nap-2 (I)	CXCR2	Neutrophils, basophils, esosinophils, NK cells, fibroblasts, megakaryocytes, endothelial cells	Monocytes, intestine, pancreas, lung
CXC	ENA-78 (I)	CXCR2	Neutrophils	Epithelial cells, platelets
CXC	IP-10 (I)	CXCR3	Monocytes, T cells, stem cells[i], endothelial cells[g], NK cells	Epithelial cells monocytes, keratinocytes, fibroblasts, spleen, thymus, lymph nodes
CXC	PF4	CXCR3	Monocytes, endothelial cells[g], stem cells[i], basophils[c], megakayocytes[i], fibroblasts	Platelets, megakayocytes
CXC	MIG (I)	CXCR3	Monocytes, act. T cells, NK cells	Macrophages, monocytes
CXC	GRO-β (I)	CXCR2	Neutrophils, basophils, epithelial cells	Mast cells, epidermal cells, heart, lung
CXC	GCP-2 (I)	CXCR1, CXCR2	Granulocytes, neutrophils	Osteocarcinoma, kidney tumor cells
CXC	SDF-1 (C)	CXCR4	Pre-B cells, lymphocytes, monocytes	Stromal cells, bone marrow, liver, muscle
CC	MIP-1α (I)	CCR1,CCR3, CCR4, CCR5	T cells, basophils[c], monocytes, eosinophils, neutrophils, mast cells[c], stem cells[i], B cells, dendritic cells, keratinocytes[m], astrocytes[m]	Fibroblasts, monocytes, lymphocytes, neutrophils eosinophils smooth muscle cells, mast cells, platelets, bone marrow stromal cells, glial cells, epithelial cells
CC	MIP-1β (I)	CCR1, CCR3, CCR5, CCR8	Monocytes, T cells, basophils[c], hematopoietic stem cells[m]	Monocytes, fibroblasts, T cells, B cells, neutrophils, smooth muscle cells, mast cells, some tumor cell lines
CC	RANTES (I)	CCR1,CCR3, CCR4, CCR5	Monocytes, T cells, basophils[c,j,n], NK cells, eosinophils, dendritic cells, mast cells[c]	T cells, epithelial cells, monocytes, fibroblasts, platelets, eosinophils
CC	MCP-1 (I)	CCR2, CCR4	Monocytes, T cells, mast cells[c], basophils[c,j], stem cells[i], eosinophils[k], NK cells, cardiac myocytes[l]	Fibroblasts, monocytes, macrophages, endothelial cells, muscle cells, heart, intestine, kidney, liver fat storing cells, bone marrow stromal cells, astrocytes, some tumor cells
CC	MCP-2 (I)	CCR1, CCR2 CCR3, CCR5	T cells, monocytes, eosinophils, basophils[c], NK cells, mast cells[c]	Tumor cells, fibroblasts, PBMCs, small intestine
CC	MCP-3 (I)	CCR1, CCR2, CCR3	Monocytes, T cells, basophils[c], eosinophils, NK cells dendritic cells, neutrophils	Fibroblasts, platelets, mast cells, monocytes, osteosarcoma
CC	MCP-4 (I)	CCR2, CCR3, CCR5	Monocytes, T cells	Small intestine, colon, lung
CC	MCP-5 (I)	CCR2	PBMCs	Macrophages, lymph nodes
CC	Eotaxin (I)	CCR3	Eosinophils	Lung, Intestine stomach, heart, thymus, liver, spleen, kidney, testes, pancreas, prostate
CC	LARC/Exodus/ MIP-3α (I)	CCR6	Lymphocytes	Liver, lung, lymph nodes, appendix, fetal liver
CC	MDC/STCP-1 (C)	CCR4	T cells, dendritic cells, NK cells, monocytes	Macrophages, dendritic cells, thymus lymph nodes, appendix, monocytes,
CC	TARC (C)	CCR4	T cells	Thymus, PBMCs
CC	I-309 (I)	CCR8	Monocytes, basophils[c], neuroblastoma cells, macrophages, neutrophils, microglial cells	T cells, mast cells,
CC	ELC (C)	CCR7	Act. T cells and B cells	Thymus, lymph nodes, appendix, spleen
CC	LKTN-1	CCR1, CCR3	Lymphocytes, monocytes, neutrophils	
CC	SLC/Exodus-2	CCR7	T cells	Lymph nodes, small intestine
CXXXC	Fractalkine (C)	CX_3CR1	Monocytes, T cells, NK cells	Heart, brain, lung, muscle, kidney, pancreas
C	SCM-1/ lymphotactin	XCR1	T cells	Thymus, T cells, spleen

[a]The expression of chemokines is shown in parentheses: I, inflammatory; C, constitutive. See Box 1 for abbreviations.

[b]The predominant *in vitro* effect of chemokines on cells is chemotaxis, but other functions on particular cell types are provided as footnotes: [c]histamine release; [d]mitogenesis; [e]angiogenesis; [f]inhibits invasion by *Plasmodium;* [g]inhibition of angiogenesis; [h]induces cellular proliferation; [i]inhibition of colony formation; [j]leukotriene synthesis; [k]internal calcium mobilization; [l]induction of ICAM-1; [m]inhibition of proliferation; [n]mediator release. These data were compiled from Refs 1,3,5–7,30,66–109.

Box 1. Glossary

ELC, Ebl1-ligand chemokine
ENA-78, epithelial-derived neutrophil attractant 78
GCP-2, granulocyte chemotactic protein 2
GRO, growth related oncogene
ICAM-1, intracellular adhesion molecule 1
IL -8, interleukin 8
IFN-γ, interferon γ
IP-10, (IFN-γ)-inducible protein 10
LARC, liver and activation-regulated chemokine
LKTN-1, leukotactin 1
LTB-4, leukotriene B4
MAPK, mitogen-activated protein kinase
MCP, monocyte chemoattractant protein
MDC, macrophage-derived chemokine
MGSA, melanoma growth-stimulating activity
MIG, monokine induced by IFN-γ
MIP, macrophage inflammatory protein
NAP-2, neutrophil-activating protein 2
NF-κB, nuclear factor κ-binding
PBMC, peripheral blood mononucleocytes
PF4, platelet factor 4
PMN, polymorphonuclear leukocytes
RANTES, regulated on activation, normal T expressed and secreted
SCM-1 single cysteine motif 1
SDF-1, stromal cell-derived factor 1
SLC, secondary lymphoid tissue chemokine
STCP-1, stimulated T-cell chemotactic protein 1
TARC, thymus and activation-regulated chemokine

