Antiviral Research 98 (2013) 158–170

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Entry inhibitors and their use in the treatment of HIV-1 infection

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ARTICLE INFO

Article history:
Received 11 February 2013
Revised 18 March 2013
Accepted 19 March 2013
Available online 28 March 2013

Keywords:
HIV
Antiretroviral
Drug
Entry
Inhibitors
Treatment

ABSTRACT

Entry of HIV into target cells is a complex, multi-stage process involving sequential attachment and CD4 binding, coreceptor binding, and membrane fusion. HIV **entry inhibitors** are a complex group of drugs with multiple mechanisms of action depending on the stage of the viral entry process they target. Two entry inhibitors are currently approved for the treatment of HIV-infected patients. Maraviroc, a CCR5 antagonist, blocks interactions between the viral envelope proteins and the CCR5 coreceptor. Enfuvirtide, a fusion inhibitor, disrupts conformational changes in gp41 that drive membrane fusion. A wide array of additional agents are in various stages of development. This review covers the entry inhibitors and their use in the treatment of HIV-infected patients.

Contents

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http://dx.doi.org/10.1016/j.antiviral.2013.03.017

1. Introduction

Viral entry is a complex, multi-step process that involves coordinated, sequential interactions between proteins on the virion and the host cell. Multiple molecular interactions crucial for entry can therefore be inhibited, resulting in a wide array of compounds under investigation with varying structures and mechanisms of action. In the case of human immunodeficiency virus (HIV), two classes of antiretroviral drugs block entry into cells: fusion inhibitors and chemokine receptor antagonists. This paper reviews current knowledge of the structure of HIV surface proteins, mechanisms of viral entry into cells, and the development of drugs to inhibit specific steps in HIV entry–collectively known as **entry inhibitors**–and clinical considerations in the use of these agents.

The only viral proteins on the exterior of the lipid membrane surrounding intact virions are the surface (SU) glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41, encoded by the viral envelope gene (*env*). As understanding their structure is essential to a full appreciation of their function in the HIV entry process, their characteristics will be briefly reviewed here. More comprehensive reviews of the molecular structure–function relationships of gp120 and gp41 have recently been published (Wilen et al., 2012). Gp120 and gp41 originate as a single polyprotein, gp160, that is cleaved in the Golgi by furin or furin-like proteases prior to being incorporated in the virion membrane (Hallenberger et al., 1992). These glycoproteins associate as a homotrimer of non-covalently associated gp41–gp120 heterodimers to form a spike approximately 110–120 Å in diameter that protrudes 120–140 Å from the virion surface (Sougrat et al., 2007; Zanetti et al., 2006; Zhu et al., 2003, 2006, 2008). Each virion displays between 8 and 14 spikes on its surface, although how many of these spikes are needed or used for entry is not known (Magnus et al., 2009; Yang et al., 2005, 2006).

2. Structure of HIV surface proteins

2.1. Gp120

Gp120 is a heavily glycosylated protein that forms the distal portion of the Env spike that protrudes from the virion surface (Fig. 1A). It contains 5 conserved domains (C1–C5) and 5 variable domains (V1–V5) (Starcich et al., 1986). The conserved regions form the core of the protein while each of the variable regions forms a distally positioned loop (Leonard et al., 1990). Upon folding, gp120 forms three key structural regions: the inner domain, the outer domain, and the bridging sheet (Kwong et al., 1998). The inner domain is conserved between HIV strains and undergoes conformational changes upon receptor and coreceptor binding that are crucial for viral entry (Finzi et al., 2010). The outer domain is more exposed than the inner domain, is heavily glycosylated and contains three of the five variable domains (V3–V5), presenting a varying and cloaked surface to the humoral immune response. The bridging sheet contains very important residues that contribute to the CD4 binding site and the coreceptor binding site (Huang et al., 2007; Kwong et al., 1998). The bridging sheet contains two of the five variable loops (V1/V2), which are heavily glycosylated and play an important role in masking the CD4 binding site prior to receptor engagement (Finzi et al., 2010; Kwon et al., 2012). In addition to their roles in evading immune responses, the variable loops also serve critical purposes in the entry process, as will be discussed in detail below.

2.2. Gp41

Gp41 comprises the proximal transmembrane portion of the Env spike and is made up of three distinct regions: the C-terminal cytoplasmic tail, the membrane spanning domain, and the large ectodomain that non-covalently interacts with gp120 and contains

