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Chemokines as Molecular Targets for Therapeutic Intervention[1]

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Despite the youth of the chemokine field, many antagonists of chemokine function have already been identified and tested at the preclinical level. These include neutralizing antibodies, peptidyl and non-peptidyl antagonists and non-specific immunosuppressive agents. These early studies suggest that chemokine agonists have the potential to regulate many diseases, ranging from HIV-1 infection and tumor growth to acute and chronic inflammation. Clinical application will depend on pharmaceutical development. Great strides have been made in defining structural domains of the chemokines involved in receptor binding and activation. The identification of receptors is rapidly progressing, but with 50 potential ligands and 15 characterized receptors, it is obvious that additional molecular studies are needed. The intriguing observation that several pathogens either use chemokine receptors as entry portals or produce chemokine decoys to subvert the immune system suggests that there is much to be learned about the immune system from studies of "virokines." Future studies should lead to the discovery and design of more effective inhibitors and antagonists with therapeutic benefit.

KEY WORDS: Chemokines; chemokine receptors; antagonists.

RATIONALE FOR TARGETING CHEMOKINES

Over 50 members of the chemokine family have been identified to date. They activate a family of G protein-coupled seven-transmembrane receptors (STM) expressed by a wide variety of cell types. Five CXC receptors have been identified that interact with the CXC subfamily of chemokines, which are characterized by having one amino acid (aa) between the first two of their four cysteines. Nine CC receptors interact with members of the CC chemokine subfamily, which lack an intervening aa between the first two of four or six cysteines (1–4). In addition, members of the defensin subfamily, which, at 4 kDa, are half the size of CC chemokines, and have six cysteines with variable numbers of intervening aa's, also interact with one of the CC chemokine receptors (5). A given receptor can interact with up to eight chemokines, and a given chemokine can interact with up to four receptors. Furthermore, cells express multiple receptors. Consequently, as is true of other cytokines, chemokines are subject to considerable redundancy in their activities. This is not the case for fractalkine, the only member of the CX3C subfamily, which interacts with CX3CR1 and lymphotactin, the only chemokine with one pair of cysteines, which interacts with XCR1. Despite the considerable overlap in receptor utilization, studies of mice with "knocked-out" chemokine genes often reveal unique selective defects indicative of specialized and nonredundant *in vivo* chemokine functions. Deletion of receptor genes usually has more wide-ranging consequences than ligand knockouts because multiple chemokine-induced responses are interfered with. It is, therefore, simpler to focus on the biological effects of activating a given receptor than to consider each individual ligand (Tables I and II).

Chemokines are multifunctional. They chemoattract a variety of leukocytes and nonleukocytic cells to sites of inflammation and injury. Chemokines also activate cells engaged in host immune responses, exhibit angiogenic or angiostatic effects, modulate hematopoiesis, promote fetal development, and regulate trafficking and homing of cells to appropriate tissue sites (Tables I and II). Overall chemokines contribute to various aspects of host defense and many of the chemokines have been detected in a wide variety of disease states involving inflammation, angiogenesis, and tissue injury and in neoplastic tissues (6). Although a number of chemokine-induced activities are essential for survival, chemokines can also mediate deleterious self-destructive effects and inhibitors can prove therapeutic. For example, chemokines contribute to self-destructive inflammation in autoimmunity,

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Table I. Consequences of CXC, CX3C, and C1 Chemokine–Receptor Interactions

Chemokine receptor	Receptor expressing cells	Chemokine ligands	Major *in vivo* receptor activities
CXCR1	Neutrophils Resting T cells EC	IL-8 > GCP2	Acute inflammation Angiogenesis Mobilizes BM neutrophils Antibacterial and antifungal host defense
CXCR2	Neutrophils Resting T cells EC	IL-8, GRO, ENA78, GCP2, NAP2	Suppress IL-4-induced IgE Fibroblasia Promotes viral replication
CXCR3	Activated T cells (TH1 > TH2), NK, B cells, monos, eos, EC	IP-10, MIG, ITAC	Chronic inflammation Immunostimulating Antibacterial host defense Angiostasis
CXCR4	Ubiquitous, including CD34+ progenitors, B cells, megakaryocytes, EC, mature DC	SDF-1α, SDF-1β	Competes with T-tropic HIV-1 Vasculogenesis and Angiogenic Myelopoietic and B cell development Lymphocyte trafficking Cerebellar and cardiac development
CXCR5	Activated T cells, B cells, monos	BLC (BLR-1 ligand)	B cell homing and seeding of 1° follicles, Peyer's patches, and germinal centers
CX3CR1	Neutrophils, activated T cells, NK, monos, microglia, astrocytes	Fractalkine (neurotactin)	Adhesion to EC Nerve repair
XCR1	Activated T cells, NK cells, thymocytes, DC	Lymphotactin	Lymphocyte trafficking

arteriosclerosis, and reperfusion injury, and they may promote tumor growth through angiogenesis. Some viruses have subverted host defense mechanisms by utilizing chemokines or their receptors to their own advantage either as sites of cell entry as in the case of HIV-1 (7), by producing chemokine receptor analogues as by HHV8, which produces an IL-8-like receptor (8), or by producing antagonists that interfere with chemokine actions as in the case of MCVI and II and the pox viruses (9). Imitation is the highest form of flattery, and viruses as well as knockout mice are teaching us the potential therapeutic benefits of targeting chemokines or their receptors. In this article we review the utilization of neutralizing antibodies, peptidyl and nonpeptidyl antagonists, and general suppressive agents that can be used to inhibit undesirable chemokine effects.