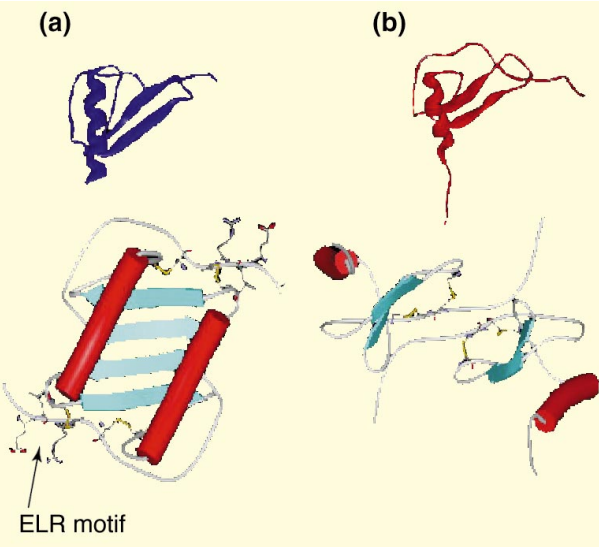


Figure 2. *Comparison of the solution structures of (a) interleukin 8 (IL-8) – a CXC ligand[64] – and (b) monocyte chemotactic protein 1 (MCP-1) – a CC ligand[65]. The figures of the monomer structures (top) were created from the dimer structures (bottom). These figure elements were retrieved from the Brookhaven data bank (http://www.pdb.bnl.gov/) and visualized by WebLab Viewer (Molecular Simulations, San Diego, CA, USA).*

presented to the receptor might be quite different from that inferred by the solution structures. Because relatively high concentrations of protein are needed for NMR structure determination, the dimeric structure is favored, but at physiologically relevant concentrations of chemokine, the monomeric protein is probably present, opening the debate as to which form of the chemokine is biologically relevant[8].

In general, the body of the chemokine seems to be primarily responsible for binding to the receptor and the GAG matrix, while the N-terminal regions are important for signaling. To understand how chemokines interact with their receptors and to facilitate the discovery of small-molecule inhibitors, several mutagenesis studies have elucidated key residues important for chemokine signaling and binding to the receptor. In the ELR-containing CXC chemokines, removal of the Glu of the ELR motif affects receptor binding to some degree but, more importantly, causes a complete loss of the protein's chemotactic and neutrophil-activating properties. As mentioned previously, Arg is the triad member most sensitive to mutation. Related CXC chemokines such as PF4 and IP10 are devoid of the ELR triad and have only weak activities for neutrophil IL-8 receptors. Substitution of the natural sequence DRL for ELR in PF4 confers IL-8-like behaviour on neutrophils. Even though mutation in the ELR triad has detrimental effects on receptor binding, the ELR sequence alone is insufficient for binding – short peptides carrying this motif do not compete with IL-8 at the CXCR1 receptor.

Site-directed mutagenesis studies on IL-8 have revealed another set of amino acids in which mutations affect receptor binding[9]. The second set (Phe17, Phe21, Ile22, Leu43) forms a solvent-accessible hydrophobic pocket that, in theory, could entropically drive the binding of IL-8 with CXCR1. In MCP-1 (a member of the CC family), the N-terminal deletion of the first eight amino acids results in a 9–76 mutant that is almost fully capable of binding to CCR2 (Ref. 10). However, no Ca^{2+} flux is observed upon mutant binding, clearly indicating that, as for the CXC chemokines, the important residues for signaling at the receptor precede the first disulphide linkage along the N-terminus. A similar result can be shown for another β-chemokine, RANTES. N-terminal deletion of the first eight

amino acids results in a mutant that prevents the infection of activated PBMCs by the HIV-1 virus by inhibiting access of the virus to CCR5 (Ref. 11).

RANTES is a typical CC chemokine that binds several chemokine receptors including CCR1, CCR3 and CCR5. Site-directed mutagenesis studies of RANTES have indicated that different sets of amino acids on the protein are responsible for interaction with the various receptors (i.e. for CCR1, R17; for CCR3, F12 and Y14; for CCR5, F12, P2 and I15). With the exception of P2, all of these residues are located on the N-loop, suggesting that the N-loop sequences confer the binding affinity and selectivity to the chemokine[11]. The P2 residue, which is necessary for RANTES binding to CCR5, sits near the N-terminus of the chemokine. However, in the dimer structure, the N-terminal arm of each chain is located near the N-loop of its partner. Perhaps this provides circumstantial evidence that the dimeric protein might be important to CCR5 binding.

Comparison of chemokine receptors with other GPCRs

Receptor structure and putative ligand-binding site(s)

By analogy with bacteriorhodopsin, members of the G-protein-coupled receptor (GPCR) superfamily (of which there are ~1000, based on genomic information) are thought to contain a seven-helical motif that traverses the cell membrane (Fig. 3) to form a central channel[12]. Together with the extracellular domains, this helical bundle is thought to provide the ligand-binding and activation sites, the precise location being determined by the nature of the ligand. All chemokine receptors share some common features, some of which are also shared by many other members of the GPCR superfamily. By inference, such features could be involved in ligand binding and receptor activation. All chemokine receptors have two conserved Cys residues, one in the N-terminal domain and the second within extracellular loop three; these seem to