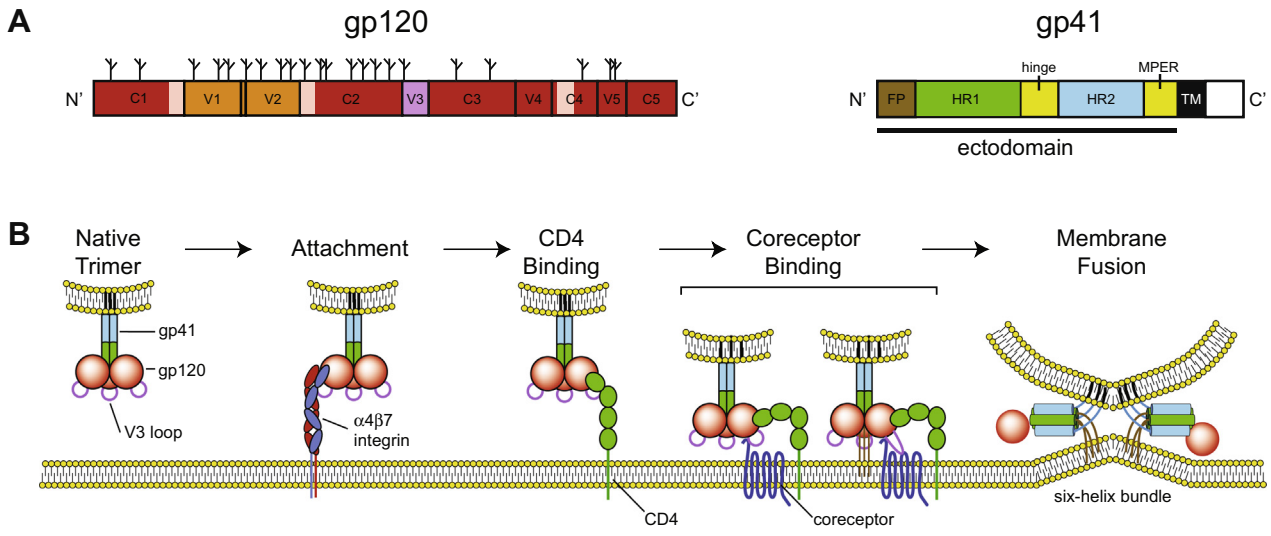


Fig. 1. (A) Schematic of the HIV gp120 and gp41 envelope proteins. Gp120 contains five conserved (C1–C5) and five variable (V1–V5) domains. Positions of conserved N-linked glycosylation sites are indicated by branched structures. The lighter regions of the C1, C2, and C4 regions represent the elements of the bridging sheet minidomain that stabilizes during CD4 binding. Gp41 consists of an N-terminal fusion peptide (FP), two heptad-repeat domains (HR1, HR2), and a transmembrane anchor (TM). The location of the hinge region and membrane proximal external region (MPER) are also shown. (B) Model of the multi-step HIV entry process. Gp120 and gp41 are located in the viral membrane (curved, top), while CD4 and coreceptor molecules are located in the host membrane (bottom). HIV entry can be facilitated by attachment to cell proteins including α4β7 integrin. HIV binding to CD4 results in conformational changes in CD4 that stabilize the bridging sheet region and form and expose the coreceptor binding site. Interaction between the N-terminus of CCR5 and the base of the V3 loop induces a conformational change in V3 that facilitates interactions between the tip of V3 and the extracellular loops of CCR5. Following engagement with coreceptor, gp120 undergoes further conformational changes that culminate with the insertion of the gp41 fusion peptide into the host membrane. Interactions between HR1 and HR2 result in the formation of the energetically favorable six-helix bundle which brings the viral and host membranes into close proximity. Formation of a fusion pore and entry of the HIV capsid into the cell mark the end of the HIV entry process.

the amino-terminal fusion peptide and two heptad repeat (HR) regions that drive fusion between the viral and host membranes (Fig. 1A). The ectodomain of gp41 is the portion that participates in membrane fusion and has 4 major structures. Starting closest to the membrane, there is a membrane-proximal external region (MPER) followed by two helical heptad-repeat regions (HR2/C-helix and HR1/N-helix), and finally the fusion peptide at the amino terminus. The MPER is a highly conserved region and is essential for successful viral fusion and infection (Muñoz-Barroso et al., 1999). The heptad-repeat regions from each gp41 in a trimer play a crucial role in viral fusion by rearranging to form an energetically stable six-helix bundle or 'coiled coil' that brings the viral membrane and the host membrane-anchored by the fusion peptide-into close proximity, as will be discussed in greater detail below.

3. HIV entry into host cells

3.1. Attachment

The initial interactions between HIV and a target cell may be facilitated by non-specific electrostatic interactions between positively charged domains on gp120 and negatively charged proteoglycans on the host cell surface (Mondor et al., 1998; Moulard et al., 2000) or by specific interactions between host proteins incorporated in the viral membrane and their ligands on target cells. For instance, interactions between intracellular adhesion molecule-1 (ICAM-1) in the HIV envelope and lymphocyte function-associated antigen-1 (LFA-1) on target cells has been demonstrated to increase attachment of HIV, leading to enhancement of viral infection (Fortin et al., 1997; Kondo and Melikyan, 2012). Additionally, interactions between gp120 and α4β7 integrin have also been proposed to promote HIV infection of target cells, particularly those in the gastrointestinal tract (Fig. 1B) (Arthos et al., 2008; Cicala et al., 2009; Nawaz et al., 2011). Once the virus is in close proximity to the host membrane with or without the assistance of attachment factors, gp120 can bind to CD4.

3.2. CD4 binding

CD4 is a transmembrane glycoprotein and belongs to the immunoglobulin superfamily. It contains four extracellular immunoglobulin domains (D1–D4), a single-pass transmembrane domain, and a short cytoplasmic tail that participates in intracellular signaling. CD4 is expressed on immune cell populations including monocytes, macrophages, and subsets of T cells and dendritic cells. The D1 domain of CD4 makes contact with the CD4-binding site of gp120, a highly conserved, carbohydrate-free region at the confluence of the inner and outer domains and the bridging sheet (Ashish et al., 2008; Kwong et al., 1998; Wang et al., 2001). Recent work by Kwon et al. suggests that the HIV core may 'sample' many conformations in the intact trimer prior to CD4 engagement (Kwon et al., 2012). Upon CD4 binding, a number of conformational changes in gp120 occur. First, the bridging sheet domain becomes an ordered 4-stranded β-sheet structure, altering the position and flexibility of the V1/V2 loop (Chen et al., 2005; Kwong et al., 1998). Second, the V3 loop extends and projects away from the virion spike (Huang et al., 2005, 2007). Third, the orientation of gp120 is altered such that the bridging sheet and V3 loop are positioned towards the host membrane where they can interact with coreceptor (Huang et al., 2005, 2007; Trkola et al., 1996; Wu et al., 1996). Unlike most other type 1 fusion machines, gp120 has evolved to bind to two receptors. CD4 binding induces a conformational transition from an unliganded state to a CD4-bound state that has the effect of creating and exposing the coreceptor binding site. The fact that the coreceptor binding site is rarely exposed prior to CD4 binding likely provides significant advantages for immune evasion.