NEUTRALIZING ANTIBODIES

The first group of chemokine inhibitors to be evaluated was neutralizing antibodies. These have the benefit, especially in the case of monoclonal antibodies, of having very specific inhibitory effects. Antibodies have the disadvantage of being orally inactive, however, they have a relatively prolonged half-life and weekly injection can have prolonged inhibitory effects. The development of recipient immune responses to administered xenogeneic antibodies can be circumvented by using fully humanized antibodies obtained from transgenic mice (10). Consequently, reports of beneficial antichemokine therapy represent more than proof of principle and can be used in man.

The first dramatic demonstration of the beneficial effects of inhibiting chemokine mediated inflammatory activities involved rabbits undergoing temporary pulmonary artery occlusion to induce hypoxia followed by reperfusion injury. Prior administration of antibody to IL-8 reduced the adhesion of neutrophils to the injured rabbit lung tissues and consequent morbidity and mortality due to this reperfusion injury (11). A similar approach using anti-IL-8 was subsequently shown to reduce the degree of stroke induced CNS damage due to occlusion of the cerebral arterial blood supply (12). Anti-MCP-1 can also reduce myocardial reperfusion injury (13), suggesting both IL-8 and MCP-1 induced by hypoxia can exacerbate posthypoxic inflammation. Anti-IL-8 treatment has also been shown to reduce the degree of inflammation and mortality of acute respiratory disease syndrome (ARDS) (12) and of acute glomerulonephritis (14). Anti-IL-8 can inhibit tumor growth and metastases by interfering with the angiogenic effects of tumor produced IL-8 (15). Anti-MIP-1α administration

Table II. Consequences of CC Chemokine and β Defensin Receptor Interactions

Chemokine receptor	Receptor expressing cells	Chemokine ligands	Major *in vivo* receptor activities
CCR1	Activated T cells, monos, NK, immature DC, eos	MIP-1α, RANTES HCC1, 2 MCP-2, 3, 4 MP1F-1	Antifungal, antibacterial, and antiviral resistance Mobilization of BM progenitors Promotes mononuclear cell adhesion Modulates myelopoiesis
CCR2	Activated T cells, monos, basophils, immature DC, mast cells, eos	MCP-1, 2, 3, 4	Chronic inflammation (TH1 > TH2) Resistance to bacterial challenge Promotes mononuclear cell adhesion Histamine release, atherogenesis
CCR3	Activated TH2 cells, monos, NK, basophils, eos	Eotaxin 1, 2 RANTES, MIP-1α MCP-2, 3, 4, HCC2	Recruits eos in allergic states Histamine release from basophils
CCR4	Activated TH2 cells, NK	TARC, MDC	Favors TH2 responses
CCR5	Activated TH1 > TH2 cells, monos, NK, immature DC	MIP-1α, MIP-1β, RANTES, MCP-2	Coreceptor for M-tropic HIV-1 Enhanced antibacterial resistance Favors TH2 responses
CCR6	Resting memory T cells, immature DC, B cells, activated neutrophils	LARC (MIP-3α) β defensins	Attracts immature DC peripherally Activates resting memory T cells
CCR7	Resting and activated TH1 cells, mature DC, B cells	SLC, ELC (MIP-3β)	Attracts naive T cells and mature DC to LN perifollicular areas
CCR8	Activated TH2 cells, monos, B cells, immature DC	I-309, TARC, MIP-1β, HCC4 (LEC)	Promotes TH2 immune responses and chronic inflammatory reactions
CCR9	Fetal thymocytes, monos, splenic DC, T cells	TECK	T cell development

can reduce chronic relapsing EAE, while anti-MCP-1 blocks the initiation of EAE (16). However, one has to be judicious in blocking chemokine functions because this can reduce needed host defense as shown by studies in which anti-MIP-2 (GRO analogue) treatment of mice exacerbated *Klebsiella pneumonia* infections (17). There are many more reports and an earlier review of the beneficial effects of antibodies to chemokines and their receptors that demonstrate their potential utility in man (4).

PEPTIDE ANTAGONISTS

Peptide antagonists of chemokines/chemokine receptors (Table III) are mostly generated based on structure–

Table III. Specific Peptide Antagonists of Chemokine Receptors

Name of the anatagonist	Target receptor	Effective *in vitro* dose	Effective *in vivo* dose
IL-8(6–72)	CXCR2	1–10 μ*M*	N.D.[a]
Antileukinate	CXCR2	10–50 μ*M*	0.1 mg/kg
RANTES(9–68)	CCR1, CCR2	50–100 n*M*	N.D.
MCP-1(9–76)	CCR2	20 n*M*	0.2 mg/kg
MCP-3(10–76)	CCR1, CCR2	50–100 n*M*	N.D.
SDF-1(1–9)	CXCR4	500 n*M*	N.D.
T22	CXCR4	300 n*M*	N.D.
AOP-RANTES	CCR1, CCR5	10 n*M*	N.D.
Met-RANTES	CCR1, CCR5	6 n*M*	N.D.

