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Review

HIV coreceptors: role of structure, posttranslational modifications, and internalization in viral-cell fusion and as targets for entry inhibitors

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Abstract

The human immunodeficiency virus (HIV) envelope glycoprotein forms trimers on the virion surface, with each monomer consisting of two subunits, gp120 and gp41. The gp120 envelope component binds to CD4 on target cells and undergoes conformational changes that allow gp120 to interact with certain G-protein-coupled receptors (GPCRs) on the same target membranes. The GPCRs that function as HIV coreceptors were found to be chemokine receptors. The primary coreceptors are CCR5 and CXCR4, but several other chemokine receptors were identified as ''minor coreceptors'', indicating their ability support entry of some HIV strains in tissue cultures. Formation of the trimolecular complexes stabilizes virus binding and triggers a series of conformational changes in gp41 that facilitate membrane fusion and viral cell entry. Concerted efforts are underway to decipher the specific interactions between gp120/CD4, gp120/coreceptors, and their contributions to the subsequent membrane fusion process. It is hoped that some of the transient conformational intermediates in gp120 and gp41 would serve as targets for entry inhibitors. In addition, the CD4 and coreceptors are primary targets for several classes of inhibitors currently under testing. Our review summarizes the current knowledge on the interactions of HIV gp120 with its receptor and coreceptors, and the important properties of the chemokine receptors and their regulation in primary target cells. We also summarize the classes of coreceptor inhibitors under development.

Keywords: HIV envelope; HIV receptor/coreceptor; Viral-cell fusion; Entry-inhibitor

1. Introduction

Within a year after human immunodeficiency virus (HIV)-1 was discovered as the causative agent for acquired immunodeficiency syndrome (AIDS), CD4 was shown to be the receptor for HIV-1 [1,2]. However, because not all CD4-positive cells allowed entry of HIV-1, the existence of a second receptor was postulated. The identification of a second fusion cofactor was accomplished in 1996 by Feng et al. [3]. This coreceptor turned out to be the receptor for the CXC chemokine SDF-1 α and was subsequently termed CXCR4. While CXCR4 functioned with HIV-1 isolates that had been adapted in the laboratory to replicate in T-cell lines, it did not function with many primary isolates. Hints that another chemokine receptor may function for these viruses came from the work of Cocchi et al. [4], who demonstrated that the CC chemokines RANTES, MIP-1α, and MIP-1β could inhibit certain primary and macrophage-tropic isolates. The chemokine receptor CCR5 was identified as the fusion cofactor for many primary isolates and macrophage-tropic viruses [5–9]. Subsequent work has revealed that between 10 and 20 G-protein-coupled receptors (GPCRs) can function as coreceptors for HIV-1, HIV-2, and/or simian immunodeficiency virus (SIV), although as yet there are no data supporting a role for these alternative or minor coreceptors in vivo [10]. Therefore, CCR5 and CXCR4 are considered the major HIV coreceptors; CD4 is now termed the primary receptor, as this molecule is where the initial contact is made between the virus envelope surface (SU) component, gp120, and the cell.

With the determination that the major determinant of viral tropism is the envelope gene and major restriction of viral infectivity is at the level of entry, a simplified nomen-

Abbreviations: CD4bs, CD4-binding site; CD4i, CD4-induced; ECI, extracellular domains I, of chemokine receptors; ECII, extracellular domains II, of chemokine receptors; ECIII, extracellular domains III of chemokine receptors; GPCR, G-protein-coupled receptor; HIV, human immunodeficiency virus; HR, heptad repeats in gp41; MAb, monoclonal antibody

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clature for viral phenotype was proposed [11]. A virus that uses CCR5 for entry is termed R5, one that uses CXCR4 for entry is termed X4, and one that uses both is classified as R5X4. While this classification system is useful, use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection.

