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Entry Inhibitors in HIV Therapy

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Preface

The entry of HIV into cellular targets is mediated by the envelope protein (Env) which studs the viral surface. A major milestone for inhibition of HIV entry was achieved in 2003 with the approval of the HIV fusion inhibitor enfuvirtide. A number of other entry inhibitors are currently being developed with diverse mechanisms of action, including (i) interfering with relatively non-specific Env-cell surface attachment factor interactions, (ii) inhibiting specific receptor and coreceptor interactions, and (iii) blocking Env transition through conformational intermediate states. Major challenges facing entry inhibitor development include the extensive sequence diversity and remarkable plasticity of Env. Env diversity can give rise to marked variability in baseline susceptibility of HIV strains to entry inhibitors, as opposed to typically minor variation in baseline susceptibilities to HIV reverse transcriptase and protease inhibitors.

Entry Inhibitors in HIV Therapy presents the current status of this relatively new and highly dynamic class of inhibitors and provides a unique overview of obstacles and considerations for HIV entry inhibition compared to other antiretroviral targets. It will be of interest to research scientists as well as clinicians.

The introductory chapters of this book provide an overview of HIV entry, entry inhibition and envelope diversity. The first chapter, by Tilton and Doms, reviews current knowledge of how Env mediates entry and presents an overview of entry inhibitors. Vergne and Peeters then discuss the challenge of genetic diversity in the HIV envelope.

Subsequent chapters of this volume feature current information on individual classes of entry inhibitors that target each step of the virus entry pathway, from attachment to membrane fusion, with an emphasis on the complex determinants of entry inhibitor susceptibility, resistance mechanisms, and how these issues create new challenges for antiretroviral therapy. Pöhlmann and Tremblay review inhibitors that block HIV cell surface attachment and Lin, Kadow and Alexander discuss inhibitors that target Env interactions with CD4. Strizki and Mosier review inhibitors of Env-coreceptor interactions and Wang and Weiss describe inhibitors that target HIV fusion. Studies of entry inhibitors as microbicides are presented by Hart and Evans-Strickfaden and the use of entry inhibitors against non-subtype B viruses is discussed by Morris, Binley and colleagues. Coakley then discusses the clinical utility of coreceptor typing and entry inhibitor susceptibility testing.

The final chapters of this book highlight the clinical use of entry inhibitors and survey antiretroviral development. Heath and Kilby review the current sta-

tus of entry inhibitors in clinical studies. The development and approval of enfuvirtide is detailed by Greenberg, then past and present drug development targets are discussed by Gulick.

In summary, this book presents a comprehensive and current overview of entry inhibitors from an expert panel of authors with diverse backgrounds and perspectives, incorporating many unrelenting successes against a backdrop of formidable challenges.

Jacqueline D. Reeves, San Francisco
Cynthia A. Derdeyn, Atlanta February 2007

Entry Inhibitors in HIV Therapy
Edited by Jacqueline D. Reeves and Cynthia A. Derdeyn

Introduction to entry inhibitors in the management of HIV infection

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Introduction

The introduction of highly active antiretroviral therapy (HAART) has dramatically improved the survival of patients infected with human immunodeficiency virus (HIV). However, HAART is complicated by the continuing emergence of drug-resistant strains of HIV and toxicities associated with the antiretroviral agents [1, 2]. Furthermore, since the combination HAART regimens are incapable of eradicating HIV infection, lifelong therapy is required to avoid disease progression [3, 4]. Together, these factors necessitate the continual development of new antiretroviral agents that can be utilized against resistant viruses or that in combination with other agents can provide superior viral suppression with less toxicity.

While all stages of the HIV life cycle are potential targets for therapeutic intervention, HAART regimens have been predominantly focused on two viral enzymes, reverse transcriptase (RT) and protease. New antiretroviral agents under development include integrase inhibitors as well as compounds that target components of the viral entry pathway. These latter compounds are collectively known as entry inhibitors, and are the subject of this volume. Entry inhibitors are varied in that they can target three different steps in the viral entry pathway: CD4 binding (Chapter by Lin et al.), coreceptor binding (Chapter by Stritzki/Mosier), or membrane fusion (Chapters by Wang/Weiss and Greenberg). Regardless of their precise mechanism, all entry inhibitors target the viral envelope (Env) protein directly or, in the case of coreceptor inhibitors, indirectly. Thus, a major challenge to the clinical use of entry inhibitors is the impressive sequence diversity of the Env protein, which contributes to the significant variation in the baseline sensitivity of HIV isolates to these compounds (Chapter Vergne/Peeters). Patient-specific variation in host factors involved in the HIV entry process may also modulate the susceptibility of HIV to entry inhibitors and the development of resistance mutations. Resistance pathways to entry inhibitors are likely to be complex, and may alter viral tropism (and hence pathogenesis and disease course) by altering the man-

ner in which Env interacts with host cell receptors. Together, these factors make entry inhibitors a particularly interesting class of antiretroviral agent that may shed significant light on HIV pathogenesis.