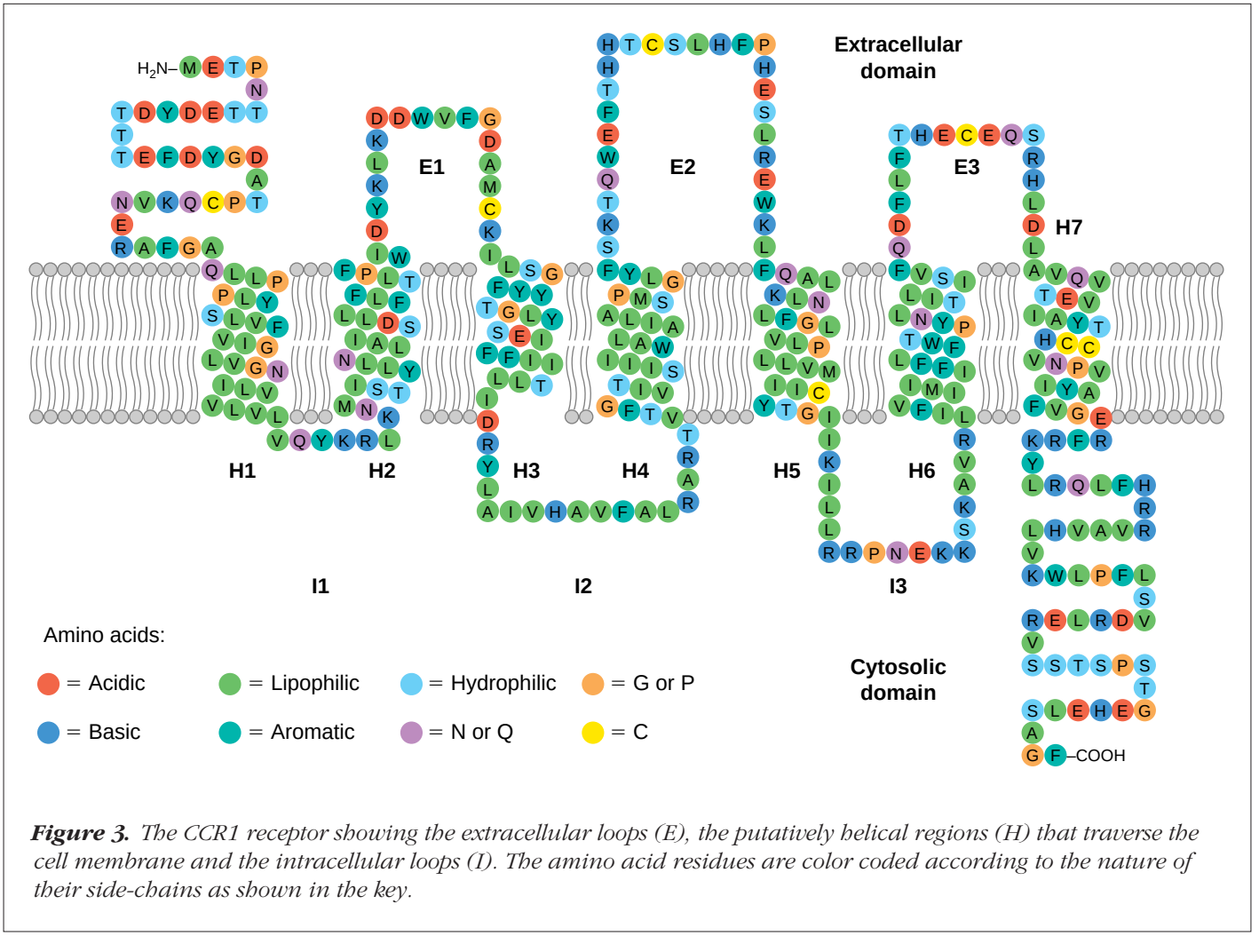


Figure 3. *The CCR1 receptor showing the extracellular loops (E), the putatively helical regions (H) that traverse the cell membrane and the intracellular loops (I). The amino acid residues are color coded according to the nature of their side-chains as shown in the key.*

be structurally forming a C→C bond that is an integral part of the ligand-recognition site. There are also two conserved acidic residues (CXCR3 excepted) within the helical bundle. The first, an Asp in helix 2, is intimately associated with receptor activation as with all GPCRs. However, unlike the GPCRs for monoamines that have a second Asp in helix 3 close to the extracellular surface, chemokine receptors have a Glu residue in a similar position but on helix 7. In common with other superfamily members, there is a conserved DRY triad at the C-terminus of helix 3 – speculatively involved in G-protein binding. Unique to the chemokine receptor subfamily, all have an acidic N-terminus with gross negative charges ranging from −1 to −6, and this might distinguish the initial recognition event from that in other GPCRs.

The binding site(s) for endogenous ligands have begun to be mapped using single-point-mutation analysis and chimeric proteins, and the residues that are critical for receptor activation are being studied[13–15]. Of the two receptors for IL-8 (CXCR1 and CXCR2), CXCR1 has high affinity only for IL-8 (and more recently, weaker affinity for GCP-2), whereas CXCR2 binds other CXC ligands (GRO, NAP-2) equally well. Chimerae between CXCR1 and CXCR2 indicated that the extracellular N-terminal domain determined ligand selectivity because receptors bearing the CXCR1 N-terminal region were selective for IL-8, while the CXCR2 N-terminal sequence, either in a CXCR1 or CXCR2 background, displayed promiscuity for other CXC chemokines. All acidic and basic residues present on the ligand-accessible surface of CXCR1 were systematically mutated to Ala, with the result that D11, R199, R203, D265, E275 and R280 were found to be critical for ligand binding[13,14]. Additional binding sites are found on the second and third extracellular loops. The integrity of the C→C bond between the N-terminal region and extracellular loop 3 is also important and, together, these might constitute a major IL-8 binding site. Surprisingly, given the role of D89 across the GPCR superfamily, little comment is available on the role of D89 in ligand-induced receptor activation.

Chimeric CCR1 and CCR2 have helped to elucidate a two-step mechanism for MCP-1 activation of CCR2 (Ref. 15). Radiolabeled MCP-1 binds with high affinity to wildtype CCR2 but not to CCR1; however, this profile is reversed in chimeras where the N-terminal sequences are interchanged, and this effect is mirrored in signaling. By contrast, substitution of the wildtype CCR1 N-terminal domain with that from CCR2 has little effect upon MIP-1α binding. Thus, for CCR2 but not for CCR1 interacting with MIP-1α or RANTES, the N-terminal domain is important both for binding and signaling, as witnessed by a >10-fold and 30-fold increase in the K_d and the IC_{50} for adenylyl cyclase inhibition, respectively. Progressive substitution of the extracellular domains in CCR2 showed that each domain contributed to signal transduction: extracellular loops 1 and 2 contribute only modestly (<10-fold combined) but all three in concert contribute markedly (30-fold). Again, in CCR1, only the third extracellular domain was especially critical for signaling.

Taken together, most studies point to a two-step model for chemokine receptor activation. The initial high-affinity recognition event apparently tethers the ligand in close proximity to the activation site from which receptor activation is triggered. This is not unlike the model currently accepted for thrombin receptor activation, albeit in that instance the ligand acts in *cis*.