2. CD4/gp120 interactions

The resolution of the crystal structure of the gp120 "core" in association with sCD4 (domains 1 and 2) and the F(ab) fragment of mAb 17b (directed against a CD4-induced epitope on gp120) provided detailed information on the molecular interactions between the virus SU envelope and its primary receptor, CD4. In addition, it shed light on the coreceptor-binding site on gp120, which normally becomes exposed subsequent to CD4/gp120 association [12,13]. It was established that CD4 is bound to a pocket in the gp120. Direct interatomic contacts are made between 22 CD4 residues and 26 gp120 residues. These include 219 van der Waals contacts and 12 hydrogen bonds. Importantly, Phe 43 and Arg 59 of CD4 were shown to make multiple contacts centered on residues Asp 368, Glu 370, and Trp 427 of gp120, which are all conserved among primate immunodeficiency viruses. The Phe 43 cavity is roughly spherical, with a diameter of about 8 Å. The phenyl ring of Phe 43 is the only non-gp120 residue contacting this cavity [13]. The gp120–CD4 interface includes two cavities, one water-filled and bounded equally by both proteins, and the other extending into gp120 interior and contacting CD4 only at Phe 43 [12]. The Phe 43 cavity is surrounded by a gp120 "ridge" containing amino acids Asp 368 and Glu 370, previously shown to be part of the neutralizing epitopes in the CD4 binding site [14]. Residues that line the Phe 43 cavity (Trp 112, Val 255, Thr 257, Glu 370, Phe 382, Tyr 384, Trp 427, and Met 475) are primarily hydrophobic. They are also highly conserved, as much as the buried gp120 hydrophobic core. Despite the lack of steric hindrance, almost no substitutions were identified in these residues, implying functional significance. While these residues do not have direct contact with CD4, they do affect gp120–CD4 interactions. Mutation of Thr 257 and Trp 427 can substantially reduce CD4 binding. Furthermore, mutation of these residues was found to reduce the binding of antibodies against the CD4 binding site [12,14]. The observed modest variations in adjacent surface-accessible residues in gp120 of some primary isolates (i.e., Pro 369, Thr 373, and Lys 432) could account for reduced recognition by CD4 binding site antibodies. In addition, many of the residues that line the cavity interact with elements of the chemokine receptor-binding region. It may be that the Phe 43 cavity and the other interdomain cavities form as a consequence of a CD4-induced conformational change (CD4i). It is important to emphasize that the above studies were conducted with gp120 (SU) that lacked several variable loops (i.e., V1/V2, V3). Therefore, additional interactions between residues in these loops and the coreceptors most likely play critical roles. However, they may not be deciphered by crystallization studies.

3. Coreceptor/gp120 interactions

AN extensive effort has been taken to map the regions and specific amino acids in CCR5 and CXCR4 that interact with gp120. Both CD4-dependent and CD4-independent interactions have been analyzed (Table 1). The main conclusions drawn to date are: (A) All the Cys residues are important for appropriate coreceptor function; most likely they are involved in disulfide bonds. (B) The N-terminal and second extracellular domain (ECII) of the coreceptors seems to be most crucial for gp120 binding and fusion. However, different strains interact with these regions differently and were affected to various degrees by site-directed

Table 1
Summary of coreceptor domain interactions with gp120 and viral fusion

Coreceptor	Virus	CD4-dependent	Contributing domains/amino acids	References
CCR5	HIV-1	+	Cytoplasmic tail not required Amino terminal: aa 1–13; YDINYY motif; D2, Y3, Y10, D11, I12; N13; Y14, Y15, S17, E18, Q21, K22 ECII: G163R↓; S184D↓; K197 ECIII: D276; Q280A↓	[17,122–129]
		–	Amino terminal; ECII	[130]
	HIV-2	+	Amino terminal, ECII	[131]
		–	Amino terminal: N13D↑; ECII essential	[132]
	SIV	+	Amino terminal: Y10, D11, Y14,	[128]
		–	Amino terminal: N13D↑;	[127,133,134]
CXCR4	HIV-1	+	Amino terminal: YDE-rich cluster, E15A↓; E32A↓; Y21A↓; E26A↓ ECI: D97A↓ ECII: D193S↓; R183A↓; Y184A↓; D187A↓; R188A↓; Y190A↓; Q200A↓ ECIII: E268A↓; Q272A↓ N-linked glycosylation (in N-terminal, ECII): not required	[17,18,135–139]
		–		[140]
	HIV-2	+		
		–	Amino terminal: involved, ECII: involved	[131,132,141]

mutagenesis of individual amino acids. (C) The role of coreceptor glycosylation is controversial. Unlike with CD4, deglycosylated CXCR4 and CCR5 are expressed normally and function as coreceptors. Furthermore, deglycosylated CXCR4 demonstrated expanded tropism, supporting binding and fusion of dual tropic and several R5 viruses [15,16]. (D) There are conserved elements in both CXCR4 and CCR5 that are involved in coreceptor function [17–19]. (E) Other posttranslational modifications of the HIV coreceptors further modulate their function by changing their surface density and by directly affecting the affinity of gp120/coreceptor interactions or coreceptor/CD4 association (see below).