[a] Not done.

function analyses of chemokine ligands. Several aa residues at or near the N termini of the chemokines are crucial for receptor binding and/or signaling. The receptor binding domain and signaling domain of a chemokine are often separated. Thus, an ideal peptide antagonist should be able to compete with the wild-type chemokine for binding to a high-affinity receptor, yet not initiate cell activation but, rather, block cell activation by the wild-type chemokine. Libraries of large synthetic and natural peptide repertoires have been used to identify specific chemokine receptor antagonists. Generally, the affinity of a peptide antagonist for a given receptor correlates with its length. The shorter the peptide, the lower the affinity. Therefore, higher concentrations of the shorter peptide antagonists are required for competitive inhibition of receptor activation by wild-type chemokines. Another intrinsic weakness of peptide antagonists lies in their potential antigenicity and susceptibility to proteolytic degradation. This can be partially compensated for by designing shorter peptide sequences at the expense of reduced receptor affinity or by modification of the sequence by adding additional chemical groups or by using D-aa's, which are resistant to mammalian enzyme degradation. Despite these limitations, many investigators have generated a variety of chemokine receptor-specific peptide antagonists that exhibit promising inhibitory effects on chemokines in various *in vitro* and animal models.

IL-8 and Related CXC Chemokines

Due to the prominent role of IL-8 in a variety of inflammatory conditions, considerable efforts have been made to develop peptide antagonists which may specifically bind to the receptors for IL-8, but do not elicit signal transduction. Moser *et al.* designed and synthesized a number of IL-8 analogues which do not contain an intact ELR motif (18). One of those analogues, termed IL-8(6-72), exhibited specific IL-8 antagonist activity. IL-8(6-72) displayed weak chemotactic activity for neutrophils at 1–10 m*M* concentrations but did not induce enzyme release or a respiratory burst. In contrast, it displaced binding of iodinated IL-8 to neutrophils and potently inhibited IL-8-induced neutrophil chemotaxis, mediator release, and respiratory burst. These results suggest that IL-8(6–72) is a partial agonist which interferes with the signaling of the parental molecule. This property of IL-8(6–72) was thought to be determined by the degree of receptor occupancy and the threshold of activation of a given signaling cascade. Of particular interest is the capability of IL-8(6–72) also to inhibit elastase release induced in neutrophils by GRO and NAP-2, two CXC chemokines with an ELR motif that share the receptor CXCR2 with IL-8. Since IL-8 is often generated together with other ELR+ CXC chemokines under various pathological conditions, it is highly desirable for an antagonist to diminish or prevent the activity of all chemokines interacting with CXCR2.

An analogue of the IL-8-related chemokine GRO/MGSA was recently described by Baly *et al.* (19). The mutant MGSA H19A showed 100–200-fold lower agonist activity on neutrophils than the wild type of GRO/MGSA, in eliciting neutrophil chemotaxis, elastase release, and up-regulation of CD18. However, in receptor binding competition experiments, H19A showed only a 13-fold decrease in its affinity for CXCR2. Furthermore, preincubation of CXR2 expressing cells with H19A inhibited cell migration, enzyme release, and calcium mobilization in response to wild-type MGSA. It appears that H19A binds to the receptor with a relatively high efficacy but is defective in inducing receptor signaling. This dissociation between receptor binding and activation is the basis for the design of specific peptide antagonists.

Regrettably, a long peptide antagonist has very limited therapeutic promise, since it is often highly antigenic and subject to rapid proteolytic cleavage, resulting in a short half-life *in vivo*. It is therefore necessary to develop shorter peptide antagonists which are less antigenic and easily modified to increase their resistance to enzyme degradation. These considerations led to the development by Hayashi *et al.* of a hexapeptide, RRWWCR, which does not bear significant homology to IL-8 or related CXC chemokines (20). The L-aa RRWWCR, with an acetylated amino terminus and amidated carboxyl terminus (Ac-RRWWCR-NH2), and the D-aa, Ac-RRWWCR-NH_2, are potent inhibitors of IL-8 binding to human neutrophils and consequently inhibited IL-8-induced neutrophil chemotaxis and enzyme release. Twenty-five-fold lower doses of Ac-RRWWCR-NH_2 were required to inhibit degranulation and rabbit skin edema in response to human IL-8 than are required for inhibition of *in vitro* chemotaxis (20). The advantage of these peptides lies in their lack of any agonist activity for IL-8 receptors and greater ease of synthesis. The D-aa peptide appears to be the more potent IL-8 receptor-specific antagonist. This may improve its therapeutic application since mammalian proteases cannot degrade D-aa peptides, thus resulting in a longer half-life *in vivo*. The peptide Ac-RRWWCR-NH_2 has been named antileukinate and has been shown to inhibit the neutrophil accumulation in rabbit lungs in response to staphylococcal enterotoxin (21). Furthermore, antileukinate also

inhibited the autocrine growth stimulating activity of MGSA/GROα for several melanoma cell lines, but not the growth of a control hepatocyte cell line which was not MGSA/GROα dependent (22). In a nude mouse model, antileukinate inhibited the growth and incidence of lung metastasis of MGSA/GROα-dependent human melanoma cells (23). Surprisingly, Fujisawa *et al.* reported an autocrine growth stimulating effect of MGSA/GROα on human umbilical cord vein endothelial cells (HUVEC), and this effect was markedly inhibited by antileukinate through the obstruction of a binding site on HUVEC for MGSA/GROα (24). These observations suggest that this specific antagonist of CXCR2 may also have angiostatic effects. Antileukinate is a promising candidate for further modification to yield more potent antagonists.