The viral Env protein

The Env protein of HIV-1 is the molecular determinant for viral attachment and membrane fusion. Env is synthesized as a single polypeptide precursor (gp160) that forms noncovalently associated homotrimers, and which is cleaved during transport to the cell surface into two subunits, gp120 and gp41. The gp120 subunit mediates receptor binding, while gp41 mediates the membrane fusion reaction. HIV-1 gp120 consists of five conserved (C1–C5) and five variable (V1–V5) domains [5], with the conserved domains contributing to the core of gp120, while the variable domains (and numerous *N*-linked glycosylation sites) are located near the surface of the molecule. The V1–V4 regions form exposed 'loops' anchored at their bases by disulfide bonds [6]

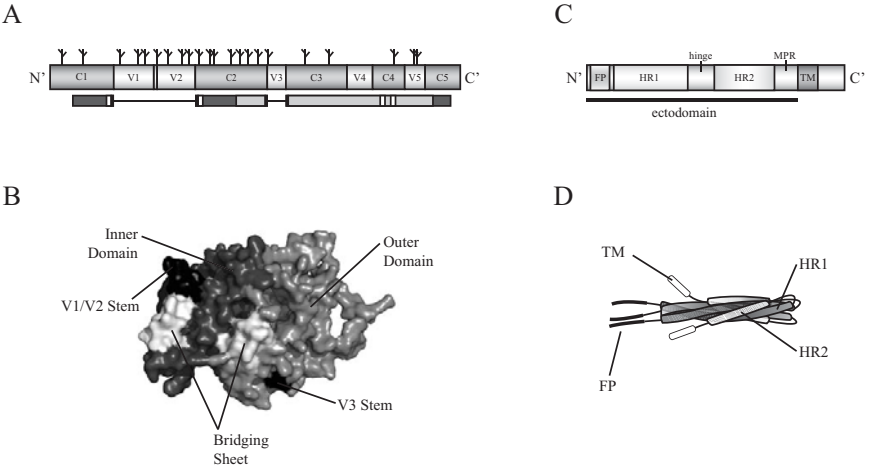

Figure 1. The HIV envelope proteins gp120 and gp41. (A) Schematic diagram of the HIV gp120 protein showing the five conserved (C1–C5) and five variable (V1–V5) domains. Positions of conserved glycosylation sites are indicated by branched chains on the diagram. The conserved "core" of gp120 with deletions of the V1/V2 and V3 loops, and truncations at the N' and C' termini, is depicted underneath the main gp120 molecule, and is shaded to match the domains indicated in the space-filling model of gp120. (B) Space-filling model of gp120 showing the major domains of the protein, including the inner domain (dark gray), outer domain (light gray), bridging sheets (white) and V1/V2 and V3 stems (black). (C) Schematic diagram of the HIV gp41 protein showing the fusion peptide (FP), heptad-repeat domains (HR1 and HR2), and the transmembrane anchor (TM). (D) A model of the gp41 protein in the post-fusion conformation, where HR1 and HR2 have interacted to form the six-helix bundle structure. Note the proximity of the fusion peptide (which has inserted into the host membrane) and the TM region (which is inserted into the viral membrane). The fusion inhibitor enfuvirtide acts by interfering with association of the HR1 and HR2 domains, blocking the formation of the six-helix bundle.

(Fig. 1A). The gp120 molecule has proven difficult to crystallize in its entirety, but several structures have been solved in recent years, including a deglycosylated HIV-1 gp120 bound to CD4 and lacking the V1–3 loops as well as containing truncations at the N and C termini [7], a similar molecule but containing the V3 loop [8], and a glycosylated form of SIV gp120 also lacking V1–3 and small portions of the N and C termini [9]. From these structures, it is evident that in its native state gp120 contains two distinct regions: an inner domain that is involved in interactions with gp41 and the formation of trimeric envelope spikes, and an outer domain that forms a large part of the exposed surface of the spikes and is heavily glycosylated. Binding of CD4 to gp120 induces significant conformational changes that result in the formation of a third domain termed the bridging sheet (Fig. 1B). This domain consists of two pairs of antiparallel β-sheets that link the inner and outer domains, and plays a major role in interacting with the viral coreceptors [10].