The exact role played by the coreceptors in triggering the conformational changes in the envelope transmembrane protein (gp41) to facilitate membrane fusion has not been deciphered. The current model of HIV entry proposes a substantial refolding and stepwise transition of gp41 from a metastable, native conformation through a pre-hairpin fusion intermediate to a thermostable, six-helix bundle structure that brings the two membranes together and results in fusion pore formation [20,21]. The six-helix bundle is created when two heptad repeat motifs (HR) in the ectodomain of gp41 self-assemble into a trimer of hairpins. The gp120 binding to cellular receptors "loosens" its association with gp41, probably resulting in the release of the hydrophobic fusion peptide at the N terminus of gp41 from a sequestered site so that it can insert into the target membrane. With the fusion peptide inserted into the target membrane and the transmembrane region embedded in the viral membrane, gp41 is likely to fold into the compact six-helix bundle, which is the most stable form, and its formation is associated with energy release [22]. Peptides mimicking the N-HR and C-HR were shown to capture gp41 fusion-active pre-hairpin intermediates [23]. Unexpectedly, CD4 alone was required and was sufficient to induce C–HR or N–HR peptide binding to gp41 [24]. However, peptide binding was clearly enhanced when Env was activated by both CD4 and coreceptors [25]. Therefore, the main role played by coreceptors may be to increase the overall avidity of viral-cell binding and to increase the number of spikes undergoing conformational changes beyond a threshold that is required to overcome the energy barrier, resulting in fusion pore formation and membrane mergence.

4. Coreceptor/CD4 interactions

In early studies in cell lines, the recruitment of coreceptors into trimolecular complexes on the surface of target cells depended on the initial binding of gp120 to its primary receptor, CD4 [26,27]. However, in the promocytic cell line U937, a low level co-precipitation of CD4 and CXCR4 was observed in the absence of soluble gp120, suggesting some constitutive association between CD4 and coreceptors [26]. Subsequently, several studies in primary cells demonstrated significant levels of preexisting CD4–coreceptor complexes that can be co-precipitated from monocytes, macrophages, thymocytes, and to a lesser degree from circulating T cells (Ref. [28–31], and M. Zaitseva, in preparation). These associations may occur in specialized membrane microdomains. They are influenced by the relative densities of CD4 and coreceptors in different cell types, and the avidity of CCR5/CD4 interactions may be stronger than that of CXCR4/CD4 [30]. Furthermore, we demonstrated that the coreceptor competition for association with CD4 may change the susceptibility of human cells to infection with X4- and R5- HIV-1 isolates, and should be taken into consideration when designing clinical trials of coreceptor-targeted inhibitors [32]. In addition, recent publication provides evidence for CD4-enhanced signaling through chemokine receptor CCR5, suggesting a functional outcome of such interactions [33].

5. Role of posttranslational modifications of coreceptors in HIV-1-mediated cell fusion

Several factors besides densities of CD4 and coreceptors may influence the efficiency of interactions between the HIV-1 envelope proteins and cellular receptors, and lead to viral entry. CCR5 and CXCR4 may be expressed in multiple conformations that are recognized differentially by anti-coreceptor antibodies [34,35]. Such heterogeneity may influence the ability of coreceptors to support viral entry and may explain cell-type specific differences in susceptibility to infection [36]. CXCR4 and CCR5 are members of the seven-transmembrane domain receptor family. As such, their function could be affected by receptor dimerization [37], by the membrane microenvironment, and by posttranslational modifications of the coreceptors.

Posttranslational modifications of CXCR4 and CCR5 include modifications of the extracellular domains (N terminal, ECI, ECII, and ECIII) or the intracellular loops. Modifications of the extracellular domains include N-linked and O-linked glycosylation and tyrosine sulfation. The intracellular loops of the coreceptors may undergo palmitoylation, phosphorylation, and ubiquitination. These three types of modifications play a major role in receptor turnover and will be discussed in relation to receptor endocytosis and recycling.

The carbohydrate moieties expressed by glycoproteins are important for stability, intracellular trafficking, expression on the cell surface, and for protein folding. Glycosylation sites are present at the N-terminal sequences of the majority of seven-transmembrane domain receptors. CXCR4 contains two potential sites for N-linked glycosylation in the N terminus and ECII loop [38]. Removal of carbohydrate moieties by endoglycosidase F digestion induced a 10-kDa shift in CXCR4 mobility in SDS-PAGE, suggesting that one, or perhaps both, potential N-linked glycosylation sites in

CXCR4 are used. Importantly, the removal of both glycosylation sites by site-directed mutagenesis did not diminish coreceptor function but rather expanded its ability to be used by several HIV-1 isolates that normally use only CCR5 [15]. The authors of the study speculated that different glycoforms of CXCR4 may exist in vivo, and that at the early stages of the infection, R5 isolates could infect target cells via non-glycosylated form of CXCR4 [17].

Unlike CXCR4, CCR5 has only one site for potential N-linked glycosylation, but it is not used [39]. Instead, Ser 6 and Ser 7 at the N terminus of CCR5 are modified by O-linked glycosylation in cell lines and in primary macrophages [40]. Importantly, the absence of O-glycans on CCR5 had minimal effect on the efficiency of HIV-1 or SIV entry, but their absence prevented binding of the CCR5 ligands MIP-1 α and MIP-1β [40].