There is considerable structural and sequence diversity across the GPCR superfamily to accommodate functional differences and also the disparate nature of the homologous ligand, from simple amines (such as acetylcholine and dopamine) through adenosine, prostaglandins and small peptides (such as angiotensin II), to the larger ligands represented by the chemokines. However, we speculate that, while the recognition event is clearly divergent, activation is conserved and always involves the helical domain most probably acting as a channel for cation (i.e. a proton or sodium ion) transfer from the extracellular fluid.

Intracelluar signaling

Chemokines signal by interaction with specific, cell-surface GPCRs and thence activation of heterotrimeric G-proteins, located intracellularly and protected from the surrounding chaos by the cell membrane. Given that there are >50 chemokines reported to date and only a dozen or so receptors, there is significant redundancy in the ligand and, not surprisingly, most ligands display promiscuity in their binding partners. The situation is further complicated by the way in which each ligand is 'presented' to the receptor because chemokines are bound tightly to various extracellular matrix proteins, such as the GAGs, heparin, chondroitin sulphate and dermatan sulphate. Thus, chemokine selectivity can be accurately mapped only in the exact physiological environment. Signaling studies for each of the receptors are far from complete and, again, experiments using convenient but nevertheless artificial systems involving transfectants should be interpreted with some caution.

Chemotactic responses to chemokines are associated with rapid and transient elevations of intracellular Ca^{2+}. After activation, chemokine receptors have altered sensitivity to repeated stimulation with the appropriate ligand. As with all GPCRs, activated chemokine receptors couple to heterotrimeric G-proteins consisting of αβγ subunits (Fig. 4). It

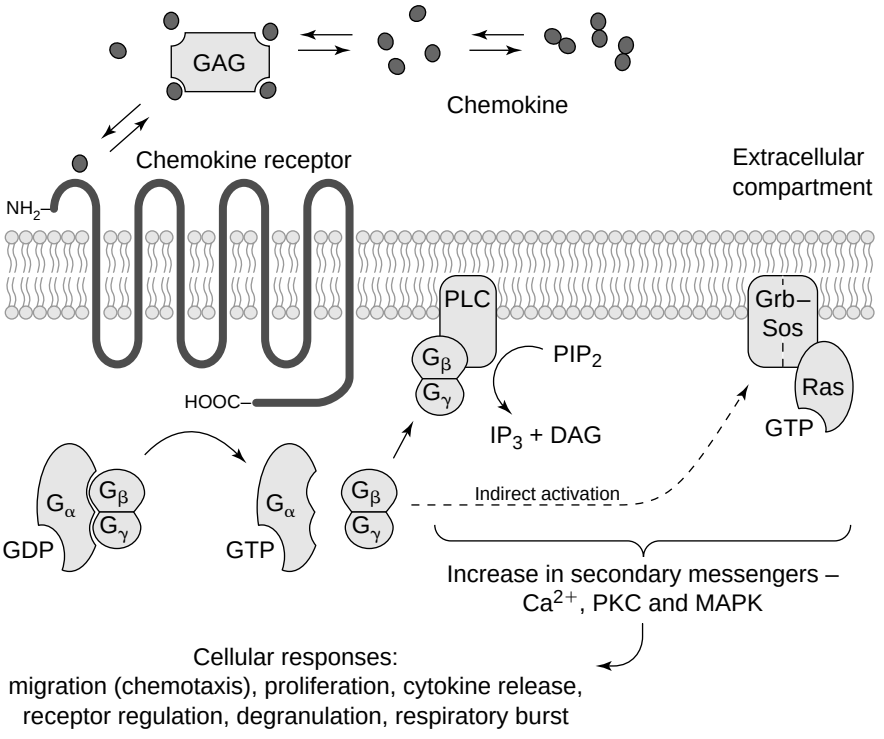


Figure 4. *Some of the important signal transduction mechanisms triggered by chemokine receptor activation. Ligand-induced activation of a chemokine receptor causes exchange of GDP for GTP on the heterotrimeric G protein and subsequent dissociation into G_α and $G_{\beta\gamma}$. The latter activates phospholipase C (PLC) directly and Grb–Sos (growth factor receptor bound protein complex) indirectly causing hydrolysis of phosphatidyl inositol and Ras activation, respectively. Other pathways not shown include $G_{\beta\gamma}$ activation of the GTP-binding protein RhoA, which in turn activates protein kinase N and integrin expression. DAG, diacylglycerol; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; PIP_2, phosphatidyl inositol diphosphate; PKC, protein kinase C.*

is the pertussis toxin-sensitive G protein[16,17], G_i, that links the receptor to PIP_2 hydrolysis through the intermediacy of the dissociated βγ subunit, that is implicated for all receptors studied to date. For example, cell lines stably transfected with CCR3 respond to eotaxin but not to any other chemokine at reasonable concentrations (<10 nM), as observed by Ca^{2+} flux measurements; at higher concentrations, RANTES and MCP-3 gave a similar response. When these transfectants were pretreated with pertussis toxin, the Ca^{2+} flux response to eotaxin but not to ATP was abolished, suggesting the involvement of G_i in transduction. Sequential stimulation of CCR3 by eotaxin but not any other chemokine caused homologous desensitization. Thus, receptor regulation represents another control point in chemokine function. Expression rates of receptor and receptor recycling also determine the response to chemokine ligands and might be modified to achieve a therapeutic benefit, as witnessed with the IL-8 receptor (see below). However, the pertussis-insensitive G protein, G_q, can also activate intracellular effector systems in other transfectant studies[18]. Finally, it is now clear that G proteins also play a pivotal role in serine/threonine kinase pathways, such as Ras/MAPK, and that chemokines have been shown to activate these processes (Fig. 4).

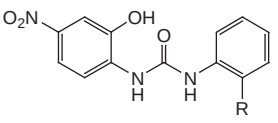
Role of chemokines in inflammatory disease

IL -8-mediated processes

While the chronic phase of the inflammatory response is characterized by extravasation and infiltration of macrophages and lymphocytes, the acute phase is mediated also by leukocytes such as neutrophils, eosinophils and basophils[19,20]. In 1987 the endogenous peptide[21], now known as IL-8, was first isolated from lipopolysaccharide-stimulated human monocytes and shown to be chemoattractive primarily for neutrophils, although it also affects other leukocytes such as T and B cells. Displacement studies with [^{125}I]IL-8 have revealed two high-affinity sites on neutrophils for IL-8: the IL-8A site, which is specific for IL-8; and the IL-8B site, at which the radiolabel can be displaced by other CXC ligands containing the ELR motif, such as NAP-2, GRO-α and MGSA. Subsequently, this was confirmed by the cloning and characterization of two distinct IL-8 receptors (IL-8R) having 77% homology.