The gp41 protein consists of three distinct domains: an unusually large cytoplasmic domain on the inside of the viral membrane, a transmembrane (TM) anchor, and an ectodomain that extends from the surface of the virion. The ectodomain is the principal determinant of membrane fusion and contains a hydrophobic, N-terminal fusion peptide that is believed to insert into the cellular membrane and two heptad repeat (HR) sequences, HR1 and HR2, which are critical to the fusion process [11, 12] (Fig. 1C). The only approved member of the entry inhibitor class of antiretrovirals, enfuvirtide (Fuzeon, T20) acts by targeting the interaction of the two conserved HR domains [13] (Fig. 1D).

A major challenge in the design of entry inhibitors that target the viral Env protein is that the structure of the native, trimeric Env is not known. Recent electron tomography studies reveal the overall dimensions of Env trimers [14, 15], but more precise information will be needed to assist in structure-based drug design efforts.

The HIV-1 entry process

Entry of HIV-1 into cells involves three distinct stages: binding of gp120 to CD4, binding of gp120 to coreceptor, and gp41-mediated fusion of the viral and host membranes. The primary receptor for HIV-1 is CD4, a member of the immunoglobulin superfamily that is expressed on monocytes, macrophages, and subsets of dendritic cells. CD4 makes contact with the gp120 molecule at a depression near the intersection of the inner domain, outer domain, and bridging sheet [7] (Fig. 2A, B). CD4 binding actually appears to induce the formation of the bridging sheet domain itself, as the two pairs of β-sheets are spatially separated in a crystal structure of the unliganded core of SIV gp120 but come together to form a four-stranded sheet in the CD4-liganded conformation [7, 9] (Fig. 2C). Additional changes in gp120 occur with CD4 binding, including movement of the V1/V2 and V3 loop structures. As a result, CD4 binding not only induces the formation of the bridging sheet, it likely enhances

