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Review

Entry inhibitors in the treatment of HIV-1 infection

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ABSTRACT

Infection of target cells by HIV is a complex, multi-stage process involving attachment to host cells and CD4 binding, coreceptor binding, and membrane fusion. Drugs that block HIV entry are collectively known as entry inhibitors, but comprise a complex group of drugs with multiple mechanisms of action depending on the stage of the entry process at which they act. Two entry inhibitors, maraviroc and enfuvirtide, have been approved for the treatment of HIV-1 infection, and a number of agents are in development. This review covers the entry inhibitors and their use in the management of HIV-1 infection.

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Contents

1. Introduction

The use of highly active antiretroviral therapy, or HAART, to treat patients infected with HIV has resulted in profound reductions in morbidity and mortality from AIDS (Detels et al., 1998; Gulick et al., 2003; Hammer et al., 1996; Palella et al., 1998). Recent studies have demonstrated that the early initiation of HAART in asymptomatic patients with higher CD4+ T cell counts leads to an improvement in survival compared with delayed therapy (Kitahata et al., 2009). HAART does not eradicate HIV infection, but rather suppresses the virus, often to below the limit of detection in patient plasma (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997), requiring sustained treatment to prevent reactivation of the virus and disease progression (Chun et al., 1999). HAART regimens have traditionally involved antiretroviral agents from three classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). While these regimens are effective in many patients, long-term toxicities, drug–drug interactions, and the emergence and transmission of drug-resistant strains of HIV limit their effectiveness (Boden et al., 1999; Lucas et al., 1999; Piscitelli et al., 1996; Wegner et al., 2000; Yerly et al., 1999). As a consequence, the identification of new drugs that inhibit viral replication is a pressing need for the treatment of HIV patients.

One stage of the HIV life cycle that presents targets for therapeutic intervention is the entry of virus into host cells. Drugs that block HIV entry are collectively known as entry inhibitors, but comprise a complex group of drugs with multiple mechanisms of action. This is a reflection of the intricate, multi-step process that HIV undergoes during entry: attachment to host cells and CD4 binding, coreceptor binding, and membrane fusion. All of these stages are mediated by the viral envelope (Env) proteins, gp120 and gp41, which are the

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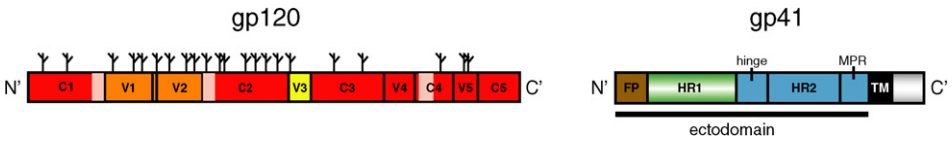


Fig. 1. Schematic of HIV gp120 and gp41 envelope proteins. The gp120 protein contains five conserved (C1–C5) and five variable (V1–V5) domains. Positions of conserved N′ linked glycosylation sites are indicated by branched structures. The lighter colored regions in the C1, C2, and C4 domains represent the bridging sheet minidomain that is formed following CD4 binding. The gp41 protein contains an N′ terminal fusion peptide (FP), two heptad-repeat domains (HR1 and HR2), and a transmembrane anchor (TM). The location of the hinge region and the membrane proximal region (MPR) are also depicted.

only viral proteins that project from the membrane of the virion. Noncovalently associated homotrimers of gp120 and gp41 subunits assemble into between 8 and 14 functional spikes on the surface of virions (Zhu et al., 2003, 2006, 2008), though it is not known how many are needed to elicit the membrane fusion process (Magnus et al., 2009; Yang et al., 2005).

Since the structure of the Env proteins is crucial to an understanding of the entry process, a brief review of their key features is essential (Fig. 1). The gp120 and gp41 proteins are initially synthesized as a single polypeptide precursor, gp160, which is cleaved during transit to the cellular membrane. The gp120 subunit of Env mediates attachment, CD4 binding, and coreceptor binding, and consists of five conserved (C1–C5) and five variable (V1–V5) domains (Starcich et al., 1986). The conserved regions form the core of gp120 and contain many of the domains critical for binding to host cells. In contrast, the variable domains are located near the surface of gp120, with V1–V4 forming exposed 'loops' that are anchored at their bases by disulfide bonds (Leonard et al., 1990). The variable loops, along with numerous glycosylation sites, provide constantly evolving epitopes for the humoral immune response, but their function is not strictly for immune evasion: the V1/V2 and particularly the V3 loop have important roles in Env binding to coreceptor. The gp41 subunit of Env encodes the molecular machinery that drives membrane fusion. The protein contains a large extracellular ectodomain, a transmembrane spanning anchor, and a large cytoplasmic domain on the inside of the virion membrane. The ectodomain contains a hydrophobic, N′ terminal fusion peptide and two heptad-repeat domains (HR1 and HR2) that are critical to the fusion process (Dubay et al., 1992; Wild et al., 1994).