An IL-8 homologue in rats or mice does not exist; hence, experiments in these species have targeted the N51/KC receptor, which is highly homologous to IL-8RB (CXCR2). Using either specific antibodies for human IL-8 or IL-8 receptor homologue knock-out mice, it has been clearly demonstrated that IL-8 is involved in the recruitment of neutrophils into tissues in the acute phase of inflammation[22]. Thus, the neutrophils from IL-8$^{-/-}$ mice kill bacteria as effectively as

wild type but fail to infiltrate tissues; apparently this process cannot be substituted by other neutrophil chemoattractants such as the complement protein C5a. Because IL-8 has been detected at diverse inflammatory sites, it is still unclear what the primary indication should be for compounds that interfere with aberrant IL-8 function. Diseases such as psoriasis, rheumatoid arthritis, glomerular nephritis, angiogenesis, adult respiratory distress syndrome, ischemic reperfusion injury and pulmonary fibrosis have all been mentioned by various investigators.



1, R = H
2, R = OH

Figure 5. *Nonpeptide CXCR2 antagonists:* ***1****, SK&F83589 and* ***2****, SB225002.*

In principle, as with all other chemokines, blockade of IL-8 function at many levels[23] can be considered (Fig. 4) from inhibition of IL-8 production through receptor blockade to interference in one or more of the IL-8-mediated intracellular signaling pathways. For example, IL-8 production is controlled primarily by the transcriptional factor NF-κB – this step is cancelled by anti-inflammatory steroids and other immunomodulators. However, agents that act at the receptor level are more specific. To date, most reports have focused on the ability of peptide fragments of IL-8 to act as functional antagonists, there being few small nonpeptide molecules described. Removal of the entire C-terminal helical domain (see above) after the fourth Cys residue attenuated, but did not eliminate, activity. By contrast, deletion of the ELR motif close to the N-terminus completely destroyed both binding to and activation of the IL-8 receptor[24]. Closer investigation[25] of truncated N-terminal peptides yielded receptor antagonists, the most potent being AA-IL-8$^{6–72}$, having a K_d of 8 nM (the K_d of IL-8 is 0.25 nM) and behaving as a functional antagonist of IL-8-induced elastase release. Subsequently, capped hexa- and heptapeptides, exemplified by Ac-RRWWCR-NH_2 and all-D-Ac-RRWWCRC-NH_2, respectively, were shown to be modest inhibitors of IL-8 binding, but in the μM range[26].

A new nonpeptide inhibitor (**1**) was discovered by high-throughput screening[27] and was optimized to produce **2** (Fig. 5), having an IC_{50} of 22 nM against CXCR2 but failing to inhibit CXCR1 at 100-fold concentration. In human neutrophils, which express almost equal numbers of each receptor, it is unclear whether chemotaxis is mediated by one or both of these receptor subtypes. In these cells, **2** inhibited GRO-α (CXCR2-selective) but not IL-8-stimulated Ca^{2+} mobilization, presumably because IL-8 can bypass CXCR2 inhibition by activating CXCR1. However, using human PMNs, chemotaxis to either IL-8 or GRO-α was inhibited (IC_{50} = 20 nM and 60 nM, respectively) indicating that neutrophil chemotaxis is mediated predominantly through CXCR2. However, whether this series of compounds acts by direct blockade at the receptor level or by a downstream event is still uncertain.

Role of chemokines and their receptors in infectious disease

Several unrelated observations implicate chemokine receptors in viral pathogenesis, although these observations have yet to be translated into a therapeutic benefit. Cytomegalovirus (CMV) possesses three open reading frames that encode putative GPCRs, designated *US27*, *US28* and *UL33*, the functions of which were unknown until it was realized that *US28* is a functional chemokine receptor with limited sequence homology to CCR1. At present, the role of this receptor in the CMV replicative cycle is unknown, but it is well known that some chemokines, for instance IL-8, can activate viral replication. Members of the poxvirus and herpesvirus families express GPCR-like receptors that are sensitive to chemokines.

Co-infection of HIV-positive individuals by CMV is a common clinical complication; indeed there is frequent histochemical evidence of dually infected cells in brain, retina and lung. The product encoded by *US28* is a functional chemokine receptor activated by RANTES, MIP-1α and MIP-1β and was recently shown to permit HIV fusion (see below) with human cell lines bearing the CD4 receptor and cotransfected with *US28* gene product. Whether *US28* plays a role in HIV pathogenesis remains controversial.

Researchers had known for over a decade that CD4 in itself was insufficient to support HIV fusion with the host immune cell, as mouse cells transfected with human CD4 and expressed on the cell surface failed to become infected with HIV. It was the discovery[28] in 1995 that the chemokines RANTES, MIP-1α and MIP-1β were the major HIV-suppressive factors produced by $CD8^+$ cells that initiated an explosion of new research to determine the mechanism and molecular target of these agents. In confirmation of their role, specific neutralizing antibodies to RANTES, MIP-1α or MIP-1β completely blocked their suppressive activity and, indeed, more recently it has been shown that some individuals who remain HIV-negative despite multiple high-risk exposures also have high levels of one or more of these chemokines. With the cloning of 'fusin'[29], now known as CXCR4, the missing co-receptor predicted to be necessary to complement the fusion process following the formation of the CD4–gp120 complex was identified. This receptor was surprisingly not sensitive to the CC chemokines listed above but, instead, was later shown[30] to

be activated by the lymphocyte chemoattractant SDF-1, suggesting that a second co-receptor remained to be discovered. In the absence of CXCR4, CD4 expressed on the surface of T helper (Th) cells or macrophages interacts with the viral envelope glycoprotein gp120/gp41, but this is, in itself, not permissive to fusion of the viral and host cell plasma membranes with subsequent viral replication. More specifically, CXCR4 supports the entry of HIV strains that emerge at late clinical stages of the infection – strains that are also distinguished by their ability to form syncytia in MT-2 cells and were previously called synctia-inducing. Because this receptor does not share RANTES, MIP-1α or MIP-1β as its ligand(s), another chemokine receptor was also thought to be involved in early-stage infection. Strains of HIV that initially establish a persistent infection are macrophage tropic, are not syncytium-inducing strains and were shown[31,32] to use CCR5 as a co-receptor for fusion. Even this explanation is a simplification because some primary clinical isolates of HIV can use CCR3, which is now known not to be found exclusively on eosinophils but is also expressed on Th2 cells and microglia. Finally, strains have been isolated, largely dual tropic, that can induce syncytial formation on cells expressing CCR2b, CCR3, CCR5, CCR8, CXCR1, CXCR4 and LTB-4.