Peptide Antagonists for CC Chemokine MCP-1

Several MCP-1 peptides have been generated based on the structure of MCP-1. Zhang *et al.* used mutagenesis studies to determine the secondary structure of MCP-1 and identified several residues which are potentially important for receptor binding and function (25). Several mutated MCP-1 analogues, termed 7ND, R24F, Y28D, and D3A, were able to inhibit monocyte migration in response to wild-type MCP-1, either by receptor competition or by forming heterodimers, which interferes with the normal function of the wild-type MCP-1. However, more potent peptide antagonists were generated by truncation of the N terminus of MCP-1. Gong *et al.* reported that the N-terminally truncated MCP-1 analogues aa 8–76, 9–76, and 10–76 desensitized calcium flux in monocytes induced by wild-type MCP-1, while these analogues were themselves biologically inactive (26). The most efficacious antagonist, MCP-1(9–76), competed for binding with an affinity only threefold lower than that of the wild-type MCP-1. MCP-1(9–76) also desensitized calcium flux in monocytes induced by MCP-3, which shares the receptor with MCP-1, but did not inhibit the signaling by CC chemokines that do not use CCR2. Thus the antagonist activity of MCP-1(9–76) is receptor specific. This peptide was evaluated as a treatment of a MPL-1pr mouse model of arthritis characterized by a monocyte-mediated chronic inflammatory response similar to human arthritis (27). Daily injection of MCP-1(9–76) prevented the onset of arthritis as measured by the degree of joint swelling and histopathology. Injection of the antagonist even after the onset of arthritis resulted in a marked reduction in symptoms and histopathology in a substantial number of the animals. In contrast, injection of the wild-type MCP-1 enhanced arthritic symptoms in mice. These results suggest a beneficial effect can be achieved using an MCP-1 receptor antagonist peptide in the treatment of an autoimmune disease despite the involvement of multiple cytokines and chemokines.

Since several chemokines may be produced at a given site of inflammation, antagonists of multiple chemokines may be more effective than antagonists of a specific chemokine to control cell migration and activation. Gong *et al.* developed such multichemokine antagonists in the form of a number of truncated analogs of RANTES, MCP-1, and MCP-3 (28). On the basis of their ability to compete for binding with their parental chemokines, three analogues were selected including RANTES(9–68), MCP-3(10–76), and MCP-1(9–76). These analogues bound to monocytic cells with a four- to six-fold lower affinity than the wild-type chemokines, but they did not induce cell migration or mediator release. RANTES(9–68) competed for the binding and biological activity of wild-type RANTES, MCP-1, and MCP-3. In contrast, native RANTES inhibited only its own binding to cells. In addition, MCP-3(10–76) also inhibited the activity of wild-type RANTES, MCP-1, and MCP-3 on monocytic cells. These observations suggest that modification of the N-terminal residues results in the generation of chemokine analogues which exhibit a broader range of receptor specificity. However, whether these deletion mutants are more effective in the treatment of disease models involving multiple chemokines remains to be determined.

Peptide Antagonists for CXCR4

CXCR4 is a receptor for SDF-1 and serves as a fusion coreceptor for the T lymphocyte tropic and laboratory adapted strains of HIV-1. Thus, the development of antagonists to CXCR4 becomes potentially useful in inhibiting HIV-1 infection. Murakami *et al.* reported a peptide termed T22 [Tyr5,12, Ly7)-polyphemusin II] consisting of 18 aa residues and an analogue of polyphemusin II isolated from the hemocyte debris of American horse shoe crabs (*Limulus polyphemus*) (29). T22 specifically inhibited the ability of T-tropic HIV-1 virus to fuse with and infect peripheral blood mononuclear cells and cell lines transfected with CD4 and CXCR4. Although the primary sequence of peptide T22 is unrelated to the CXCR4 ligand SDF-1, mechanistic studies revealed that T22 attenuated CXCR4-mediated calcium flux in response to SDF-1, but not CCR2-mediated signaling

induced by MCP-1. Since CXC chemokines have been proposed to possess a core structure of three antiparallel β sheets, it was postulated that the anti-CXCR4 property of T22 may be based on its one antiparallel β-sheet structure maintained by two disulfide bonds in T22 (29). The CXCR4 specific inhibitory activity of T22 was confirmed by Tamamura *et al.* (30).