2. The HIV entry process

Infection of target cells by HIV is a complex, multi-stage process involving attachment to host cells and CD4 binding, coreceptor binding, and membrane fusion (Fig. 2). The initial interaction between HIV and a target cell may be facilitated by nonspecific interactions between positively charged domains on the gp120 protein and negatively charged proteoglycans on the cellular membrane (Mondor et al., 1998; Moulard et al., 2000) or by specific interactions with cell surface lectin binding proteins such as DC-SIGN. Such attachment factors, while not needed for infection, can enhance the efficiency of virus infection (Geijtenbeek et al., 2000). The primary receptor for HIV is CD4, a member of the immunoglobulin superfamily that is expressed on monocytes, macrophages, and on subsets of T cells and dendritic cells. HIV interaction with CD4 occurs at a structurally conserved, recessed surface on gp120 that is formed by epitopes that are discontinuous in the primary protein sequence (Kwong et al., 1998). Unlike many other regions of gp120, this CD4-binding site consists of residues that are highly conserved and devoid of carbohydrate, properties that make it a logical target for inhibitors of gp120-CD4 binding. Upon engagement of CD4, gp120 undergoes a dramatic conformational shift that has several important consequences. First, two sets of β-sheets that are spatially separated in unbound gp120 are brought together by CD4 binding into a four-stranded β-sheet minidomain called the bridging sheet (Chen et al., 2005; Kwong et al., 1998). Second, CD4 binding results in movement and exposure of the V1/V2 and V3 loop structures. Third, binding of CD4 changes the orientation of gp120 such that the bridging sheet and the V3 loop are directed towards the host cell membrane, where they can subsequently interact with coreceptor (Huang et al., 2005; Trkola et al., 1996; Wu et al., 1996). Thus, CD4 binding is a prerequisite to the formation and exposure of the coreceptor binding site of gp120.

In humans, the primary coreceptors for HIV-1 are the chemokine receptors CCR5 and CXCR4, members of the seven-transmembrane G protein-coupled receptor family (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Oberlin et al., 1996; Zhang et al., 1998). These proteins are integral membrane proteins with seven transmembrane helices, an extracellular N′ terminus and three extracellular loops (ECLs) that form a small pocket. The N′ terminus of chemokine receptors contains sulfated tyrosine residues and elements within and around ECL2 that are critical for gp120 binding (Atchison et al., 1996; Edinger et al., 1997; Rucker et al., 1996). These spatially separated domains of CCR5 interact with distinct regions of gp120: the N′ terminus with the bridging sheet and the base of the V3 loop, and ECL2 with the tip of the V3 loop (Basmaciogullari et al., 2002; Cormier and Dragic, 2002; Cormier et al., 2001; Hoffman et

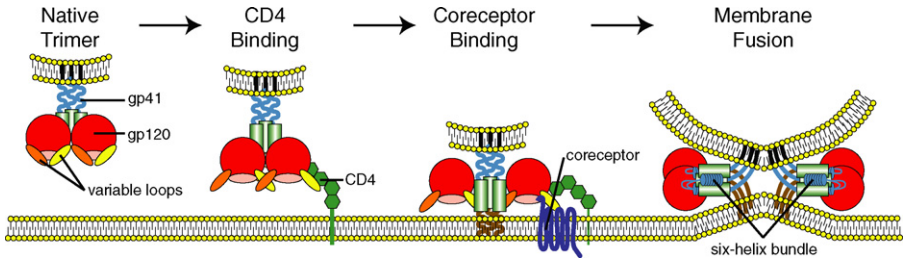


Fig. 2. Model of the multi-step process of HIV entry. The CD4 and coreceptor molecules are located in the host membrane (bottom), while the gp120 and gp41 proteins are associated with the viral membrane (curved, top). HIV entry is initiated by attachment of gp120 to CD4, which results in conformational changes in gp120 that result in the formation of the bridging sheet minidomain and extension of the V3 loop. The interaction between gp120 and coreceptor involves interactions between (1) the base of the V3 loop and adjacent regions and the N′ terminus of the coreceptor and (2) the crown of the V3 loop and the extracellular loops of the coreceptor. Following engagement of coreceptor, gp120 undergoes further conformational changes that allow for the insertion of the gp41 fusion peptide into the host membrane. Interaction between the HR1 and HR2 domains results in the formation of the six-helix bundle which brings the host and viral membranes into close proximity and creates a fusion pore, allowing entry of the HIV capsid into the host cell.