3.3. Coreceptor binding

In 1996, after a decade-long pursuit, the coreceptors most often used for HIV entry in vivo were determined to be CCR5 and CXCR4 (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). CCR5 and CXCR4 are both seven-pass G-protein coupled receptors (GPCRs) that contain an extracellular N-terminal tail, three intracellular (ICL1, ICL2, ICL3) and extracellular loops (ECL1, ECL2, ECL3), and a C-terminal cytoplasmic tail. As chemokine receptors do not project as far from the cell surface as CD4, bending of the CD4 receptor after engagement is required for interaction with coreceptor. Binding of gp120 to CCR5 involves two separate interactions. First, the N-terminus of CCR5 binds to the bridging sheet of gp120 and the base of the V3 loop. Four sulfonated tyrosine residues (at positions 3, 10, 14, and 15) in the N-terminus have been shown to be important in gp120 binding (Farzan et al., 1998, 1999; Huang et al., 2007; Rabut et al., 1998). Binding of sulfated tyrosines alters the conformation of the V3 loop, converting it from a flexible loop to a rigid β-hairpin (Huang et al., 2007). This transition likely facilitates the second interaction between the ECLs of CCR5-particularly ECL2-and the tip of the gp120 V3 loop. This interaction is structurally less well defined but contact between these regions is critical for HIV entry (Lee et al., 1999; Platt et al., 2001; Samson et al., 1997; Wu et al., 1997). The relative dependence on the two interaction domains appears to vary for different strains of HIV. Binding of gp120 to CXCR4 is thought to occur in a similar manner but may be less dependent on the N-terminus (Basmaciogullari et al., 2002; Chabot et al., 1999; Doranz et al., 1999; Lin et al., 2003; Lu et al., 1997). The V3 loops of viruses that interact with CXCR4 tend to be more positively charged, particularly at positions 11, 24, and 25; this observation has led to algorithms to predict viral tropism based on sequencing data. Although these algorithms have only ~80% concordance with phenotypic tests, recent studies have shown that population sequencing or deep sequencing of HIV *env* can predict virologic response to CCR5 antagonists in patients (Kagan et al., 2012; McGovern et al., 2012; Swenson et al., 2011).

3.4. Fusion

Binding of gp120 to coreceptor triggers a second conformational change in the receptor that results in exposure of the gp41 fusion peptide and its insertion into the membrane of the host cell. These conformational changes also enable the heptad repeat regions HR1 and HR2 to undergo a highly energetically favorable rearrangement into a six-helix bundle or 'coiled coil' structure in which the HR2 domains pack in an antiparallel manner into the grooves of an inner 3-coil HR1 core (Chan et al., 1997; Weissenhorn et al., 1997). This results in the cellular and viral membranes being brought into close proximity and initiation of a fusion pore that allows the viral capsid to enter the cell.

3.5. Location of viral entry

The cellular location where HIV fusion occurs is still debated: microscopic studies of HeLa-derived target cells indicated that endocytosis of viral particles is required for full fusion (Miyauchi et al., 2009), whereas a recent paper examining cell-to-cell transfer of HIV did not demonstrate a requirement for endocytosis during infection of primary CD4+ T cells (Permanyer et al., 2012). These data are important for consideration of entry inhibitors because achieving sufficient concentrations of entry inhibitors may be more

difficult to achieve in endosomal compartments. Similarly, the focused release of virus across a virological synapse that occurs during cell–cell transfer or *trans*-infection from uninfected dendritic cells to CD4+ T cells may limit the effectiveness of entry inhibitors compared to cell-free viral infection. However, these modes of transmission may provide additional targets for inhibition. For instance, *trans*-infection from immature DCs to CD4s has been demonstrated to rely on DC-SIGN–gp120 interactions (Geijtenbeek et al., 2000). Recent studies suggest that interactions between sialyllactose-containing gangliosides incorporated into the HIV lipid membrane and the Siglec-1 receptor on mature dendritic cells strongly enhance *trans*-infection in an envelope-independent manner (Izquierdo-Useros et al., 2012; Puryear et al., 2012). Inhibiting these interactions may retard HIV transmission and spread in vivo.

4. Entry inhibitors

Since HIV entry is a complex, multi-stage process involving multiple sequential interactions between gp120 and gp41 and host surface proteins, there are a large number of potential targets to impede the entry process. As a consequence, entry inhibitors are a heterogeneous group of compounds with multiple mechanisms of action. Here, we will focus primarily on compounds that are or have been tested in clinical trials of HIV-infected patients.

4.1. Drugs blocking the gp120-CD4 interaction

A number of strategies for blocking interactions between gp120 and CD4 have been pursued. Development of recombinant, **soluble CD4** (**sCD4**) molecules to serve as molecular decoys for cell-associated CD4 was an early attempt at inhibiting HIV entry and was successful at high doses in vitro. However, sCD4 was relatively ineffective when administered to patients in clinical trials, as primary isolates of HIV were found to be significantly more resistant to sCD4 than laboratory adapted strains (Daar et al., 1990). In 1998, X-ray crystallographic structures of gp120 in association with CD4 provided molecular detail of the highly conserved CD4-binding site of gp120, guiding efforts to develop small molecular inhibitors to interrupt their interaction (Kwong et al., 1998; Wyatt et al., 1998). **BMS-378806** was an early compound blocking CD4-gp120 interactions and was discontinued in phase 2 clinical development (Fig. 2A). More recently, it has demonstrated success in blocking infection when used as a topical microbicide (Veazey et al., 2005). A subsequent compound, **BMS-448043**, demonstrated efficacy in reducing plasma HIV-1 RNA from treatment-naïve subjects but was discontinued due to poor pharmacokinetics. **BMS-626529** and its prodrug, **BMS-663068**, are currently being explored in phase 2 clinical studies in combination with optimized background therapy. Administration of BMS-663068 alone or in the presence of ritonavir in 50 patients resulted in a 1.21–1.73 log10 decline in plasma HIV RNA levels and was well tolerated (Nettles et al., 2012). HIV gp120 clones from patients demonstrated a >6 log10 difference in susceptibility to this drug, indicating that patient responses may be variable and highly dependent on circulating isolates (Nowicka-Sans et al., 2012). Resistance to BMS-626529 often involves the M426L mutation (Charpentier et al., 2012; Soulié et al., 2013). BMS-448043 and BMS-626529 appear to stabilize a conformation of gp120 that is incapable of binding to CD4 (Ho et al., 2006), whereas BMS-378806 and other compounds may interfere with conformational changes in gp120 that result in gp41 exposure (Madani et al., 2004).