Tyrosine sulfation is a posttranslational modification common to many secreted and membrane-bound proteins. It plays an important role in protein–protein interactions that enhance leukocyte adhesion. A tyrosine-rich region, a potential site for tyrosine sulfation, is present at the N-terminal regions of several coreceptors, including CCR5 and CXCR4 [41]. Prevention of tyrosine sulfation substantially decreased HIV-1 entry without affecting CCR5 expression [40,41]. Based on the critical role of tyrosine sulfation in viral entry, a new peptide inhibitor that mimics the sulfated region of CCR5 was generated and was shown to successfully block R5 virus entry in vitro [42]. All studies on sulfation of tyrosine residues in CCR5 and CXCR4 have been performed using cell lines transfected with coreceptor genes. Therefore, it is still not clear whether susceptibility and/or specificity of primary cells to HIV-1 entry is attenuated by the presence or absence of sulfated moieties. The enzyme tyrosyl protein sulfotransferase (TPST) generates this modification in the Golgi compartment, and it is not known whether the activity of TPST enzymes is cell-type-specific or could be altered under physiological conditions, thus contributing to heterogeneity of coreceptor sulfation and function.

In summary, glycosylation and sulfation of the extracellular coreceptor domains may contribute to the efficacy and specificity of fusion by providing larger binding interface and by increasing the overall negative charge, facilitating the electrostatic interactions with gp120.

6. The role of coreceptor internalization and recycling in fusion

Multiple studies with mutated and truncated forms of CXCR4 and CCR5 demonstrated that coreceptor internalization is not required for HIV entry. However, the rate of receptor internalization and recycling can control its surface density and fusion potential. In addition, the protective effect of therapeutic agents targeting the HIV coreceptors depends not only on their ability to bind but also to deplete coreceptor molecules from the cell surface [43–45].

The classical pathway of GPCR internalization is mediated by agonist-induced endocytosis via clathrin-coated pits. Endocytosis is promoted by a highly conserved mechanism mediated by β-arrestin (reviewed in Refs. [46–48]). It is thought that following endocytosis, GPCRs can be targeted to two different pathways: receptors may recycle back to the cell membrane or directed to lysosomes for degradation (reviewed in Ref. [49]). Much of the current knowledge on GPCRs internalization was derived from studies on β_2-adrenergic and γ-opioid receptors. Determination of the mechanisms underlying trafficking and internalization of CXCR4 and CCR5 has only recently begun. In the absence of ligand, the level of surface coreceptor expression is determined by a balance between the rate of internalization and the rate of replacement (recycling or new synthesis). CXCR4 and CCR5 undergo significant spontaneous endocytosis followed by recycling to the cell surface [50,51]. Spontaneous internalization of CXCR4 leads to its redistribution from the membrane to the endocytic compartment but not to lysosomes for degradation [50]. In addition, large intracellular stores of CXCR4 were found in various leukocyte subsets [52]. Following physiological stimuli or cell culture, the intracellular pool of CXCR4 was shown by us [53] and by others [54] to translocate to the cell surface. Nonspontaneous internalization of coreceptors can be induced by specific agonists (chemokines) or by chemokine analogues, and, in T cells, by strong cell activation. Specific agonists induce internalization of both CXCR4 and CCR5, while only CXCR4 undergoes internalization in response to phorbol-ester [51,52,55–57].

Following SDF-1-induced receptor internalization, a significant proportion of CXCR4 is translocated to the lysosomes and is targeted to the degradative pathway [58] with only a small proportion of CXCR4 being recycled back to the cell surface depending on the cell type [50]. At the same time, CCR5 agonists induce its redistribution to endosomal vesicles followed by recycling to the cell surface [51]; so far there have been no reports indicating that CCR5 is subject to lysosome-dependent degradation.

Rapid phosphorylation of serine residues within the cytoplasmic tails of many GPCR including CXCR4 and CCR5 is an essential step in receptor internalization following ligand binding [56,59–61]. Phosphorylation of serine residues can be mediated by GPCR kinases (GRK) or by protein kinase C (PKC) [62,63]. Importantly, phosphorylation of serine residues in CCR5 is kinase-specific: Ser 337 is exclusively phosphorylated by a PKC-mediated process, while GRK phosphorylates Ser 349 (see below) [63]. Phosphorylated serine residues in the C terminus of CCR5 provide a binding site for β-arrestin, an intracellular adaptor molecule that facilitates endocytosis, [61]. β-arrestin was also shown to associate physically with phosphorylated CXCR4 [64]. Two distinct sites for β-arrestin binding were detected within CXCR4, one in the third intracellular loop and one at the C terminus [65]. However, while binding of β-arrestin to the third intracellular domain promotes recep-