al., 1999; Huang et al., 2007). The relative dependence on the N′ terminus compared with the ECL2 loop appears to vary for different R5 viruses. The N′ terminal interaction has now been investigated using a crystal structure between gp120 and an unusual sulfated antibody, 412d, that mimics the N′ terminus of CCR5 (Huang et al., 2007). These studies suggest that prior to coreceptor binding, the V3 loop of gp120 is flexible and located close to the target cell membrane. Engagement of the N′ terminus by gp120 requires the formation of a conserved sulfotyrosine binding pocket and converts the V3 stem from a flexible structure into a rigid β-hairpin. In contrast, the interaction between the tip of the V3 loop and ECL2 is less well defined, but contact between these regions is particularly important for HIV entry (Lee et al., 1999; Platt et al., 2001; Samson et al., 1997; Wu et al., 1997). These data are consistent with a crystal structure of gp120 in which the V3 loop is found to extend nearly 30 Å from its base towards the cellular membrane, where it could presumably make contact with the ECLs of the coreceptor (Huang et al., 2005). Binding of gp120 to CXCR4 appears to occur in a similar fashion (Basmaciogullari et al., 2002; Chabot et al., 1999; Doranz et al., 1999; Lin et al., 2003a), although the V3 loop of viruses that utilize CXCR4 tend to be more positively charged, particularly at positions 11, 24, and 25 of V3 (De Jong et al., 1992; Fouchier et al., 1992; Milich et al., 1993; Xiao et al., 1998).

Binding of gp120 to coreceptor is thought to trigger further conformational changes in the envelope trimer that result in the exposure of the hydrophobic fusion peptide of gp41 and its insertion into the host cell plasma membrane. Following insertion of the fusion peptide, the heptad repeat regions HR1 and HR2 of gp41 undergo a highly energetically favorable rearrangement in which they fold back on each other. In a functional trimer spike, this forms a six-helix bundle structure where the three HR1 domains form a central coiled-coil and the three HR2 domains wrap around in an anti-parallel direction around the central coil (Chan et al., 1997; Weissenhorn et al., 1997). This structural rearrangement brings the transmembrane region of gp41, which is embedded in the viral membrane, into close proximity to the fusion peptide, which is inserted into the host cell membrane. This juxtaposition results in the formation of the fusion pore, allowing the viral capsid to enter the cell. The entry process of HIV has traditionally been thought to occur at the plasma membrane of the cell, but recent evidence suggests that endocytosis of viral particles may be required for full fusion (Miyauchi et al., 2009). These data are important for the consideration of entry inhibitors since an endocytic process would presumably impede the delivery of effective drug concentrations to their targets at the appropriate stages of entry.

3. Entry inhibitors

As the entry of HIV into target cells is a complex, multi-step process, the effort to identify pharmacological agents that can interfere with entry has resulted in a heterogeneous group of compounds that act at multiple stages of the entry process and have distinct mechanisms of action. Generally, the group of entry inhibitors can be subdivided into classes of agents that act at different stages of entry: attachment and CD4 binding, coreceptor binding, and fusion. Currently, only antagonists that block CCR5 binding (maraviroc) and fusion (enfuvirtide) have been approved by the FDA for treatment of HIV-infected patients, although strategies to inhibit other aspects of HIV entry are under development.

3.1. Drugs blocking the gp120–CD4 interaction

A number of diverse strategies for blocking the interaction between gp120 and CD4 have been pursued. In contrast to cell-associated CD4, which is required for HIV entry, soluble CD4 (sCD4) was found to inhibit HIV entry at high doses *in vitro*. However, clinical administration of this protein did not reduce viral loads in HIV-infected patients, and detailed analysis revealed that the levels of sCD4 achieved in patients were not sufficient to inhibit primary HIV isolates (Daar et al., 1990). Nevertheless, the observation that sCD4 could inhibit HIV entry has led to a class of sCD4 derivatives and CD4 mimics, including the PRO-542 CD-IgG2 tetrameric fusion protein and the NBD-556 and NBD-557 compounds (Allaway et al., 1995; Arthos et al., 2002; Martin et al., 2003; Schon et al., 2006; Trkola et al., 1995) (Fig. 3). These compounds appear to work by inducing a short-lived activated state of gp120 that spontaneously and irreversibly converts into a non-functional conformation. In contrast, the activated intermediate of gp120 generated by cell-surface CD4 is far more stable (Haim et al., 2009).