A number of studies have focused on generating peptides derived from the structural analysis of SDF-1. The three-dimensional structure of SDF-1 was determined by NMR spectroscopy, which revealed SDF-1 to be a monomer with a disordered N-terminal region (31). The 1–8 aa residues in this region form an important receptor binding site. However, only Lys-1 and Pro-2 seemed to be directly involved in receptor binding. Modification of Lys-1 and or Pro-2 resulted in the loss of agonist activity of SDF-1. Such a mutated SDF-1 analogue acts as a potent antagonist of CXCR4 by competitively interfering with the binding of wild-type SDF-1. Furthermore, a dimeric peptide derived from residues 1–9 (P2G) was also a CXCR4 antagonist (32). Heveker *et al.* generated a series of peptide domains of SDF-1, each comprising 13 aa residues, spanning the whole SDF-1α sequence (33). The antiviral and signaling properties of SDF-1 were retained by a 13-aa peptide corresponding to its amino terminus. As reported by Crumps *et al.*, removal of the first two residues yielded an antiviral SDF-1 analogue that also antagonized SDF-1-induced CXCR4 signaling. The investigators also identified a single-mutation analogue of SDF-1 which exhibited anti-HIV-1 activity but did not activate CXCR4 or antagonized the CXCR4 signaling induced by wild-type SDF-1. However, this anti-HIV-1 analogue of SDF-1 competed with monoclonal anti-CXCR4 antibody 12G5 for binding to CXCR4. These results indicated that the amino terminus of the SDF-1 is sufficient for signaling through CXCR4 and for inhibition of HIV-1 entry. However, these activities could be dissociated in a peptide analogue. Therefore, peptide analogues derived from different domains of SDF-1 can antagonize selected activities and dissociate different functions of CXCR4.

Agents that Down-Regulate Chemokine Receptors as Potential Therapeutics

CCR5 and CXCR4 are crucial coreceptors for HIV-1 fusion with the host cells by interacting with viral envelope proteins during infection. Studies with N-terminally modified CC chemokine RANTES, AOP-RANTES, which is a potent antagonist of CCR5, suggest that receptor internalization and inhibition of receptor recycling may represent a promising approach to inhibition of HIV-1 infection (34, 35). The mechanism of HIV-1 inhibition by AOP-RANTES is based on a rapid decrease in cell surface expression of CCR5 on monocytes, macrophages, and T lymphocytes. More importantly, AOP-RANTES inhibited the subsequent recycling of internalized CCR5 to the cell surface, while the native RANTES did not. This endows AOP-RANTES with a greater than wild-type RANTES capacity to inhibit HIV-1 infection.

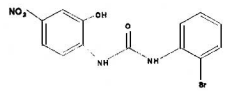
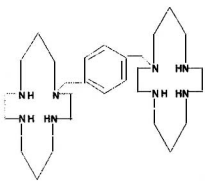
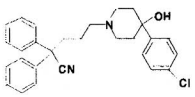
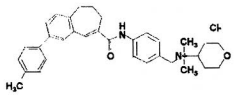
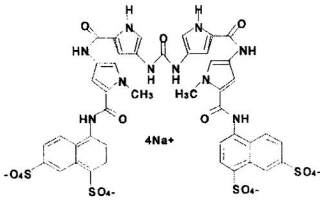
Extension of recombinant human RANTES by a single aa methionine at the N terminus also is sufficient to produce a potent and selective antagonist of binding and cell activation by chemokines that share the receptors with RANTES, such as MIP-1α and MCP-3 (36). Met-RANTES was shown to inhibit actin polymerization, release of oxygen intermediates, and calcium flux by human eosinophils stimulated with native RANTES, MCP-3, and eotaxin (37). Therefore Met-RANTES may be an effective inhibitor of the accumulation of potentially self-destructive eosinophils in allergic diseases. In contrast, the addition of a methionine to the CXCR4 ligand SDF-1β resulted in an analogue which was more potent than wild-type SDF-1β in inducing calcium flux by CXCR4 expressing cells (38). Met-SDF-1β also induced the internalization of CXCR4, but with a potency comparable to that of native SDF-1β. The differences in receptor interaction between Met-RANTES and Met-SDF-1β suggests that the functional consequences of modifying the N-termini is dependent on individual chemokines.

A very creative approach favoring the retention of chemokine receptors CCR5 and CXCR4 has been described (39, 40). RANTES, MIP-1α, or SDF-1α genetically engineered to express an endoplasmic reticulum retrieval peptide, KDEL, at the C termini can retain newly synthesized coreceptors CCR5 or CXCR4 intracellularly, possibly by forming complexes. Lymphocytes transfected with these modified chemokines (termed intrakines) are viable and resistant to HIV-1 infection. These observations further support the feasibility of using a receptor internalization and degradation strategy to prevent HIV infection.