tor internalization, binding of β-arrestin to the C terminus of CXCR4 interferes with receptor coupling to G proteins [65]. Recently, it has been shown that in GPCR signaling, β-arrestins perform an additional function as scaffold proteins by recruiting components of MAPK cascade and thus coupling GPCR to down-stream G-protein-independent signaling events, including activation of ERK1/2 and JNK/SAPK [66]. Additional mechanisms may provide fine-tuning of phosphorylation. For example, phosphorylation of the serine residues at the C terminus of CCR5 was shown to be dependent on the presence of palmitoylated cysteine residues [61,67]. It was suggested that this lipid modification may attenuate the activity of GRK by controlling the access to serine residues within the C terminus of CCR5 [61].

Down modulation of CXCR4 can be induced by SDF-1 and phorbol esters and is mediated through either one of two internalization signals: the serine-rich domain and Ser/IleLeu (di-leucine) motif within the cytoplasmic tail of CXCR4 [55,56]. Lack of di-leucine motif in CCR5 may explain the inability of phorbol esters to induce CCR5 internalization [62]. This motif was previously shown to function as endocytosis signal in CD4 and in the γ-subunit of the TCR complex, and is believed to promote interaction with AP2 clathrin adaptor complex [68–70]. It was suggested that CXCR4, unlike CCR5, has a capacity to interact with two distinct sets of endocytic adaptors, the AP2 complex and β-arrestins. Both of these interactions are likely to be regulated through phosphorylation of distinct sets of serine residues within its cytoplasmic tail [56]. As discussed earlier, internalization of CXCR4 occurs in response to either phorbol ester treatment or SDF-1 binding and is mediated via clathrin-coated vesicles. However, CXCR4 was reported to recycle back to the cell surface after phorbol-ester removal and to enter degradative pathway after activation with SDF-1 [50,55]. Therefore, it is reasonable to suggest that selective engagement of the adaptor molecules may determine the fate of endocytosed CXCR4—degradation or recycling.

The ubiquitin/proteasome system and lysosomes are two major intracellular machineries responsible for protein turnover. In cell lines, proteasome inhibitors reduced CXCR4 internalization, thus implicating a role for the ubiquitination machinery in the trafficking of CXCR4 [71]. However, in other experimental systems, CXCR4 degradation was sensitive to lysosomotrophic but not proteasomic inhibitors [58]. In addition, rapid ubiquitination of the cytoplasmic tail of CXCR4 was shown to occur upon agonist treatment, and this modification was suggested to serve as a sorting signal for lysosome-mediated degradation of surface CXCR4 [58]. Three lysine residues, potential sites for ubiquitin binding, are located next to a di-leucine motif within the SSLKILSKGK degradation signal in the cytoplasmic tail of CXCR4. Based on studies of CXCR4 mutants, it was suggested that agonist-induced phosphorylation of serine residues within the degradation motif enables binding of ubiquitin molecules to neighboring lysine residues, and this association promotes interaction with yet unknown adaptor molecules that sort CXCR4 for lysosomal degradation [58]. Studies from our laboratory suggested that ubiquitination might contribute to the observed heterogeneity of CXCR4 species detected in human primary cells [31]. Our data further suggest that even in the absence of SDF-1 binding, ubiquitination of CXCR4 plays a role in intracellular trafficking of fusion-competent isoforms of CXCR4 and may induce conformational changes favoring the association of CXCR4 with CD4 in primary cells (Ref. [31] and M. Zaitseva, unpublished observations). Furthermore, CXCR4 turnover may be controlled not only by ubiquitination of the receptor itself but also by the ubiquitination status of associated adaptor molecules, as was demonstrated recently for β_2-adrenergic receptors [72].

According to a widely accepted model, dephosphorylation of a GPCR within the acidic milieu of the endosomal vesicle is a crucial step for subsequent recycling to the cell surface [73]. Two phosphatases, SHP2 and SHIP, have been reported to associate with CXCR4 constitutively and upon activation [74,75] and therefore may be possible candidates for dephosphorylation of endocytosed CXCR4. However, recent data demonstrated that, in some cases, dephosphorylation of GPCR does not require trafficking of the receptor to endosomes. Importantly, it was shown that at low concentration of the agonist, CCR5 is phosphorylated by PKC and then is rapidly dephosphorylated at the plasma membrane by an unknown GPCR phosphatase (GRP) that is active at neutral pH and therefore differs from the endosome-associated GRP [63]. The rapid endosome-independent dephosphorylation of CCR5 was suggested to provide a mechanism to maintain cell-membrane receptors in a non-phosphorylated and therefore signaling-competent status [63].