Other small-molecule inhibitors of the CD4-gp120 binding interaction are the BMS-378806 and BMS-488043 compounds. These agents also target the conserved CD4-binding site of gp120, but their precise mechanism of action is unclear. Some studies suggest that these compounds compete with sCD4 for binding to gp120 (Ho et al., 2006; Lin et al., 2003b), while others indicate that they do not block sCD4 binding (Schon et al., 2006) and may exert their antiviral effects by preventing conformational changes in gp120 upon CD4 engagement (Si et al., 2004). The clinical utility of BMS-806 is limited by a low genetic barrier to resistance, as 1–2 amino acid changes of gp120 result in 40–500-fold resistance to drug (Lin et al., 2003b). Amino acids that confer resistance to BMS-806, including Trp 112, Thr 257, Ser 375, Phe 382, and Met 426, line the 'phenylalanine 43 cavity' on gp120 that is involved in stabilization of the CD4-bound conformation of gp120 (Madani et al., 2004). Although BMS-806 was discontinued in phase II clinical development, is has shown success in an animal model as a potential topical microbicide (Veazey et al., 2005).

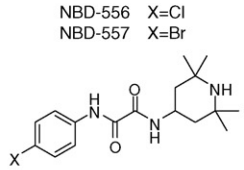
An additional strategy for blocking the interaction between CD4 and gp120 is to target the CD4 receptor using antibodies. The humanized antibody ibalizumab (TNX-355) binds to the D2 domain of CD4 and blocks CD4-induced conformational changes in gp120 (Moore et al., 1992). This agent has been demonstrated to reduce viral loads and increase CD4 T cell counts in combination with optimized background therapy (Kuritzkes et al., 2004), but is not orally bioavailable. This agent is currently in phase II studies.

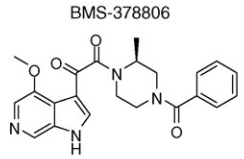
3.2. Drugs blocking the gp120–coreceptor interaction

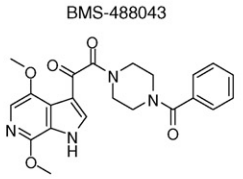
The discovery of CCR5 and CXCR4 as the critical coreceptors for HIV entry (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Oberlin et al., 1996; Zhang et al., 1998) was rapidly followed by the identification of a subset of individuals that were homozygous for an inactivating deletion for CCR5, Δ32-*ccr5*, which conferred high-level resistance to HIV-1 infection without significant immunological consequences (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Coupled with the observation that patients heterozygous for Δ32-*ccr5* had delayed rates of disease progression (Dean et al., 1996; Huang et al., 1996; Michael et al., 1997; Rappaport et al., 1997; Samson et al., 1996), these findings indicated that pharmacological blockade of the gp120–CCR5 interaction could be an effective and well-tolerated strategy for inhibiting HIV infection.

A number of naturally occurring ligands for the CCR5 receptor block HIV infection, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Cocchi et al., 1995). These chemokines exert their antiviral effects by blocking Env binding to CCR5 and by inducing the internalization of CCR5 from the cell surface (Alkhatib et al., 1997), but have potentially undesirable agonist activity on CCR5. Several RANTES derivatives, including AOP-RANTES, NNY-RANTES,