Although the details of chemokine receptor function in viral fusion and entry are unknown, it is useful to speculate on mechanisms because this might help in the design of novel antiviral agents acting by blocking the fusion process. When the viral envelope glycoprotein binds to CD4, it is assumed that a conformational change occurs, resulting in the exposure of the V3 loop in a form complementary to the chemokine-receptor binding site (Fig. 6). In turn, the transmembrane subunit of envelope protein, gp41, can now present its hydrophobic N-terminus to the host cell membrane in a manner reminiscent of hemagglutinin-mediated fusion during influenza virus infection. This begs the question as to whether a chemokine receptor agonist or antagonist would be an effective anti-HIV therapy. Chemokine receptor blockade by designing a small-molecule antagonist acting in the helical domain will probably be the most pragmatic approach. This is supported by studies on $CCR5^{-/-}$ individuals who have been shown to be resistant to macrophage-tropic HIV infection. However, if the known ligands act by downregulation of the receptor, an agonist profile would seem to be more appropriate.

Rather complex molecules, previously known to inhibit the replication of HIV-1 and HIV-2 but by an unknown mechanism, have now been recognized as inhibitors of CXCR4, the co-receptor for T-cell-tropic viruses. The prototype derivative **3** (Fig. 7) does not inhibit viral envelope gp120 binding to the CD4 receptor[33], suggesting that its role in the fusion/uncoating process is probably mediated through a cofactor receptor. An escape mutant of HIV-1 LAI with decreased sensitivity to **3** had multiple mutations within or close to the V3 loop. While replication of the wildtype virus was inhibited by SDF-1 (the endogenous ligand for CXCR4), **3**-resistant virus was no longer inhibited by that ligand. More recently, **3** has been shown to inhibit SDF-1 binding to CXCR4, completely blocking Ca^{2+} signaling at 0.1 nM with no detectable binding to gp120; furthermore, it does not itself cause signaling. In addition, the molecule has no effect on RANTES signaling via CCR5. An 18-amino acid peptide (H-RRWCYRKCYKGYCYRKCR-NH_2), cyclized by means of two internal disulphide bonds (C4→C17 and C8→C13), also blocks T-cell-tropic HIV-1 infection at the level of cell fusion[34], presumably by interaction with CXCR4. Similarly, a D-Arg-based nonapeptide (all D-Ac-RRRRRRRRR-NH_2) blocked[35] viral fusion by interaction directly with the same cofactor receptor, whereas replication of macrophage-tropic or dual-tropic strains utilizing CCR5 was unaffected. Other structures (**4**–**6**) that putatively interact at CCR5 are shown in Fig. 7 (Refs 36–38).

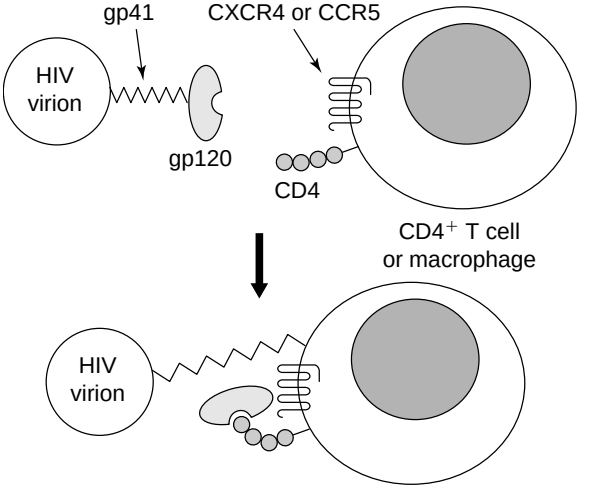


Figure 6. *Hypothetical model for HIV infection mediated through gp120, CD4 and the chemokine cofactor receptor (either CXCR4, CCR5 or others). Following the initial interaction between gp120 and CD4, a conformational change is assumed to expose the V3 loop on gp120 for binding to the GPCR acting as cofactor. Interestingly, no signaling is observed upon receptor binding of gp120, thus there is speculation of the biochemical process involved. This binding event facilitates gp41 binding to the host cell membrane to initiate the fusion process.*

Diseases implicating CCR1

Upregulation of CCR1 ligands and the visual recruitment of cell subtypes known to carry CCR1 have been extrapolated to provide the indications of CCR1 in disease. This receptor is found on neutrophils, monocytes, eosinophils, B and T cells and predominantly shows biological effect in response to MIP-1α and RANTES, its two principal ligands. The search for small-molecule CCR1 antagonists has been initiated primarily for two disease implications: rheumatoid arthritis (RA) and multiple sclerosis (MS). Both of these inflammatory diseases are marked by increases in the chemokines MIP-1α and RANTES and consequent macrophage infiltration during the disease progression. Increases in synovial MIP-1α and RANTES levels as well as CCR1 RNA in the peripheral blood and synovial fluid have been detected in patients with RA (Ref. 39). Anti-MIP-1α or anti-RANTES, coupled with anti-IL-8, have shown a reduction in the infiltration of leukocytes to the synovium in both the adjuvant induced arthritis (AIA) or the collagen induced arthritis (CIA) animal model of RA (Ref. 40). In the experimental autoimmune encephalomyelitis (EAE) model in the Lewis rat (an animal model of MS), levels of MIP-1α and RANTES were shown to correlate with both the acute disease and the incidence of relapse[41]. Moreover, anti-MIP-1α but not anti-RANTES had beneficial effects during both the number and severity of the relapses.