An additional strategy is provided by the monoclonal antibody **ibalizumab** (TNX-355) that binds to the D2 domain of CD4. Residues within the D1 domain have also been implicated in the binding, suggesting that this antibody may in fact target the D1–D2 interface of CD4 (Song et al., 2010). In contrast to monoclonal antibodies predominantly targeting D1, ibalizumab is not immunosuppressive. It has demonstrated efficacy in phase 2 studies with optimal background therapy (Khanlou et al., 2011). Ibalizumab is often referred to as a 'post-attachment' inhibitor as it does not prevent gp120 binding to CD4 but blocks subsequent interactions with coreceptor. The loss of a potential N-linked glycosylation site in the V5 loop of gp120 has been associated with resistance (Pace et al., 2013).

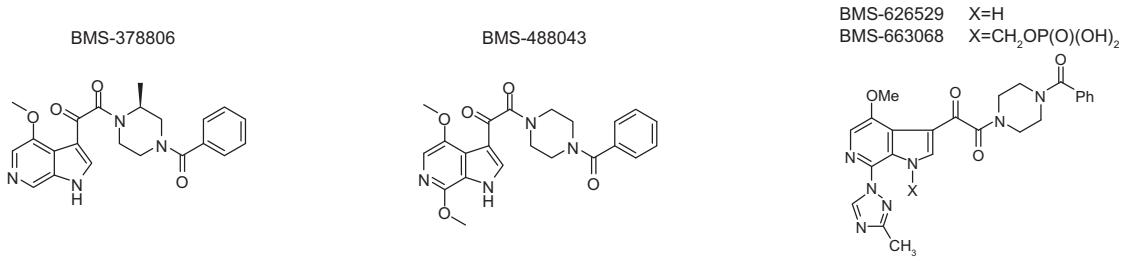
4.2. Drugs blocking the gp120-coreceptor interaction

In 1996, the coreceptors for HIV were identified as the chemokine receptors CXCR4 and CCR5 (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). This discovery was rapidly followed by the observation that a cohort of patients homozygous for an allele with a deletion in CCR5, *ccr5Δ32*, expressed virtually no CCR5 on the surface of their cells and were highly resistant to HIV-1 infection without significant immunological sequellae (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). In addition, it was found 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 suggested that blocking the gp120-CCR5 interaction with drugs could be an effective and well-tolerated strategy for inhibiting HIV entry.

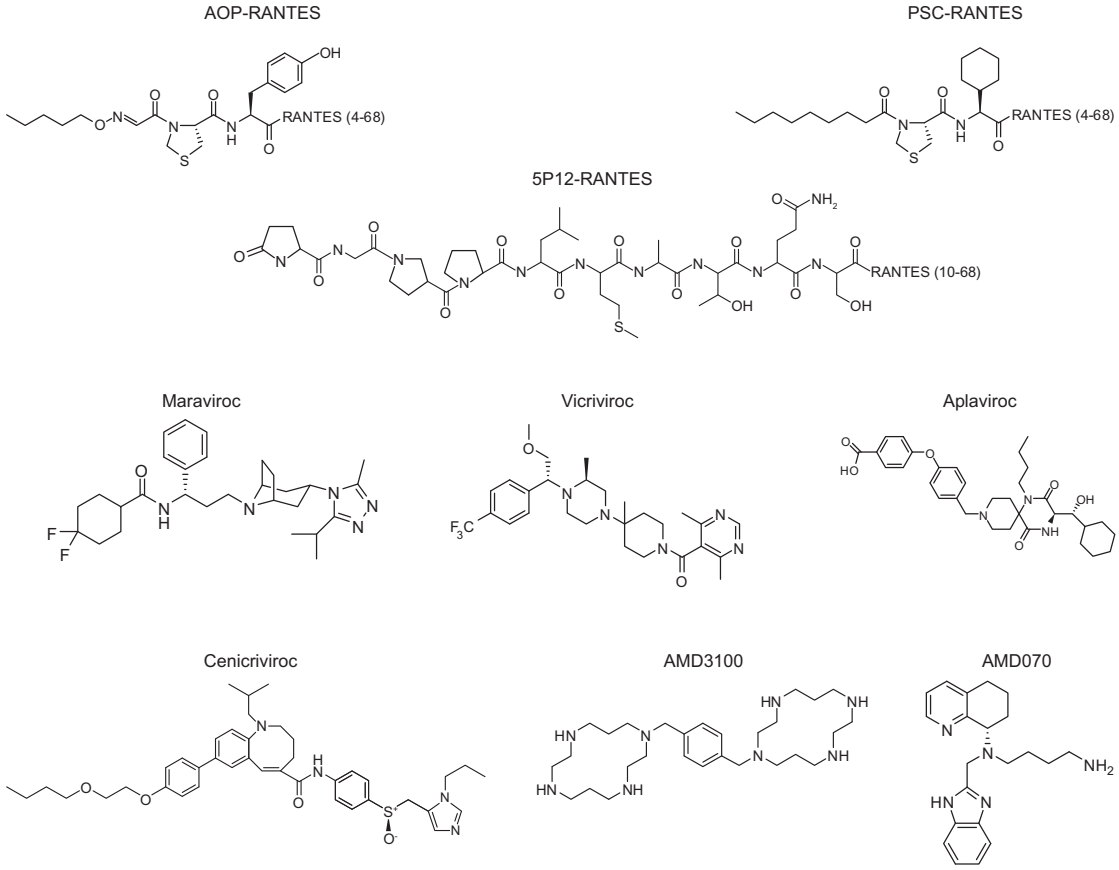
The natural ligands for CCR5 block HIV infection, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Cocchi et al., 1995). These chemokines appear to have a dual effect upon the CCR5 receptor: they induce internalization of CCR5 shortly after exposure, but over time CCR5 is recycled to the cell surface where the chemokines can also act as competitive antagonists for gp120 (Alkhatib et al., 1997). A number of RANTES derivatives, notably **AOP-RANTES**, **PSC-RANTES**, and **5P12-RANTES** have been developed either to maximize intracellular retention of HIV or to reduce agonist activity on CCR5 while maintaining anti-HIV activity (Fig. 2B) (Gaertner et al., 2008; Mosier et al., 1999; Simmons et al., 1997). PSC-RANTES and 5P12-RANTES have been explored for use as topical microbicides (Cerini et al., 2008; Lederman et al., 2004; Veazey et al., 2009), as discussed in more detail below.