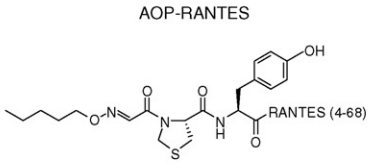
A. Drugs blocking gp120-CD4 binding

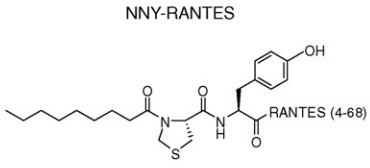


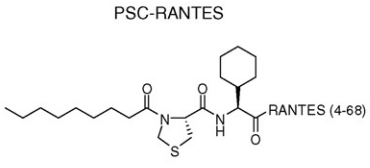


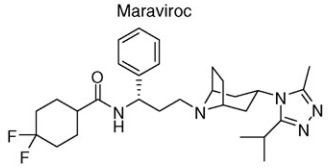


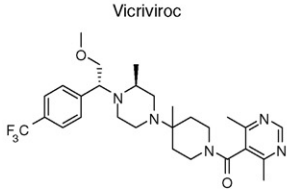
B. Drugs blocking gp120-CCR5 binding

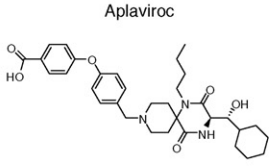




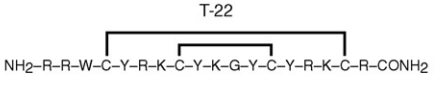


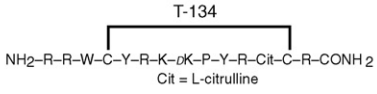


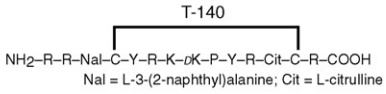




C. Drugs blocking gp120-CXCR4 binding

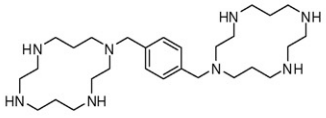


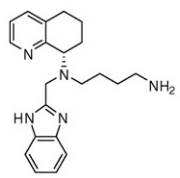




ALX-40-4C

AcHN–(dR)9–CONH2





D. Drugs blocking gp41-mediated membrane fusion

Enfuvirtide

NH2-F-W-N-W-L-S-A-W-K-D-L-E-L-L-E-Q-E-N-K-E-Q-Q-N-Q-S-E-E-I-L-S-H-I-L-S-T-Y-Ac

PIE7

NH2-dK-dG-dA-dC-dD-dY-dP-dE-dW-dQ-dW-dL-dC-dA-dA-COOH

Fig. 3. Structures of small-molecule and peptidic inhibitors of HIV entry.

and PSC-RANTES, have been developed in an effort to maintain anti-HIV activity while reducing or eliminating the agonistic effects on CCR5 (Mosier et al., 1999; Simmons et al., 1997). PSC-RANTES is in development as a potential microbicide for HIV (Cerini et al., 2008; Lederman et al., 2004). These agents compete with gp120 for binding to coreceptor and therefore are competitive antagonists of HIV infection.

Another strategy for inhibiting gp120–coreceptor interactions has been demonstrated by a group of small molecular compounds that bind to a hydrophobic pocket in the transmembrane helices

of CCR5 and are believed to exert their antiviral effects by altering the conformation of the extracellular loops that HIV interacts with during coreceptor binding. These agents do not bind to the same binding site as gp120, making them allosteric rather than competitive inhibitors. Many small-molecule antagonists have demonstrated efficacy against HIV replication *in vitro*, and three of these agents have been tested extensively in humans. Aplaviroc (GW873140) was tested in phase IIb studies before reports of idiosyncratic hepatotoxicity halted its development in 2005 (Nichols et al., 2008). Vicriviroc (SCH-D, SCH-417690) is currently in phase III clinical trials, and is a second-generation compound based on ancriviroc (SCH-C), which was discontinued after being associated with an elongated QT cardiac interval in clinical trials (Strizki et al., 2005; Tagat et al., 2004). Maraviroc (UK-427857) was approved in 2007 by the FDA for the treatment of HIV-infected patients with viral replication and HIV strains resistant to multiple antiretroviral agents. Maraviroc is the result of medicinal chemistry optimization of the compound UK-107543 and is effective against CCR5-using (R5-tropic) HIV strains in the low nanomolar range (Dorr et al., 2005). It is administered orally twice daily, with dosages that vary depending on the presence of strong CYP3A inducers or inhibitors in the antiviral regimen.

Antibodies that block the CCR5 receptor and prevent HIV infection have also been developed. PRO-140 is a humanized mouse anti-CCR5 antibody that prevents gp120 from engaging CCR5 but does not block CCR5 ligand activity. It has demonstrated potent efficacy against CCR5-tropic HIV strains both *in vitro* and in HIV-infected adults (Jacobson et al., 2008; Trkola et al., 2001). It is currently in phase II clinical trials.

Drugs that target the interaction between gp120 and the second primary coreceptor for HIV, CXCR4, have also been developed. Unlike CCR5, CXCR4 is essential for multiple physiological processes. In mice, knockout of the CXCR4 gene or its ligand, CXCL12 (SDF-1), is embryonic lethal due to defects in vascularization, hematopoiesis, cardiogenesis, and abnormal cerebellar development (Tachibana et al., 1998; Zou et al., 1998). In humans, heterozygous truncating mutations in the cytoplasmic tail of CXCR4 have been associated with WHIM syndrome, an immunodeficiency syndrome characterized by warts, hypogammaglobulinemia, infection, and myelokathexis (Hernandez et al., 2003).