NONPEPTIDYL ANTAGONISTS

This section focuses on small nonpeptidyl antagonists of chemokine function that target chemokine receptor(s) and their *in vitro* and *in vivo* effects and potential

Table IV. Nonpeptidyl Antagonists of Chemokine Receptors

Common name	Structure	Target(s)	Mode of action	Effective dose *in vitro*	Effective dose *in vivo* (animal model)
SB225002		CXCR2	Block ligand binding to CXCR2	20-60 n*M*	5.5 mg/kg/min (rabbit)
Bicyclam		CXCR4	Block ligand binding to CXCR4	1-10 n*M*	Not determined
4-Hydroxy piperidine compounds		CCR1	Block ligand binding to CCR1	41-61 n*M*	Not determined
TAK-779		CCR5	Block ligand binding to CCR5	1.0-32 n*M*	Not determined
Distamycin A derative NSC 651016		CCR1, CCR3, CCR5, CCR8, CXCR4	Induces receptor internalization and, at higher concentrations, blocks ligand binding	Anti-HIV-1; 1-5 μ*M* or 0.1-100 n*M* induces chemokine receptor internalization; 200 μ*M* blocks ligand binding	100 mg/kg/3× daily (mouse)

therapeutic uses. The interaction of the chemokines and their receptors offers a target for the identification of selective small molecular antagonist to further define biochemical functions and to treat pathophysiological conditions. Small nonpeptidyl antagonists, unlike peptide-based antagonists, have the advantage of not inducing a neutralizing immune response. Another advantage of this class of antagonists is that, by modifying the delivery methods, bioavailability and toxicity problems can be reduced. The biggest challenge to the development of small nonpeptidyl antagonists is the initial identification of the prototype compounds; fortunately several programs have been successful at identifying antagonists of chemokine function. (Table IV).

Selective Antagonists

SB225002. The first selective nonpeptide antagonist for CXCR2, SB225002, was reported in 1998 (41). SB225002 was identified using a combination of high-throughput screening based on inhibition of ligand binding and targeted drug design. The chemical formula for SB225002 is *N*-(2-hydroxy-4-nitrophenyl)-*N'*-(2-bromophenyl urea). SB225002 selectively inhibited IL-8- and GROα-induced calcium mobilization by CXCR2 transfectants with 50% inhibitory concentrations (IC_{50}) of 20 and 40 n*M*, respectively. In contrast, SB225002 did not inhibit IL-8-induced calcium mobilization by CXCR1 transfectants. SB225002 inhibited neutrophil migration to both IL-8 and GROα *in vitro*, with IC_{50} values in the 20–60 n*M* range and inhibited neutrophil migration to IL-8 in a rabbit model following intravenous administration. The development of SB225002 will provide an opportunity to dissociate CXCR1 and CXCR2 signals and assess their respective contributions to leukocyte recruitment in inflammatory disease. SB225002 has potential clinical applications as a suppressor of neutrophil recruitment in inflammatory conditions such as adult respiratory distress syndrome, reperfusion injury, chronic bronchitis, and asthma. Since

both CXCR1 and CXCR2 contribute to angiogenesis, this agent will probably not be an inhibitor of wound repair or tumor vascularization (Salcedo *et al.*, unpublished observation).

Bicyclam. The bicyclams were shown to have anti-HIV activity almost a decade ago (42). Antiviral activity was recently shown to be due to interaction with the HIV-1 co-receptor, CXCR4 (43,44). The prototype compound AMD3100 is the octahydrochloride dihydrate of 1,1′-[1,4-phenylene-bis(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane. The antiviral bicyclams target only T-cell trophic (X4) virus and induce the development of an AMD3100-resistant viral strain (45, 46), which also abrogated the inhibitory effect of exogenously added SDF-1. Resistance to both AMD3100 and SDF-1 is due to modification of the gp120 V3 loop (47). Recent tissue culture studies suggest that prolonged AMD3100 treatment will cause a shift to the less virulent R5 virus, but further *in vivo* studies are needed (48). The IC_{50} for AMD3100 antiviral activity *in vitro* was 1–10 n*M*. Inhibition of binding by anti-CXCR4 was also observed in this range. Inhibition of SDF-1α-induced calcium flux and receptor binding required about 10-fold less AMD3100. The *in vivo* efficacy of AMD3100 has not been reported. The potential for AMD3100 to be used as an anti-HIV-1 and HIV-2 therapeutic is obvious. Because CXCR4 and its ligand, SDF-1, are essential for embryonic vascular development (49, 50), it is tempting to speculate that AMD3100 may interfere with the angiogenic effect of SDF-1 in tumor neovascularization and endothelial cell migration (51).

4-Hydroxypiperidine Compounds. Recently, Hesselgesser *et al.* reported the identification of a 4-hydroxypiperidine compound that selectively inhibits ligand binding to CCR1 (52). Several 4-hydroxypiperidine compounds with IC_{50} values less than 5 μM were identified by a high-throughput assay that measured inhibition of MIP-1α binding to CCR1. The prototype molecule has the chemical formula of (2-2-diphenyl-5-(4-chlorophenyl)piperidin-lyl) valeronitrite and is referred to as compound **1.** The IC_{50} values for compound **1** inhibition of MIP-1α and RANTES binding to CCR1 were 41 and 61.5 n*M*, respectively. The IC_{50} values for MIP-1α or RANTES binding competition to CCR1 were 2.1 and 21.5 n*M*, respectively. Compound **1** also inhibits MIP-1α- and RANTES-induced cellular acidification and migration in the same 41–61 n*M* concentration range. Compound **1** did not inhibit ligand binding to 18 other surveyed STM G protein-coupled receptors including CCR5, CXCR2, and CXCR4. The *in vivo* efficacy of compound **1** has not been reported. Thus, compound **1** is a potent, selective *in vitro* antagonist of CCR1. CCR1 is one of the most promiscuous CC chemokine receptors, having eight reported ligands; compound **1** can be used to demonstrate the domains of CCR1 commonly used by each of these ligands to transduce the chemotactic signal. Compound **1** has the potential to address the role of MIP-1α and RANTES binding to CCR1 versus CCR5 in several pathophysiological conditions such as rheumatoid arthritis and asthma (53–55). Due to the role of MIP-1α in experimental autoimmune encephalomyelitis (EAE), one can speculate that compound **1** will be an important therapeutic for the treatment of chronic inflammatory conditions in the central nervous system such as multiple sclerosis (56). MIP-1α also participated in the activation of T cells in acute graft-versus-host disease, suggesting that compound **1** may also be an effective inhibitor of graft rejection (57).