Small molecular inhibitors of CCR5 have also been developed, which bind to a hydrophobic pocket in the transmembrane domains of CCR5 and alter the conformations of the extracellular loops required for HIV to enter cells. Unlike RANTES derivatives, these compounds do not bind to the same site as gp120 making them allosteric inhibitors of the gp120-CCR5 interaction. A number of compounds have been effective against HIV replication in vitro, and several have been tested extensively in humans. **Aplaviroc** (GW873140) reached phase 2b clinical trials before being halted in 2005 due to cases of idiosyncratic hepatotoxicity (Nichols et al., 2008). **Vicriviroc** (SCH-417690, SCH-D) reached phase 3 clinical trials but development was stopped after failure to demonstrate superiority to optimized background therapy in two double-blinded trials (Caseiro et al., 2012). The compound **Cenicriviroc** (TBR-652) is an antagonist of both CCR5 and CCR2, providing the drug with both anti-viral and anti-inflammatory properties (Klibanov et al., 2010; Lalezari et al., 2011; Marier et al., 2011). Since immune activation is associated with disease progression and all-cause mortality in HIV infection (Froebel et al., 2000; Giorgi et al., 1993; Liu et al., 1998; Sandler et al., 2011; Strategies for Management of Antiretroviral Therapy (SMART) Study Group et al., 2006), anti-inflammatory CCR2 blockade may have additional beneficial effects for HIV-infected patients. **Maraviroc** (UK-427857) is the product of medicinal chemical optimization of the compound UK-107543 effective in the low nanomolar range

A. Drugs blocking gp120-CD4 binding



B. Drugs blocking gp120-coreceptor binding



C. Drugs blocking gp41-mediated membrane fusion

Enfuvirtide

Ac-Y-T-S-L-I-H-S-L-I-E-E-S-Q-N-Q-Q-E-K-N-E-Q-E-L-L-E-L-D-K-W-A-S-L-W-N-W-F-NH_2

Sifuvirtide

Ac-S-W-E-T-W-E-R-E-I-E-N-Y-T-K-Q-I-Y-K-I-L-E-E-S-Q-E-Q-Q-D-R-N-E-K-D-L-L-E-NH2

VIRIP L-E-A-I-P-M-S-I-P-P-E-V-K-F-N-K-P-F-V-F

VIR-576 L-E-A-I-P-C-S-I-P-P-E-F-L-F-G-K-P-F-V-F-x2

Fig. 2. Structures of small-molecule and peptidic HIV entry inhibitors.

against HIV isolates that use the CCR5 coreceptor for entry (Dorr et al., 2005). As one of two entry inhibitors currently approved for treatment of HIV-infected patients in the Unites States and Europe, its use in the management of HIV infection will be discussed in further detail below. However, it is important to note that blockade of CCR5 by maraviroc may be of use in other clinical settings as well. For instance, maraviroc has been found to inhibit *Staphylococcus aureus* leukotoxin ED-mediated killing of CCR5+ cells (Alonzo et al., 2013) and to reduce leukocyte chemotaxis in graft-versus host disease (Reshef et al., 2012).

Several drugs blocking the gp120-CXCR4 interaction have also been developed, although none are currently approved for the management of HIV infection. The bicyclam analog **AMD3100** (Plexifor, Mozobil) has been extensively analyzed in patients. Originally selected due to its potent activity against CXCR4-using HIV strains in vitro, clinical development as an antiretroviral was halted following a lack of efficacy and cardiac abnormalities (Hendrix et al., 2004). Surprisingly, patients treated with AMD3100 demonstrated a mobilization of CD34+ stem cells into the peripheral blood (Liles et al., 2005). The drug has been used to mobilize hematopoietic stem cells for transplantation in cancer patients. A large number of clinical studies involving AMD3100 are currently underway, including for the management of hematopoietic disorders and malignancies (www.clinicaltrials.gov). **AMD070** is a third-generation CXCR4 antagonist that inhibits CXCR4-using strains comparably to AMD3100 (Stone et al., 2007). Although this compound is no longer in active development due to liver histological changes observed in preclinical toxicity studies (Moyle et al., 2009), several derivatives of AMD070 are being explored (Skerlj et al., 2011a, b).

4.3. Drugs blocking gp41-mediated membrane fusion

In the early 1990s, 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). Crystal structures of the post-fusion conformation of gp41 in 1997 subsequently revealed how these peptides actually work; namely by interfering with the packing of HR2 domains into the grooves of an HR1 coiled coil to form the six helix bundle driving membrane fusion (Chan et al., 1997; Weissenhorn et al., 1997).

In 2003, the fusion inhibitor **enfuvirtide** (T20) was the first entry inhibitor approved for the management of HIV infection (Fig. 2C). It is a linear, 36 amino acid synthetic peptide based upon the heptad repeat 2 (HR2) sequence of gp41. As described above, the HR2 region of gp41 packs in an antiparallel manner into the grooves on an inner HR1 coiled-coil to form the six helix bundle; enfuvirtide disrupts this interaction by competing with gp41 HR2 for binding to HR1. Enfuvirtide demonstrated anti-HIV activity in clinical trials (Kilby et al., 2002; Lalezari et al., 2003) although there is a wide range in sensitivity of primary patient isolates to the drug (Derdeyn et al., 2000; Melby et al., 2006; Reeves et al., 2002). Another 36 amino acid synthetic peptide, **sifuvirtide**, also contains residues from the HR2 domain and partially overlaps in sequence with enfuvirtide. Sifuvirtide has demonstrated enhanced potency against a wide range of primary and laboratory-adapted strains of HIV compared to enfuvirtide (He et al., 2008; Liu et al., 2011). Importantly, sifuvirtide has also demonstrated efficacy against some HIV strains resistant to enfuvirtide (Liu et al., 2011). Further clinical studies of sifuvirtide may be performed. As enfuvirtide and sifuvirtide are peptide drugs, they must be administered by subcutaneous injection. Development of orally bioavailable fusion inhibitors remains a goal in pharmacological development, and a pocket formed by the HR2 residues W628, W631, and I635 has been identified as a potential target for small molecule inhibitors (Chan et al., 1998). *D*-peptides, which unlike natural *L*-peptides are not digested by proteases and are therefore suitable for oral delivery, have potential as pocket-specific inhibitors of entry (PIEs) (Welch et al., 2007).