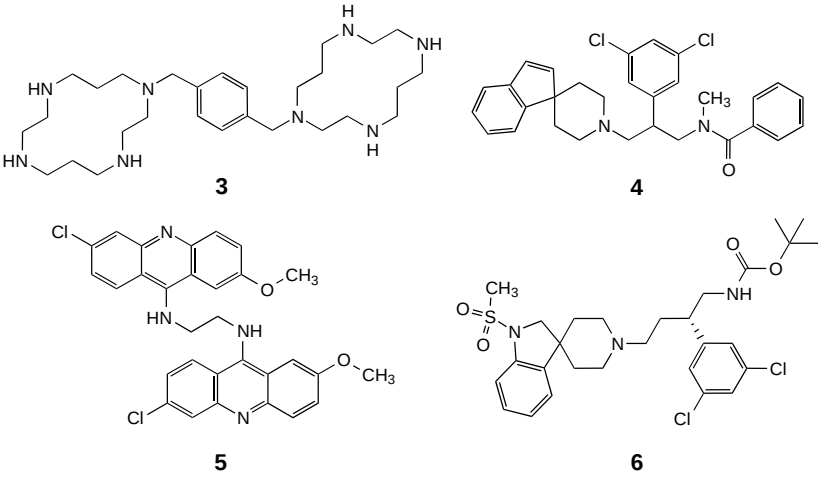


Figure 7. *Putative CCR5 anatgonists:* ***3****, JG3100;* ***4****, Merck;* ***5****, SmithKline Beecham CCR5 antagonist; and* ***6****, Merck.*

Recently, five companies have disclosed efforts towards the development of small-molecule CCR1 antagonists (Fig. 8)[42–46]. The molecules are similar in that most (**7–10** and **12**) contain a benzhydryl group a certain distance away from a basic nitrogen, which presumably interacts with one of the key carboxylates in or near the transmembrane domain of the GPCR. Although potent binding and functional inhibitors have been observed *in vitro*, no reports have yet claimed successful small-molecule *in vivo* activity in either of the aforementioned animal models. This is in contrast to the peptide-based inhibitor Met-RANTES[47], which has shown activity in several animal models. Met-RANTES has been shown to delay the onset and severity in the CIA model[48], as well as showing a reduction in the proteinurea and cellular infiltration in IgG-induced glomerular crescentic nephritis. It has also proved to be an effective inhibitor in foot-pad swelling induced by either MIP-1α or RANTES, and inhibited cellular infiltration and bronchial hyper-responsiveness in the ovalbumin-sensitized mouse. Phenothiazines are potent inhibitors[49] of RANTES binding monocytic THP-1 cell membranes and prevent RANTES-induced chemotaxis of THP-1 cells, presumably through interaction at CCR1.

Development of CCR2 antagonists

As with the development of small-molecule inhibitors of CCR1, the diseases for which a CCR2 antagonist is indicated have been characterized by an increase in the principal ligands found in the tissues affected by the disease. MCP-1 is the predominant ligand for CCR2. In humans there are two splice variants of the receptor, designated CCR2a and b, identical except for the truncation of the C-terminal end in CCR2b. Chemotaxis and Ca^{2+} mobilization is only observed upon interaction with the CCR2b variant, indicating that this is the functional form of the receptor. To help elucidate the role of MCP-1 *in vivo*, transgenic mouse models that have elevated levels of MCP-1 restricted to either thymus, brain or pancreas, have shown the infiltration of unactivated monocytes into these tissues. Although MCP-1 is important to the recruitment of these cells, additional factors are necessary for their activation[50]. Another transgenic mouse that constitutively expresses elevated levels of MCP-1 has an impaired ability to recruit monocytes when challenged, possibly owing to receptor sensitization or downregulation. These results are consistent with the report of Newton that small molecules can apparently inhibit the binding of [^{125}I]MCP-1 to CCR2 via its downregulation[51]. Further studies on CCR2-knockout mice have indicated that the CCR2$^{-/-}$ animals have an impaired ability to recruit monocytes when challenged by either thioglycollate injected into the peritoneum

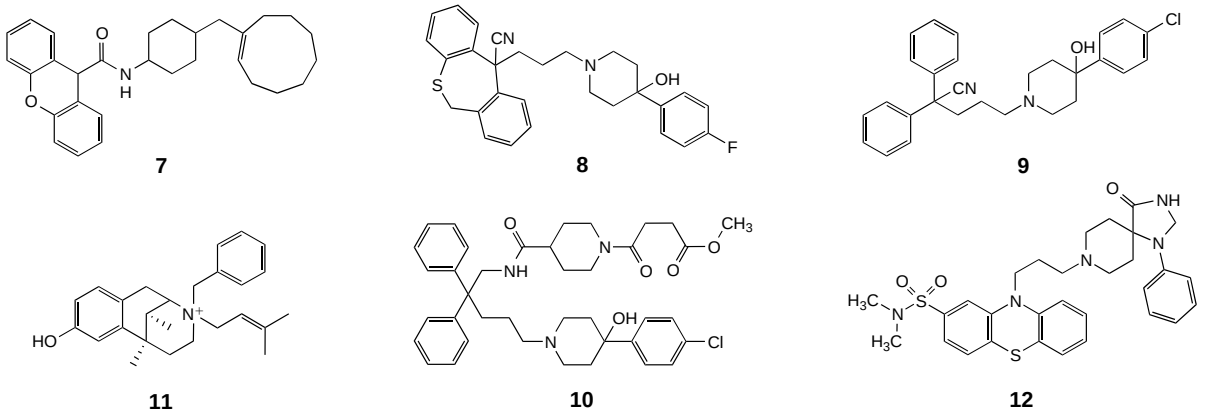


Figure 8. *CCR1 antagonists:* **7**, *Banyu Pharmaceuticals [IC_{50} = 1.8 nM (binding)];* **8**, *Leukosite [IC_{50} = 200 nM (^{125}I-RANTES), IC_{50} = 360 nM (^{125}I-MIP-1α)];* **9**, *Berlex Biosciences [IC_{50} = 40 nM (^{125}I-MIP-1α)];* **10**, *Takeda Industries [IC_{50} = 50 nM (^{125}I-RANTES), IC_{50} = 90 nM (^{125}I-MIP-1α)];* **11**, *Takeda Industries [IC_{50} = 200 nM (^{125}I-RANTES)]; and* **12**, *Rhône-Poulenc Rorer.*

or PPD-bead-induced lung granuloma. These studies have further suggested that activation of CCR2 might be involved in Th1-type cytokine responses[52].