TAK-779. Baba *et al.* have identified a small nonpeptidyl antagonist of RANTES binding to CCR5, TAK-779, for which the chemical formula is *N,N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2H-pyran-4-aminium chloride (58). TAK-779 was identified by a high-throughput screening method based on the inhibition of RANTES binding to CCR5-transfected Chinese hamster ovary cells. TAK-779, like compound **1**, but unlike SB225002, bicyclams, and distamycin A derivatives, is an asymmetric compound. TAK-779 has been shown to inhibit RANTES, MIP-1α, and MIP-1β binding to CCR5 with an IC_{50} of 1.0–1.5 n*M* and to inhibit RANTES-induced calcium mobilization at 10 n*M*. The *in vitro* selectivity of TAK-779 for inhibition of HIV-1 R5 fusion was demonstrated by comparison to the anti-HIV X4 activity of AMD3100. Like AMD3100, the antiviral activity of TAK-770 is at least 10-fold greater than the chemokine antagonist activity. The *in vivo* efficacy of TAK-779 is being evaluated. There are several chemokines shared by CCR1 and CCR5: RANTES, MIP-1α, and MIP-1β (2). TAK-779 and compound **1** could be used in combination to demonstrate the importance of CCR1 versus CCR5 in pathophysiological conditions. In addition to having anti-R5 HIV-1 activity, TAK-779 may be beneficial in the treatment of rheumatoid arthritis, asthma, HHV8-encoded chemokine pathology, uveitis, and allergy (53–55, 59–61).

Distamycin A Derivative. Ureido analogues of distamycin were shown to have antitumor activity several years before a connection to the chemokine receptors was made (62–64). Interaction with chemokine receptors was suggested by the discovery of anti-HIV activity for many of the analogues (65). Several of the analogues had anti-HIV-1 activity, with IC_{50} values of 1–10 μM, but due to synthesis and availability concerns, NSC 651016

became the prototype molecule. NSC 651016 is a symmetric molecule with the chemical formula 2,2′[4,4′-[[aminocarbonyl]amino]bis[*N*-4′-di[pyrrole-2carboxamide-1,1′-dimethyl]]-6,8 naphthalenedisulfonic acid]-hexasodium salt. Unlike AMD3100 and TAK-779, NSC 651016 inhibits both R5 and X4 HIV-1 infection with an IC_{50} of 1–3 μM. It also inhibited HIV-2 and SIV infection, although at higher IC_{50} levels, 13.9 and 26 μM, respectively. When tested for anti-HIV activity in a mouse hollow fiber model, no organ or cellular toxicity was observed at doses of 100 mg/kg body weight given three times daily, a dose that resulted in a 99% reduction in HIV-1 p24 production (66). *In vitro* studies indicated that NSC 651016 selectively blocked chemokine binding and calcium mobilization mediated by CCR1, CCR3, CCR5, and CXCR4, but not the fMLP receptor (FPR), CCR2 or CXCR1, at 200 μM. A more physiological result was the inhibition of MIP-1α-induced chemotaxis, with an IC_{50} of 0.1 nM (67). Further mechanistic studies showed that NSC 651016, unlike the other nonpeptidyl chemokine receptor antagonists, induces receptor internalization after binding (67). The peptidyl-based R5 antagonists, AOP-RANTES and NYY-RANTES, appear to drive HIV-1 rapidly to select X4 virus, suggesting the development of a more virulent HIV-1 infection (68). Recent studies suggest that NSC 651016 may be able to inhibit ligand-resistant HIV-1 strains because it uses different receptor domains (69). Although NSC 651016 must be injected, it has a long *in vivo* half-life and, therefore, has the potential to be a broad-spectrum anti-HIV-1 therapeutic. Additionally, NSC 651016 may have therapeutic effects in many inflammatory conditions, such as graft-versus-host disease. It will be interesting to evaluate other distamycin A analogues with receptor targets distinct from that of NSC 651016.

Emerging Inhibitors of Signal Transduction

Hamycin. Hamycin is a complex of polyene antibiotic compounds produced by *Streptomyces pimprina* that is sometimes used as an antifungal. Manna *et al.* investigated the ability of hamycin to inhibit IL-8-induced biological functions (70). Their work demonstrated that hamycin blocked IL-8 association with its receptor, resulting in decreased IL-8-induced calcium mobilization, chemotaxis, and superoxide production. At 5 μg/ml hamycin appears to inhibit IL-8-induced neutrophil responses, with little or no effect on fMLP or PAF-induced responses, thereby demonstrating the selective nature of the hamycin effect. However, at 10 μg/ml hamycin inhibited all of the tested chemoattractants, suggesting a narrow range of selective function. The mechanism by which hamycin blocks IL-8-induced responses appears to be by distorting the cellular membrane, causing the IL-8 receptors to be structurally modified resulting in reduced binding of IL-8. Hamycin could potentially be used as a general antiinflammatory if the organ toxicity and bioavailability problems associated with polyene antibiotics are addressed (71–74).