Several polypeptide mimics of the natural ligand for CXCR4, CXCL-12, have been developed. These compounds, including T-22, T-134, T-140, and ALX40-4C, act by binding specifically to the CXCR4 receptor and preventing gp120 binding (Arakaki et al., 1999; Doranz et al., 2001; Tamamura et al., 1994, 1998). ALX40-4C was tested in humans prior to the identification of CXCR4 as a coreceptor for HIV, and despite being well-tolerated did not have a significant effect on reduction of HIV viral loads. However, the majority of patients in this study were later found to have CCR5-tropic strains of HIV (Doranz et al., 2001).

Small-molecule antagonists and partial agonists of CXCR4 are also under development. The bicyclam analog AMD3100 demonstrates potent activity against CXCR4-using (X4-tropic) strains of HIV *in vitro*, although clinical development as an antiretroviral agent was halted due to cardiac abnormalities and a lack of significant viral load reduction (Hendrix et al., 2004). Interestingly, patients treated with AMD3100 were found to have increased mobilization of CD34+ stem cells into the peripheral circulation (Liles et al., 2005), and the drug was approved by the FDA as a hematopoietic stem cell mobilizer for transplantation under the trade names Plerixafor and Mozobil. The compound AMD070 is a third-generation small molecule CXCR4 antagonist that is orally bioavailable and inhibits X4-tropic strains of HIV with similar potency to AMD3100 (Stone et al., 2007), but development has been halted due to liver histological changes in preclinical toxicity studies. Although no CXCR4 antagonists are in active clinical trials for the treatment of HIV-1 infection, several are in development and have demonstrated potent inhibitory effects on X4-tropic strains (Iwasaki et al., 2009; Murakami et al., 2009).

3.3. Drugs blocking gp41-mediated membrane fusion

Pharmacological agents that disrupt gp41-mediated membrane fusion, collectively called fusion inhibitors, were the first entry inhibitors to be approved for the treatment of HIV infection. Synthetic peptides corresponding to the HR1 and HR2 domains of gp41 were found to have potent antiviral effects (Jiang et al., 1993; Wild et al., 1992). These agents were initially analyzed during epitope-mapping experiments designed to identify targets for vaccine development (Matthews et al., 2004). However, biochemical and crystallization studies subsequently revealed their true mechanism of action: prevention of the formation of the six-helix bundle by competing for binding to the HR1 and HR2 domains on gp41 (Chan et al., 1997; Weissenhorn et al., 1997; Wild et al., 1994).

The fusion inhibitor enfuvirtide (T-20) was approved by the FDA for treatment-experienced, HIV-infected patients in 2003. Enfuvirtide is a linear, 36 amino acid synthetic peptide with a sequence identical to part of the HR2 region of gp41 (Wild et al., 1993), and competes for binding to HR1. It has demonstrated potency against HIV in clinical trials (Kilby et al., 2002; Lalezari et al., 2003), although there is considerable variability in the enfuvirtide sensitivity of primary virus strains (Derdeyn et al., 2000; Melby et al., 2006; Reeves et al., 2002). A number of other next-generation peptidic fusion inhibitors are under investigation, several of which have improved pharmacodynamics and efficacy compared with enfuvirtide (Dwyer et al., 2007; Lalezari et al., 2005b). Additionally, certain agents are active against some enfuvirtide-resistant strains of HIV (Dwyer et al., 2007; He et al., 2008), and fusion inhibitors that bind to different functional domains of gp41 can have synergistic effects (Pan et al., 2009). However, since peptidic fusion inhibitors are not orally bioavailable and must be administered via injection, the development of small-molecule inhibitors of gp41-mediated fusion remains a goal in drug development. An alternative approach that has shown considerable potential is the generation of D-peptide pocket-specific inhibitors of entry (PIEs) that bind to gp41 and which, unlike natural L-peptides, are not digested by proteases and have the potential for oral bioavailability (Welch et al., 2007).

4. Clinical considerations

Entry inhibitors hold considerable potential for the treatment of HIV infection, particularly in patients harboring viruses resistant to RT and protease inhibitors. Since entry inhibitors target multiple stages of the HIV entry pathway and operate via distinct mechanisms of action, they represent a diverse collection of drugs that will require a high degree of clinical acumen for their optimal use. As maraviroc and enfuvirtide are the currently approved members of this group of antiretroviral agents, several of the clinical challenges of the CCR5 antagonists and fusion inhibitors will be discussed here. However, as more drugs that inhibit HIV entry become available, especially those that target additional stages of the entry pathway, the complexity of this group of agents will undoubtedly increase.