7. Coreceptor-targeted therapies: modified chemokines, small molecule inhibitors, and intrakines

Chemokine receptors constitute an important target for the development of anti-HIV-1 therapies. As stated above, the ability of CC chemokines to block HIV-1 infection was one of the factors that implicated chemokine receptors as coreceptors for HIV-1 [4]. The main mechanism of antiviral activity of chemokines is based on chemokine-induced internalization of chemokine receptors into early endosomes [43,44,51]. On the other hand, inefficient internalization of CCR5 in macrophages and transfected adherent cell lines correlated with low HIV-1 inhibitory activity by chemokine analogues in these cells [45].

Infusion of native chemokines in vivo as therapeutic agents to control viral spread is compromised by a short half-life (less than 10 min) and their potential proinflammatory effects [76]. Therefore, novel strategies have been employed to overcome these limitations [77]. They include

but are not limited to modified chemokines, small molecules that target chemokine receptors, intrakines, and monoclonal antibodies.

7.1. Modified chemokines

The major goal in designing modified forms of chemokines is to create coreceptor antagonists that interact with the receptor with high enough affinity to prevent HIV-1 entry by blocking relevant epitopes and/or inducing coreceptor down modulation. At the same time, it is important that these antagonists are incapable of inducing signaling. In search for chemokine antagonists that can satisfy these criteria, RANTES, MIP-1α, and SDF-1 were subjected to chemical modifications or were truncated. Several derivatives of RANTES that bind to CCR5 and inhibit infection of lymphocytes and cells of the macrophage lineage with R5 viruses were identified: truncated forms of RANTES that lack two or six N-terminal residues, RANTES(3–68) and RANTES(9–68) [78,79], and a derivative of RANTES that was created by chemical modification of the amino-terminus, aminooxypenetene (AOP)-RANTES [80]. All three modified molecules were shown to induce less chemotaxis than intact RANTES. However, their anti-HIV effects differed, with RANTES(9–68) being less effective and AOP-RANTES and RANTES(3–68) being more potent in blocking infection with R5 viruses than unmodified RANTES [78,80]. In addition, several recombinant analogues of RANTES with substitution of selected residues in the N-terminal region were generated [81]. C1.C5-RANTES and L-RANTES showed a dramatically reduced ability to trigger intracellular calcium mobilization while exhibiting an increased antiviral activity against R5 primary isolates, suggesting that minor alterations of RANTES at the N terminus play a major role in determining chemotactic and antiviral activity and that the antiviral and signaling functions of RANTES can be uncoupled [81]. To further improve RANTES-based agonists, the fusion protein between RANTES and human IgG3 (RANTES-IgG3) was generated [82]. Even though RANTES-IgG3 was no more effective than intact RANTES in inhibiting of HIV infection, the authors pointed out certain desirable properties that were provided by this product: IgG fusion proteins have extended half-lives in vivo and molecules with IgG heavy chain moieties may be able to cross the placenta and potentially induce fetal protection [82,83]. Two MIP-1α derivatives with potent CCR5 antagonist activity were recently described. AOP-MIP-1α was shown to be about 10-fold more active than AOP-RANTES at inhibiting HIV entry [84]. LD78-α, an isoform that differs from MIP-1α only in three amino acids, is more potent in preventing viral entry than native MIP-1 α and RANTES. However, unlike AOP-MIP-1 α, LD78- α induces signaling [84,85]. In addition to modified agonists of CCR5, truncated form of the plasmatic human CC chemokine 1, HCC-1(9–74), displayed potent antiviral activities by inducing down-regulation of CCR5 on primary cells [86]. However, the effect of HCC-1(9–74) on viral replication was donor-cell-dependent, sensitive to HIV-1 isolate variations [87].

Some attempts have been made to generate an optimized CXCR4 antagonist. Addition of an N-terminal methionine residue to SDF-1 α resulted in increased antiviral activity [88]. However, the long-term effects of Met-SDF-1 α on infection are unknown and could be compromised by its enhanced signaling activity [88].

Chemokine analogues exert their antiviral inhibitory activities primarily by inducing internalization of chemokine receptors [44,51,84,87]. Both RANTES and AOP-RANTES induced down modulation of cell surface CCR5 and its targeting to early endosomes [44,51]. However, only AOP-RANTES prevented CCR5 recycling to the cell surface [44], resulting in a more long-lasting depletion of coreceptors. In addition, AOP-RANTES showed reduced capacity to induce chemotactic responses. The ability to induce effective internalization of CCR5, coupled with reduced proinflammatory signaling, makes AOP-RANTES an attractive candidate for development as antiviral therapy. However, it should be noted that RANTES and RANTES analogues were shown to enhance the infectivity of HIV-1 in vitro in some systems, possibly due to chemokine-induced cell activation [87,89,90]. In addition, *N*-nonanoyl-RANTES (NNY-RANTES) was shown with the 242 R5 virus to select for coreceptor switch to CXCR4-using viral variants in vivo in the hu-PBL-SCID mouse system [91]. Therefore, it may be important to develop a strategy that could prevent rapid selection of viruses with alternative coreceptor usage by simultaneously targeting CCR5 and CXCR4 coreceptors [92].