An alternative strategy to inhibit fusion is suggested by findings from Münch and colleagues, who isolated a peptide from human blood that potently blocked HIV-1 infection (Münch et al., 2007). This peptide, termed virus inhibitory peptide (**VIRIP**), corresponds to the C-proximal region of α1-antitrypsin. Rather than inhibiting six-helix bundle formation by preventing HR1-HR2 interactions, VIRIP was found to block entry by binding to the fusion peptide and preventing its insertion into the host cell membrane (Münch et al., 2007). An optimized dipeptide derivative of VIRIP, **VIR-576**, was administered to 18 HIV-infected patients in phase 1/2 clinical trials and resulted in a 1.23 log10 decline in viral load over 10 days of monotherapy (Forssmann et al., 2010). Resistance to these peptides has been observed after long-term passage in culture (Bonjoch et al., 2013).

5. Clinical considerations

As entry inhibitors are a diverse group of compounds targeting multiple stages of the HIV entry process, their optimal use will require a high degree of clinical acumen. Moreover, the variability of the HIV *env* gene and the ability of the virus to utilize multiple coreceptors for entry complicate the use of entry inhibitors in patients. However, these agents hold considerable potential for the treatment of infected patients, particularly those with resistance to reverse transcriptase, protease, or integrase inhibitors. Since maraviroc and enfuvirtide are the two entry inhibitors currently approved for the treatment of HIV-infected patients, several of the clinical challenges in their use will be discussed here.

5.1. Identifying suitable patients for treatment

A primary challenge in the optimal use of entry inhibitors is that the diversity of the HIV *env* gene results in a wide array of primary viral sequences and Env structures to be inhibited. This diversity has two important consequences. First, the susceptibility of primary HIV isolates to entry inhibitors can vary by several orders of magnitude, a much larger range than is seen with agents targeting reverse transcriptase, protease, or integrase (Derdeyn et al., 2000; Melby et al., 2006; Nowicka-Sans et al., 2012; Reeves et al., 2002). Patients harboring pre-existing resistance to entry inhibitors have also been identified (Tilton et al., 2010a). While *env* diversity clearly contributes to the variability in sensitivity to entry inhibitors, host factors have also been implicated (Pugach et al., 2009). Second, studies have shown that resistance occurs by different pathways in different patients. This suggests that mutations that lead to drug resistance are dependent on the context of the patients' *env* genes and that efforts to identify signature drug resistance mutations based on *env* sequence are unlikely to be successful. Together, these consequences of viral *env* diversity complicate the clinician's ability to identify patients in whom entry inhibitors are likely to be effective.

HIV can use multiple coreceptors for entry in patients–most commonly CCR5 and CXCR4, although alternative coreceptor use has also been reported (Jiang et al., 2011). Maraviroc is effective only against HIV strains using CCR5 for entry. The presence of CXCR4-using viruses is associated with virologic failure due to outgrowth of these strains during therapy (Gulick et al., 2007; Lalezari et al., 2005; Westby et al., 2006). As a consequence, patients should undergo tropism testing prior to treatment. The Trofile assay from Monogram Biosciences is a phenotypic assay that has been used extensively to viral tropism in clinical trials. Genotypic assays to determine HIV coreceptor usage based on *env* sequence have been

shown to predict patient response to maraviroc therapy (Kagan et al., 2012; McGovern et al., 2012; Swenson et al., 2011), but typically only have ~80% concordance with tropism as measured by the Trofile assay (Archer et al., 2012; Gonzalez-Serna et al., 2012; Swenson et al., 2013). Although the appearance of CXCR4-tropic HIV has been associated with accelerated CD4+ T cell loss and progression to AIDS in untreated patients (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), CXCR4-tropic viruses that emerge during pharmacological blockade of CCR5 are rapidly replaced by CCR5-tropic viruses upon termination of therapy (Gulick et al., 2007; Lalezari et al., 2005; Westby et al., 2006). Indeed, a study treating patients with predominantly CXCR4-tropic, dual-mixed (CCR5/CXCR4), or non-typable virus with maraviroc found that CD4+ T cell counts increased in all groups at weeks 24 and 48 compared to baseline, indicating that CXCR4-tropic HIV emergence was not associated with rapid CD4+ T cell loss or disease progression in the context of CCR5 blockade (Saag et al., 2009).

Importantly, the disease stage of patients is related to the coreceptor usage of HIV. Transmitted forms of HIV predominantly use CCR5 for entry and CCR5-tropic isolates dominate during early infection. However, CXCR4-tropic HIV is more common in patients with advanced disease (de Roda Husman et al., 1997; Scarlatti et al., 1997; Schuitemaker et al., 1992); fewer of these patients may be suitable candidates for maraviroc treatment. In addition, CCR5-tropic viruses isolated from patients at later stages of disease have demonstrated decreased sensitivity to entry inhibitors compared to viruses isolated from patients early in HIV infection (Karlsson et al., 2004; Koning et al., 2003, 2005; Parker et al., 2013; Repits et al., 2005), suggesting that maraviroc and other CCR5 antagonists may have optimal utility if given early in infection.

A final consideration in determining which patients are suitable candidates for entry inhibitor therapy are CD4+ T cell counts at initiation of therapy. Maraviroc has been demonstrated to result in higher virological suppression in patients with >50 CD4+ T cells/mm^3 (Schapiro et al., 2011). Enfuvirtide has demonstrated virological efficacy and immune reconstitution in patients with <50 CD4+ T cells/mm^3 when added to a tenofovir-based HAART regimen (Bonora et al., 2012).

5.2. Viral resistance

A primary clinical concern in the use of all antiretroviral agents is the development of drug-resistant HIV isolates. As maraviroc and enfuvirtide target different stages of the HIV entry process, it is perhaps not surprising that viruses demonstrating resistance of one drug typically maintain sensitivity to the other (Ray et al., 2007; Tilton et al., 2010a,b).