One challenge common to all of the entry inhibitors is that they target the HIV envelope either directly or, in the case of coreceptor inhibitors, indirectly. The Env protein is highly diverse and can exhibit dramatic variability between patients. This diversity has two important consequences. First, viral resistance to entry inhibitors may occur via different pathways in different patients, depending on the envelopes they harbor prior to therapy. This may complicate efforts to identify patients that are resistant to

these agents. Second, the baseline sensitivity of patients to entry inhibitors can differ by several orders of magnitude, a much larger range than has been seen with other classes of antiretrovirals (Derdeyn et al., 2000; Melby et al., 2006; Reeves et al., 2002). While much of this variability can be attributed to diversity of the viral envelope, host factors are also involved in susceptibility to entry inhibitors, furthering the potential differences in efficacy between patients (Pugach et al., 2009).

As mentioned previously, HIV can utilize two different coreceptors for entry, CCR5 and CXCR4. Maraviroc and other CCR5 antagonists are only active against R5-tropic strains of HIV, and as a consequence, patients must undergo tropism testing prior to therapy. Currently, the Trofile assay from Monogram Biosciences is the only CLIA and clinically validated test for viral tropism prior to treatment with CCR5 antagonists, and the Enhanced Sensitivity form of the assay can detect the presence of X4-using viruses as low as 0.3% of the circulating population. Patients with detectable levels of viruses that use CXCR4 for entry are not candidates for CCR5 antagonist therapy, since viruses using CXCR4 have become the dominant circulating strains in patients harboring dual-mixed (R5/X4-tropic) viruses prior to therapy (Gulick et al., 2007; Lalezari et al., 2005a; Westby et al., 2006). The outgrowth of X4-tropic HIV is of clinical concern because when these viruses occur naturally during the course of infection their appearance is associated with accelerated CD4+ T cell loss and disease progression (Connor et al., 1997; de Roda Husman et al., 1997; Karlsson et al., 1994; Maas et al., 2000; Richman and Bozzette, 1994; Scarlatti et al., 1997; Schuitemaker et al., 1992). However, whether X4-tropic viruses that emerge under conditions of pharmacological blockade of CCR5 will also result in rapid CD4+ T cell loss and progression to AIDS remains unclear. In the majority of patients that had an outgrowth of X4-tropic viruses while on CCR5 antagonist therapy, the circulating strains of virus reverted to predominantly R5-tropic virus following cessation of therapy (Gulick et al., 2007; Lalezari et al., 2005a; Westby et al., 2006), suggesting that X4-tropic viruses may be less fit than their R5-tropic counterparts. The emergence of X4-tropic viruses as a result of CCR5 antagonist therapy and the consequences on disease progression will need to be closely monitored.

The disease stage of patients on CCR5 antagonist therapy is an additional complicating factor in their use. Currently, maraviroc is approved for patients with multi-drug-resistant HIV isolates, which tend to be patients in later stages of disease. However, X4-tropic viruses are more common in patients with advanced disease (de Roda Husman et al., 1997; Scarlatti et al., 1997; Schuitemaker et al., 1992), increasing the possibility that patients will fail therapy as a result of outgrowth of CXCR4 using viruses. Additionally, R5-tropic viruses that have been isolated from patients in later stages of disease have shown decreased sensitivity to entry inhibitors compared with viruses from patients at earlier disease stages (Karlsson et al., 2004; Koning et al., 2005, 2003; Repits et al., 2005). These data indicate that CCR5 antagonists might be more effective if utilized to treat patients at earlier stages of disease, and clinical trials of CCR5 antagonists on treatment-naïve patients are in progress. As CCR5 inhibitors become more established antiretroviral drugs, the indications for their use will likely change to maximize clinical benefit.

A substantial concern affecting all classes of antiretroviral agents, including the entry inhibitors, is the development of drug-resistant HIV strains. Since maraviroc and enfuvirtide have different mechanisms of action, it is not surprising that viruses gain resistance to these compounds in fundamentally different ways. Consequently, viruses that become resistant to one type of entry inhibitor can maintain sensitivity to entry inhibitors that act at distinct stages of HIV entry (Ray et al., 2007).