GENERAL SUPPRESSIVE AGENTS

Steroids/Prostaglandins

Many of the established antiinflammatory therapies also regulate some aspect of chemokine-induced leukocyte infiltration. Sozzani *et al.* have recently reviewed the role of proinflammatory cytokines such as LPS, TNF, and IL-1 and anti-inflammatory agents such as glucocorticoids on CC chemokine production and receptor expression (75). They suggest that there is a balance between increased receptor expression induced by proinflammatory cytokines and decreased receptor expression induced by glucocorticoids. The net result depends on the degree of each treatment. In fact, a single concentration of dexamethasone inhibits the expression of the murine GRO homologues KC and MIP-2 but increases the expression of the murine homologue for ENA-78, LIX (76). Standiford *et al.* showed that IL-8 expression by peripheral blood monocytes could be inhibited by either PGE2 or dexamethasone, but only dexamethasone inhibited IL-8 production by macrophages (77). Physiological concentrations of estrogen inhibit MCP-1 expression and MCP-1-induced chemotaxis, however, other estrogen receptor ligands, tamoxifen, and clomiphene blocked these effects (78–80). Therefore, steroid or prostaglandin therapy can either increase or decrease inflammation, depending on the targeted system.

Cytokines

Antiinflammatory cytokines suppress the production of most chemokines. The two cytokines most widely associated with this suppressive effect are IL-10 and TGFβ (81–83). However, TH2 cytokines IL-4 and IL-13 have also been shown to reduce MCP-1 and MIP-1α expression by activated epithelial cells and macrophages (84, 85). A notable exception to the IL-10-induced reduction of chemokine expression is HCC-4, whose expression is increased by IL-10 (86). Presumably the

antiinflammatory cytokines suppress chemokine expression at the messenger RNA level (87). A rather unique interaction between chemokines and TGFβ is that PF4 selectively binds to the TGFβ type 1 receptor (88). Since, TGFβ has been shown to induce cell migration, it is difficult to correlate this unique observation with the inhibitory effects of TGFβ.

Cross-Talk with Other G Protein-Coupled Receptor Agonists and Antagonists

The G protein-coupled receptors are preeminent pharmacological targets and many agonists and antagonists have been identified. Recently, an agonist, IB-MECA, for the A_3 adenosine receptor was shown to suppress MIP-1α production by activated monocytes (89). Further, in a murine collagen-induced arthritis model, IB-MECA reduced the degree of joint inflammation. The mechanism of IB-MECA action has not been explored, but several of the purine nucleotide receptor agonists regulate the interface between the immune system and the central nervous system (90).

Molecules that activate intracellular second messengers through unrelated receptors also may cause deactivation or desensitization of chemokine receptors, a process defined as heterologous receptor desensitization (91, 92). It has been shown that stimulation of a classical G protein-coupled STM receptor named FPR, by the bacterial chemotactic formyl peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), elicits a cascade of intracellular signaling events including increased cAMP and activation of protein kinase C (PKC). A recent study of various cAMP modulating drugs indicated that most inhibited chemokine expression and function (93). PKC activation by fMLP is associated with functional attenuation of heterologous STM receptor responses to other chemoattractants, including chemokines (92).

Several synthetic peptide domains derived from the HIV-1 envelope protein gp120 or gp41 have similarly been shown to be potent chemotactic agonist for the fMLP receptor FPR and its variant FPRL1 (94–96). One of the synthetic peptide domain derived from the V4–C4 region of the HIV-1 gp120, termed F-peptide, was shown selectively to activate FPRL1 in human phagocytic cells and to induce down-regulation of CCR5 and CXCR4 expression and function in monocytes (96). An acute-phase protein, serum amyloid A (SAA) at the high concentrations seen during acute phase responses, has also been identified as an agonist of the receptor FPRL1 and potently desensitizes monocyte or neutrophil responses to chemokines (97, 98). Thus, down-regulation of the function and/or expression of chemokine receptors by heterologous desensitization may represent an additional approach to the design of antiinflammatory and HIV-1 agents. Heterologous desensitization does not involve agonist occupancy of the receptor and does not lead to arrestin-mediated receptor internalization. Since heterologous desensitization has been reported to occur between a number of STM receptors, it has been postulated to play an important role in orchestrating the host cell response to multiple stimulants. For example, activation of two STM opiate receptors, δ and μ, by morphine or its endogenous analogue met-enkephalin resulted in a decrease in phagocyte migration to a number of chemokines (91). Further, activation of δ and μ opiate receptors induced chemokine receptor phosphorylation without a change in the level of cell surface expression or agonist-induced calcium mobilization (91). These results suggest that the suppression of chemokine receptor function could be achieved indirectly by agents that indirectly perturb the integrity of chemokine receptor signaling machinery.

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