5.2.1. Resistance to maraviroc

Resistance to maraviroc can occur through two predominant pathways: the outgrowth of pre-existing CXCR4-tropic HIV isolates and the emergence of CCR5-tropic viruses that can infect cells in the presence of drug (Gulick et al., 2007; Lalezari et al., 2005; Tilton et al., 2010a,b; Westby et al., 2006, 2007). As outgrowth of CXCR4-tropic HIV strains has been discussed above, we will focus here on the mechanisms and consequence of resistance by CCR5-tropic HIV. Studies with maraviroc and other CCR5 antagonists, both in vitro and in vivo, have revealed several key trends. First, resistance to these drugs appears to occur predominantly through the use of drug-bound receptor for entry characterized by a non-competitive mechanism in which further increases in drug concentration do not reduce entry efficiency (Pugach et al., 2007; Tilton et al., 2010a,b; Trkola et al., 2002; Westby et al., 2007). However, viruses that demonstrate resistance via a competitive mechanism, as evidenced by a shift in IC50 values without reduction of maximal percent inhibition at high concentrations, have also been reported (Ratcliff et al., 2013). Second, resistance determinants have been mapped to multiple regions of gp120, particularly the V3 loop, as well as gp41 (Anastassopoulou et al., 2009; Baba et al., 2007; Kuhmann et al., 2004; McNicholas et al., 2010; Ogert et al., 2008; Putcharoen et al., 2012; Tilton et al., 2010b; Tsibris et al., 2008; Westby et al., 2007; Yuan et al., 2011). Importantly, there was minimal overlap of resistance-conferring mutations, indicating that the resistance pathway utilized is heavily dependent upon the context of the viral *env* gene and the CCR5 antagonist utilized. Resistance patterns also depend upon the antagonist used; maraviroc and vicriviroc selected for different patterns of resistance in the V3 loop of gp120 (Berro et al., 2012). Third, resistant viruses often demonstrate altered interactions with CCR5, particularly with reliance on residues in the N-terminal or ECL2 regions. CCR5 antagonist-resistant viruses differ in which CCR5 residues are critical for entry (Henrich et al., 2012; Roche et al., 2011; Tilton et al., 2010b), supporting the conclusion the V3 can mutate in a variety of ways to recognize different residues on drug-bound coreceptor. Fourth, the degree of cross-resistance to other CCR5 antagonists varies. Maraviroc and other coreceptor antagonists induce conformational changes to the extracellular loops of CCR5 but have minimal impact on the N-terminus, as demonstrated by antibody binding studies to drug-free and drug-bound receptors. The ECL2 loop changes vary by antagonist: for instance, aplaviroc strongly inhibits binding of the monoclonal antibody 45529 and completely abrogates binding of 45531 to ECL2, whereas vicriviroc, maraviroc, and TAK-779 show similar and more modest effects on antibody binding (Tilton et al., 2010b). However, these differences appear to be significant: resistant viruses isolated from patients treated with vicriviroc are often cross-resistant to maraviroc (Putcharoen et al., 2012), whereas the converse is not true (Tilton et al., 2010b; Westby et al., 2007). These studies have important ramifications for future clinical use of entry inhibitors, as they imply that the order in which CCR5 antagonists are given to patients may impact the emergence of cross-resistant viruses perhaps by selecting for different patterns of V3 resistance (Berro et al., 2012). From a drug-development perspective antagonists inducing significantly different changes in ECL2 compared to maraviroc may require drastic alterations in the way the virus recognizes CCR5 for cross-resistance to emerge, such as a dependence on the N-terminus of CCR5 alone (Pfaff et al., 2010).

While in vitro and in vivo studies have extensively characterized the development of resistance of CCR5 antagonists, it is important to note that many cases of virological failure in patients are not explained by coreceptor switching to CXCR4 or by mutations that confer reduced susceptibility to drug (Hardy et al., 2010; Kitrinos et al., 2009; McNicholas et al., 2010; Tilton et al., 2010b; Tsibris et al., 2008). The cause of these treatment failures remains unclear.

5.2.2. Resistance to enfuvirtide

Enfuvirtide resistance mutations have been investigated in patients failing therapy, and in contrast to maraviroc, several 'signature' resistance mutations have been identified. These mutations cluster in the HR1 domain where enfuvirtide binds to gp41 and include G36D, V38A, N42D, and N43D/Q (Poveda et al., 2004; Wei et al., 2002; Xu et al., 2005). Mutations in *env* outside of HR1 appear to play little role in enfuvirtide resistance (Baatz et al., 2011). While these resistance mutations decrease sensitivity to enfuvirtide, they are associated with reduced efficiency of fusion and enhanced susceptibility to neutralizing antibodies (Reeves et al., 2005). Compensatory mutations in the HR2 domain of gp41 can restore viral fusion kinetics while retaining enfuvirtide resistance (Ray et al., 2009). Importantly, patients that continue enfuvirtide treatment despite the presence of resistance mutations

retain CD4+ T cell increases from therapy (Melby et al., 2007; Soria et al., 2008), potentially due to antiviral effects under conditions of incomplete virologic suppression (Deeks et al., 2007).

5.2.3. Viral consequences of resistance

Mutations in the Env protein that confer resistance to entry inhibitors can impact viral fitness and tropism. As mentioned above, mutations in HR1 that confer resistance to enfuvirtide reduce fusion efficiency; these are compensated for by additional mutations in HR2. The majority of Envs that are resistant to coreceptor antagonists demonstrate incomplete inhibition in the presence of drug, suggesting that the virus cannot use drug-bound CCR5 as efficiently as the native form (Kuhmann et al., 2004; McNicholas et al., 2010; Ogert et al., 2009; Tilton et al., 2010a; Westby et al., 2007). However, some patient-derived Envs that have developed resistance to CCR5 antagonists have adapted to preferentially use the drug-bound form of the receptor. These viruses have improved entry efficiency and kinetics in the presence of maraviroc and vicriviroc, respectively (Putcharoen et al., 2012; Tilton et al., 2010b). Interestingly, a virus that developed complete resistance to vicriviroc in a patient on a phase 2b clinical trial reverted to a sensitive phenotype following treatment discontinuation, suggesting a fitness cost to the resistance mutations in the absence of drug (Tsibris et al., 2008, 2012). This contrasts with in vitro data indicating a lack of fitness cost of coreceptor antagonist resistance (Anastassopoulou et al., 2007). Finally, there is evidence that CCR5-tropic viruses resistant to CCR5 antagonists can have altered tropism on primary CD4+ T cells and macrophages in the presence and absence of drug, suggesting that the consequences of resistance may be complex (Pfaff et al., 2010; Roche et al., 2011).