Resistance to maraviroc and other CCR5 antagonists can occur via two primary pathways: outgrowth of X4-tropic virus or the presence of viruses that can utilize CCR5 for entry in the presence of drug (Gulick et al., 2007; Lalezari et al., 2005a; Westby et al., 2006). As outgrowth of CXCR4 using viruses has been discussed previously, we will focus on the drug-resistant, R5-tropic viruses in this section. Since the CCR5 antagonists are relatively new antiretroviral agents, there is a paucity of data regarding resistance in patients. However, HIV strains that are resistant to CCR5 antagonists have been studied extensively *in vitro*, and several key trends have emerged. First, drug-resistant R5-tropic viruses can utilize the antagonist-bound conformation of CCR5 for entry (Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Second, resistant viruses typically show a non-competitive mechanism of resistance in which their ability to use the drug-bound receptor is not affected by further increases in drug concentration (Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Third, the regions of envelope that are responsible for resistance to CCR5 antagonists have mapped to multiple regions of the gp120 and gp41 proteins (Anastassopoulou et al., 2009; Baba et al., 2007; Ogert et al., 2008; Westby et al., 2007), suggesting that the resistance pathway utilized may vary depending on the viral envelope and the CCR5 antagonist used in treatment. Unfortunately, this indicates that developing genotypic screening assays to test for resistance, as is done prior to RT or protease inhibitor therapy, may not be viable for CCR5 antagonists. However, viruses are under a number of different selection pressures *in vivo* that do not act upon *in vitro*-derived resistant viruses, including the humoral immune system, constant high-level drug exposure, and complex target cell environments. Although it is likely that patient-derived viruses will emerge that can also utilize drug-bound forms of CCR5, this has not been formally demonstrated. A recent report from a study with the CCR5 antagonist aplaviroc has identified some patients with clinical failure that maintained primarily R5-tropic HIV strains (Kitrinos et al., 2009). These patients, and others failing CCR5 antagonist therapy, will need to be studied in detail to identify how HIV utilizes evolves resistance to these drugs *in vivo*.

Resistance to enfuvirtide has been investigated in patients that have failed treatment with these agents and, in contrast to CCR5 antagonists, several mutations indicative of resistance have been identified. These mutations cluster within the HR1 domain of gp41, the binding site for enfuvirtide, and include G36D, V38M, N43D/Q (Poveda et al., 2004; Wei et al., 2002; Xu et al., 2005). Although these mutations decrease the susceptibility of gp41 to enfuvirtide, they also decrease the efficiency of the fusion reaction and increase susceptibility to neutralizing antibodies (Reeves et al., 2005). However, compensatory mutations in HR2 can restore viral fusion kinetics while retaining enfuvirtide resistance (Ray et al., 2009). Unexpectedly, patients that continue treatment with enfuvirtide despite the presence of resistant viruses have been reported to maintain increased CD4+ T cell levels (Melby et al., 2007; Soria et al., 2008). Although the exact cause of the CD4+ T cell benefit to patients with enfuvirtide-resistant virus has not been determined, the drug has demonstrated antiviral effects in the setting of incomplete viral suppression (Deeks et al., 2007).

One additional clinical consideration with entry inhibitors is the synergistic use of multiple agents. Most entry inhibitors – both pharmaceutical agents and antibodies – have distinct windows during HIV entry at which they are active. For instance, enfuvirtide is effective only after CD4 binding and prior to gp41-mediated membrane fusion. Agents that slow viral entry kinetics expand these windows of viral sensitivity and increase the potency of drugs or antibodies. Importantly, many entry inhibitors appear to slow the overall rate of HIV entry, making the virus more susceptible to other entry inhibitors as well as neutralizing antibodies. Synergism has been demonstrated between monoclonal antibodies, coreceptor antagonists, and enfuvirtide (Ji et al., 2007; Reeves et al., 2005; Tremblay et al., 2000).

5. Future directions

The ability of entry inhibitors to block infection of target cells makes them attractive agents for use in microbicide therapy. Studies of HIV transmission have indicated that relatively few viruses are responsible for the initiation of infection in a new host (Keele et al., 2008), and strategies to block infection of cells at this stage may be particularly effective. A number of entry inhibitors have shown efficacy in animal transmission models as microbicide therapy (Lederman et al., 2004; Veazey et al., 2008, 2005). Microbicide strategies to prevent HIV transmission will be discussed in detail in subsequent chapters of this review series.

The success of antiretroviral therapy in improving and extending the lives of patients infected with HIV has been remarkable. However, due to the requirement for lifelong therapy, toxicities associated with these agents, and the emergence of drug-resistant strains of HIV, novel agents that target other aspects of the HIV life cycle are required. Entry inhibitors are a heterogeneous group of drugs that act at multiple points in the HIV entry pathway and present unique clinical challenges for their optimal use. The approval of maraviroc and enfuvirtide have demonstrated that blocking HIV entry is an effective strategy for reducing HIV replication in patients, and have provided additional clinical options for treating infected patients. As additional entry inhibitors become available, the complexity of this group of drugs will increase, but so will the options for the treating – and blocking – HIV infection.

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