Future design of therapies based on chemokine analogues may be confounded by several factors: (A) there is a large degree of variability in the sensitivity of primary isolates to chemokine-mediated inhibition [93]; (B) chemokine binding is augmented by surface proteoglycan molecules, which vary among cell types [94]; and (C) down modulation of CCR5 may free surface CD4 molecules for association with CXCR4, making the cells more susceptible to infection with cytopathic X4 viruses [32].

7.2. Small molecule antagonists of coreceptor function

Small molecule antagonists of coreceptor function represent a recently emerging group of antiviral drugs that are designed to interfere with the initial stages of viral envelope binding to the cell membrane. The primary mechanism of their antiviral activity does not rely on down modulation of the coreceptors but seems to be due to receptor occupancy. The advantage of small molecule-based inhibitors is that they are unlikely to induce signaling and therefore to indirectly augment viral entry or to induce inflammation. Several important small molecules have been described so far. A small-molecule CCR5

antagonist, TAK-779, was shown to selectively inhibit R5 virus replication with laboratory-adapted and clinical isolates without any cytotoxicity to the host cells [95]. It was further shown that TAK-779 inhibits HIV-1 replication at the membrane-fusion stage and that the binding pocket for TAK-779 on CCR5 is located near the extracellular surface of the receptor within a cavity formed between transmembrane helices 1, 2, 3, and 7 [96]. Another candidate for blocking CCR5/envelope interactions is SCH-C [97]. This oxime-piperidine compound specifically inhibited infections with R5 HIV-1 in in vitro cell cultures and in vivo in SCID mice reconstituted with human thymic tissue. Importantly, SCH-C has a serum half-life of 5–6 h, which makes it a good candidate for the development of antiviral therapies [97].

Several small molecules demonstrated potent inhibitory activity against the coreceptor function of CXCR4. The bicyclam AMD3100 efficiently blocks cell/cell fusion and HIV-1 entry of both X4 and X4R5 viruses by binding to a conserved region of CXCR4 [98,99]. Importantly, even though AMD3100 prevents SDF-1 from binding to its receptor, AMD3100 by itself does not cause signaling [98]. It has been suggested that AMD3100 induces conformational alterations of the receptor [100]. The results obtained with AMD3100 compound are reminiscent of those generated with a cationic peptide derived from a horseshoe crab blood cells, T22 ({Tyr^{5,12}, Lys^{7}}-polyphemusin II) [101]. T22 was shown to inhibit replication of both laboratory-adapted and primary isolates of HIV-1 by binding to the N terminus and to two extracellular loops of CXCR4 [101,102]. Importantly, T22 did not induce signaling or CXCR4 down modulation at the concentrations required for inhibition of infection (200 nM) and was shown to interfere with very early events of viral/cell or cell/cell fusion at the stage of lipid mixing [102]. AMD3100 and T22 both inhibit the binding of the CXCR4-specific antibody 12G5 and of SDF-1 to CXCR4 [103]. However, recently it was shown that T134, a T22 derivative, efficiently inhibits replication of an AMD3100-resistant HIV-1 isolate, therefore suggesting that the sites of AMD3100 and of T22 activity on CXCR4 only partially overlap [104]. Further development of T22-based therapies will result in synthesis of T22 analogues that exhibit reduced cytotoxic effect on the host cells and at the same time preserve high anti-HIV activity [105].

The molecular mechanisms underlying the high-affinity binding of small molecules to coreceptors have not been investigated in detail. Small molecules can inhibit by steric hindrance to prevent interaction of gp120 with the receptor, or by inducing a global conformation change unfavorable to coreceptor function. In this regard, some light have been shed on the activity of the CXCR4 antagonist ALX40-4C. It was shown that the binding site of ALX40-4C partially overlaps with that of 12G5 and SDF-1 [106]. Importantly, it was further demonstrated that successful binding of SDF-1 and of ALX40-4C to CXCR4 is critically dependent on positively charged residues present in the synthetic peptide inhibitor as well as the beta-sheet region of SDF-1 [107]. The important role of electrostatic interactions between coreceptor and inhibitor was further demonstrated for AMD3100, in which interactions between positively charged residues (analogous to the highly basic V3 loop of the gp120 in X4 viruses) and negatively charged amino acid residues located in transmembrane domains 4, 6 and 7 of CXCR4 [103] were demonstrated.