5.3. Entry inhibitors in combination therapy

Combination antiretroviral therapy has traditionally consisted of two reverse transcriptase inhibitors and a third drug from a different class, typically a protease inhibitor. The addition of entry inhibitors, integrase inhibitors, and new reverse transcriptase and protease inhibitors to the clinical arsenal has expanded treatment options to reduce toxicities (drug-sparing regimens), combat multidrug resistant HIV strains (salvage therapy), and to intensify antiretroviral treatment. In particular, maraviroc has been studied in non-nucleoside reverse transcriptase inhibitor (NNRTI) and ritonavir-boosted protease inhibitor sparing regimens and been found to be effective and well-tolerated (Bonjoch et al., 2013; Mills et al., 2013; Mora-Peris et al., 2013). In addition, maraviroc appears to have reduced toxicity on adipose cells in vitro (Díaz-Delfín et al., 2013) and improves lipid profiles in patients with dyslipidemia (Bonjoch et al., 2013; MacInnes et al., 2011). Maraviroc has also been investigated as part of salvage regimens (Calcagno et al., 2011; Imaz et al., 2011; Reuter et al., 2010; Weimer et al., 2013), but use trails that of other antiretroviral agents such as raltegravir, darunavir, and etravirine (Willig et al., 2013). Finally, maraviroc intensification as part of efforts to increase CD4+ counts in patients with poor immune reconstitution despite control of viral replication resulted in only extremely modest CD4+ gains (~30 cells/mm^3/year and <20 cells/mm^3/24 weeks, respectively) in two recent clinical studies (Cuzin et al., 2012; Wilkin et al., 2012).

5.4. Synergistic action of multiple entry inhibitors

An additional clinical consideration with the use of entry inhibitors is the synergistic use of multiple drugs. There are several mechanisms through which multiple entry inhibitors may yield enhanced antiviral activity. First, as demonstrated with enfuvirtide, resistance mutations can alter the kinetics of stages of the HIV entry process and increase the potency of drugs or antibodies. Second, if more entry inhibitors are approved for the use in HIV-infected patients, it is feasible that the number of resistance mutations required within gp120 and gp41 may significantly impact the fitness of the virus or be incompatible with the formation of a meta-stable Env trimer required for entry. Synergism has already been demonstrated between coreceptor antagonists, enfuvirtide, and monoclonal antibodies (Ji et al., 2007; Reeves et al., 2005; Tremblay et al., 2000).

5.5. Future directions

5.5.1. Microbicide development

A large number of entry inhibitors are at various stages of development for the treatment of HIV-infected patients. In addition, efforts are underway to use these agents to prevent HIV transmission through the development of effective microbicides. In particular, RANTES derivatives and maraviroc have demonstrated potent inhibition of viral transmission in non-human primate models (Lederman et al., 2004; Veazey et al., 2010). Resistance to these RANTES derivatives can occur through coreceptor switching to CXCR4 (Nedellec et al., 2011), but resistance of CCR5-tropic Envs remains controversial. A study examining *env* sequences in a SHIV (SF162-p3)-infected macaque pretreated with PSC-RANTES identified two mutations that enabled replication at high doses of drug (Dudley et al., 2009). However, introduction of these mutations into the parental SF162 or macaque-adapted SF162-p3 *envs* was not found to alter sensitivity to PSC-RANTES using a single-cycle assay for resistance (Nedellec et al., 2010). Analysis of macaques infected despite maraviroc-based microbicide treatment did not identify drug-resistant viruses, implying the infections arose from incomplete viral inhibition rather than the presence and selection of drug-resistant variants (Tsibris et al., 2011). Microbicide development efforts with these compounds continue and would provide an important tool for preventing HIV transmission.

5.5.2. Inhibition of HIV entry through gene therapy approaches

The functional cure of an HIV-infected patient with acute myelogenous leukemia by bone marrow transplantation using cells from a *ccr5Δ32* homozygous patient has reinvigorated interest in the eradication of HIV infection (Allers et al., 2011; Hütter et al., 2009). However, chemo- and radio-ablative treatments followed by allogeneic bone marrow transplantation carry significant risks to the patient. An alternative strategy is to genetically modify a patient's hematopoietic stem cells or CD4+ T cells to disrupt the *ccr5* or *cxcr4* genes using zinc-finger nucleases (ZFN) or transcription activator-like effector nucleases (TALEN) (Holt et al., 2010; Maier et al., 2013; Perez et al., 2008; Wilen et al., 2011). A recent study has demonstrated ZFN-mediated homologous recombination to simultaneously knockout the CCR5 locus and insert a cassette of restriction factors to inhibit viruses that do enter cells. The resultant cells were resistant to both CCR5- and CXCR4-tropic HIV variants (Voit et al., 2013). This exciting field of HIV research has been recently reviewed (Didigu and Doms, 2012).

6. Conclusion

The advent of combination antiretroviral therapy has transformed the HIV epidemic from a nearly universally fatal disease to a medically managed condition in which patients have near normal life expectancies. Antiretroviral drugs initially targeted the reverse transcriptase and protease enzymes, but over the past decade additional classes of drugs targeting the HIV entry process and the integrase enzyme have become available for the management of HIV-infected patients. Entry inhibitors represent a diverse group

of compounds targeting multiple stages of the HIV entry process and currently contain two clinically approved drugs–maraviroc and enfuvirtide–that target gp120-CCR5 interaction and gp41-mediated fusion, respectively. Primarily due to the ability of HIV to use multiple coreceptors for entry and the diversity of the HIV *env* gene, identifying patients that will respond effectively to treatment is a challenge and the optimal use of these drugs will require a high degree of clinical acumen. As more entry inhibitors are approved for the treatment of infected patients, the complexity of this group of compounds will increase, but so will the opportunities for the synergistic use of these compounds and the treatment of HIV infection.

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