Several inflammatory diseases are marked by specific site elevations of MCP-1. These include atherosclerosis, RA, MS, glomuleronephritis, asthma and stroke. For example, in RA, the infiltation of monocytes and macrophages can be observed in the synovial lining of the arthritic joint. The synovial fluid contains increases in a variety of chemokines including MCP-1, a key ligand for CCR2. Elevated levels of this chemokine are probably responsible for the monocyte infiltration into the synovial tissue. Antibodies to MCP-1 have exhibited a reduction in swelling in both the AIA and CIA models of the disease in rat; however, the effect seen is relatively small (30% reduction in swelling observed in CIA)[53]. A more dramatic effect has been seen with the MCP-1 antagonist, MCP-1$^{9-76}$, in the MRL-*lpr* mouse arthritis model, providing a 50% reduction of the overall histopathological score 28 days after early onset of the disease[54]. In an animal model of crescentic nephritis, an influx of T cells, macrophages and neutrophils to the kidney is observed along with the expression of both MCP-1 and RANTES. The development of the disease begins with inflammation of the kidney, the endothelial cells transform into crescents that, over time, become larger and fibrotic, ultimately squashing the glomeruli. In humans, the inflammatory phase of the disease is relative short, and the development of therapeutic agents that block the formation of the endothelial crescents and resulting fibrosis is critical. Although Met-RANTES is capable of reducing renal inflammation, only anti-MCP-1 is able to reduce both the inflammation and the development of fibrosis in the kidney in an animal model of the disease[55]. Furthermore, anti-MCP-1 blocks renal cell proliferation. It is hypothesized that a CCR2 antagonist might help reverse or halt the progression of the disease.

Three companies have reported studies in the initial development of CCR2 antagonists[56–58]. Compounds **14** and

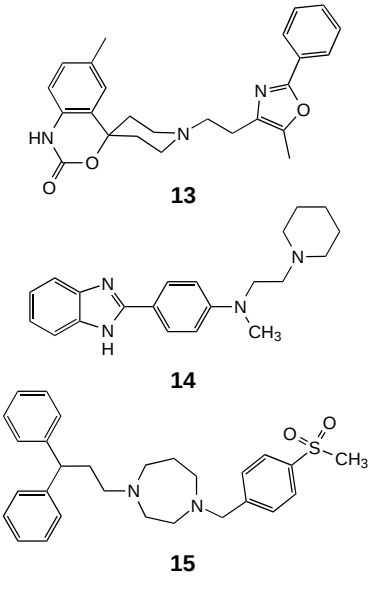
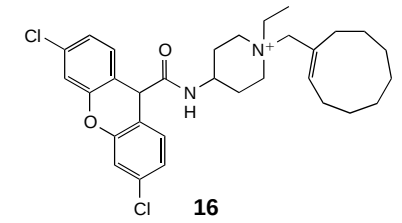


Figure 9. *CCR2 antagonists:* **13**, *Roche Bioscience [K_i = 89 nM (MCP-1), IC_{50} = 210 nM (chemotaxis-THP-1 cells)];* **14**, *Warner Lambert [IC_{50} = 1.1 μM (binding)];* **15**, *Teijin-CombiChem [IC_{50} = 1.8 μM (chemotaxis)].*



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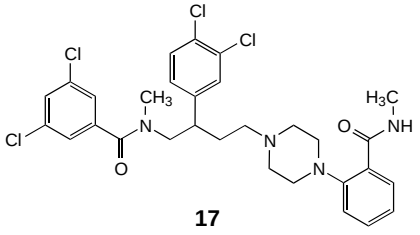

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Figure 10. *CCR3 antagonists:* ***16****, Banyu Pharmaceuticals (IC_{50} = 0.74 nM);* ***17****, Merck (binds CCR3 and CCR5).*

15 (Fig. 9) are seemingly weak inhibitors of MCP-1 binding, while **13** appears to be the most advanced of the three. Again, similar to CCR1 antagonists, the compounds share hydrophobic groups some distance away from a basic nitrogen functionality. It has been postulated that this basic nitrogen interacts with a key anionic residue in or near the 7-transmembrane region of the receptor, as found with antagonists of the monoamine receptors. This has recently been disclosed: site-directed mutagenesis studies indicated that **13** makes a key interaction with E291 on helix 7 of CCR2 (Ref. 58). No successful *in vivo* studies have yet been reported with these small molecules.

Eotaxin receptor and asthma

The accumulation of eosinophils in tissues is a characteristic feature of IgE-mediated allergic reactions such as allergic asthma, rhinitis and eczema. In the lung, the immediate bronchoconstriction in response to an allergic stimulus involves mast-cell degranulation and the release of constrictor agents. After several hours there follows a massive influx of eosinophils and a marked hyperresponsiveness to constrictor mediators, resulting in chronic inflammation of the airways. This late response might be inhibited by suppression of the lung eosinophilia.

It was a traditional pharmacological experiment that revealed the nature of an agent responsible for eosinophil recruitment into lung[59]. Fluid recovered by bronchoalveolar lavage of an inflammed guinea pig lung selectively induced local accumulation of eosinophils when injected into the skin. The endogenous chemoattractant was shown to be eotaxin and it, together with its receptor, was later[60–62] cloned from human cDNA. Unlike other CC chemokines, eotaxin displays high fidelity for CCR3 and the receptor shows some selectivity for that ligand, albeit binding RANTES and MCP-3 but with lower affinity. Taken together, this suggests a highly discriminating mechanism by which eosinophils are selectively recruited in inflammation and an opportunity for intervention in those diseases where eosinophils contribute to pathogenesis. To date, only Merck[63] and Banyu[43] have reported potent antagonists of CCR3 (Fig. 10).

Concluding remarks

Will this become a fruitful area for the discovery of new drugs against 'inflammatory' disease or will the early promise dissipate rapidly, as it has done in other fields such as the prostaglandin research of the 1970s? As it is only within the past year or so that potent, low molecular weight, nonpeptide antagonists have become available, further *in vivo* studies are necessary to determine their therapeutic potential. However, the design of ligands for GPCRs, both agonists and antagonists, has a long tradition of furnishing highly successful drugs (~40% of the top 200 synthetic drugs act at GPCRs), unencumbered by cell permeability issues. It is only a matter of time before new drug entities will emerge from the clinical programs for the variety of indications discussed above.

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