One of the problems with the small molecule coreceptor inhibitors described to date if they were to move into the clinic is that they cannot be taken orally and must be injected. A recent description of KRH-1636, an antagonist of CXCR4 that blocks infection of primary cells by primary and laboratory-adapted X4 viruses with similar efficiencies to those of AMD3100 but that is absorbed through the duodenum, would have an advantage for clinical use [108].

In summary, a progress has been achieved in the development of antiviral therapies based on small molecule antagonists of coreceptors. It is important to note, however,

Table 2
Summary of activity of small molecules that inhibit HIV entry

Inhibitor	Coreceptor	Ligand activity	Chemical	Active concentration[a]
SDF-1	CXCR4	Ligand	Chemokine	0.2–5 mg/ml; 30 nM
Met-SDF-1	CXCR4	Antagonist	Mod. Chemokine	1.0–2.8 mg/ml
AMD-3100	CXCR4	Antagonist	Bicyclam	2–7 ng/ml; 20 nM
KRH-1636	CXCR4	Antagonist	LMW	18–152 nM
ALX40-4C	CXCR4	Antagonist	Peptide (9 aa)	3–20 nM
T22	CXCR4	Antagonist	Peptide (18 aa)	5–290 nM
T140	CXCR4	Antagonist	Peptide (14 aa)	0.18–12 nM
T134	CXCR4	Antagonist	Peptide (14 aa)	2–74 nM
RANTES	CCR5	Ligand	Chemokine	61 nM; 480 ng/ml
AOP-RANTES	CCR5	Antagonist	Mod. Chemokine	0.3–3 nM
NYY-RANTES	CCR5	Antagonist	Mod. Chemokine	40–300 nM
TAK-779	CCR5	Antagonist	LMW[b]	10–100 nM
SCH-351125 (SCH-C)	CCR5	Antagonist	Oxime-piperidine	2.3–13.4 nM
SCH-350581 (AD101)	CCR5	Antagonist	Oxime-piperidine	0.3–1.5 nM
T20	Fusion	NR[c]	Peptide	0.07–0.31 μg/ml; 2–40 nM

[a] Values are ranges and depend on the assay, cells, and viruses used.
[b] Low molecular weight compound.
[c] NR, not relevant.

that primary viral isolates were shown to generate escape mutant variants during long-term passage in the presence of a CCR5-specific small molecule inhibitor [109]. Interestingly, the escape mutant was not able to use CXCR4 or any other tested coreceptor and was still dependent on low levels of CCR5, suggesting that newly emerged virus was able to use a drug-bound form of the receptor [109]. The properties of some of the small molecule inhibitors are summarized in Table 2.

7.3. Intrakines

One of the recently developed strategies designed to inhibit binding of the viral envelope to the cell surface is based on the trapping of the chemokine receptor in the cytoplasm by the intracellular expression of chemokines engineered to contain an endoplasmic reticulum (ER) retention signal [110,111]. Such "intrakines" have been prepared with SDF-1 and RANTES, and their genes are transduced into PBMC using retroviral vectors. Intrakines function by binding to the newly synthesized chemokine receptors and trap them in the ER. PBMC transduced with SDF-1- or with RANTES-based intrakines are resistant to infection with X4 or R5 viruses, respectively, but these cells retained their basic biological functions [110–112]. Antiviral therapies that involve intrakines may have potential for inactivating CCR5 safely, since homozygous inactivation of this receptor has not been associated with loss of immune function [113,114]. In contrast, CXCR4 is broadly expressed in cells of both the immune and the central nervous systems (CNS), and both SDF-1 and CXCR4 were shown to play an essential role in hematopoiesis, in cerebral development in mice [115,116], and in thymocyte development in humans [117]. In addition, intrakine-transduced adult mice exhibited impaired lymphopoiesis and myelopoiesis [118], suggesting that in vivo inactivation of CXCR4 by intrakines may not be safe.

7.4. Anti-coreceptors monoclonal antibodies (mAb)

Several CCR5-specific mAb were described with very potent anti viral activity in vitro [34,119,120]. However, their further development as in vivo therapies will be confounded by the development of anti-mouse IgG response and the potential selection of virus variants with altered tropism. Nevertheless, under conditions of short-term treatment such as child birth, coreceptor-specific mAbs may be used as part of inhibitor-cocktail to block mother to child intrapartum transmission.

7.5. New microbial-derived coreceptor inhibitors

Recently, it was found that cyclophilin C-18 from the parasite *Toxoplasma gondii* binds specifically to CCR5 [121]. Importantly, C-18 was found to block binding of R5 HIV-1 envelope and to inhibit R5 virus infection of activated PBMC without CCR5 down modulation (H. Golding, submitted for publication). Studies will test the suitability of this microbial-derived protein as a coreceptor antagonist in vivo.

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