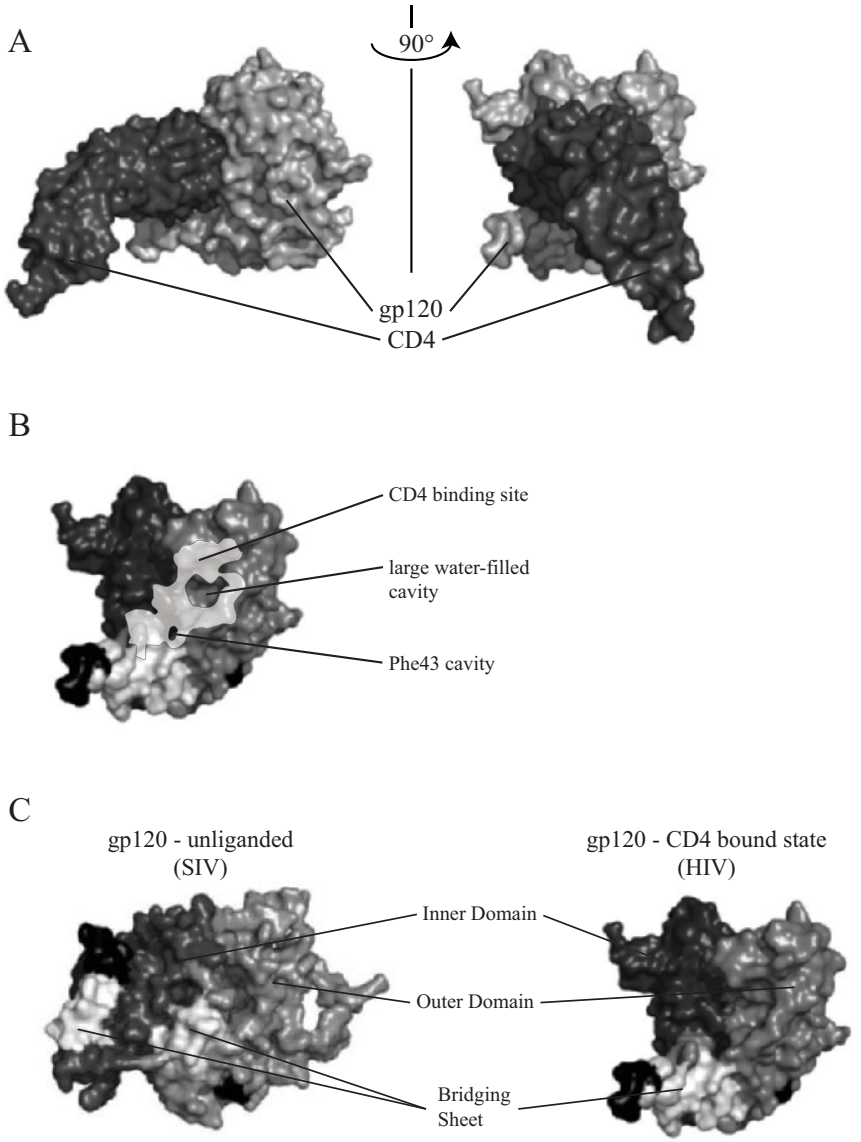

Figure 2. Space-filling models of gp120 interactions with CD4. (A) Models depicting the most distal immunoglobulin domain of the CD4 receptor (black) binding to CD4 gp120 (gray). (B) The CD4-binding site on the surface of gp120 (white highlight) overlaps the inner domain, outer domain, and the bridging sheet domain (white) of gp120. Two water-filled pockets, a large cavity and the Phe43 cavity, are conserved features of the CD4-gp120 interface and are thought to be important in the conformational changes in gp120 that accompany CD4 and coreceptor binding. (C) Interaction between gp120 and CD4 results in major conformational changes in gp120. The structure of gp120 in an unliganded form (left) shows the positions of the inner domain (dark gray), outer domain (light gray) and the β-sheets that comprise the bridging sheet (white). Note that in the unliganded state (left), the bridging sheet β-sheets are spatially separated, but come together to form a four-stranded domain in the CD4-bound conformation (right).

exposure of this region and orients it and the V3 loop towards the target cell membrane where they can engage the viral coreceptor [16, 17]. Unlike other regions of the gp120 molecule, the residues contacting CD4 are highly conserved and are devoid of carbohydrate [7]. These properties make the CD4-binding face of gp120 a logical target for small molecule inhibitors of gp120-CD4 binding.

In humans, the major coreceptors for HIV-1 are the chemokine receptors CCR5 and CXCR4, both of which are members of the seven-TM G protein-coupled receptor family [18–25]. These receptors are integral membrane proteins with a small extracellular pocket formed by three loops between TM segments. The N-terminal segment of the receptor also extends into the extracellular space. Both regions are involved in binding to gp120. The two main regions of gp120 that are involved in binding to coreceptor are the coreceptor-binding site formed by the bridging sheet and adjoining regions, and the V3 loop [26–29]. Several of the amino acids in the coreceptor-binding site are among the most highly conserved residues between HIV-1, HIV-2, and SIV [10, 30]. In contrast, the V3 loop is defined as one of the variable domains of gp120, but the length of the V3 loop is strictly conserved, with most HIV-1 isolates containing between 34 and 36 residues. V3 has a GPGR or GPGQ motif that forms a β-turn in the loop, a region that comprises the center of the 'tip' or 'crown' of V3. Binding of gp120 to the pocket of CCR5 appears to be dependent on the residues present at the 'crown' of the V3 loop [29]. Contact between residues in the tip of CCR5 and extracellular loop 2 have been shown to be particularly important for HIV entry [31–34]. These data are consistent with a recent 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 chemokine receptor pocket [8].

On the coreceptor molecules, the N terminus of CCR5 is rich in sulfated tyrosines and is highly acidic [35]. Mutagenic studies have indicated that these sulfotyrosines in the N-terminal extracellular region of CCR5 interact with gp120 by binding to conserved residues at the base of the V3 loop and may also make contact with the coreceptor binding site [28]. Indeed, sulfated peptides corresponding to this region inhibit infection by CCR5-tropic viruses [36, 37]. Binding of CXCR4 to gp120 appears to occur in a similar fashion [26, 38–41].

Binding of gp120 to coreceptor is believed to trigger further conformational changes in the envelope trimer that enable gp41 to mediate the fusion of viral and cellular membranes [42]. The structural rearrangements triggered by binding to CD4 and coreceptor are believed to allow the glycine-rich, hydrophobic fusion peptide at the N-terminal region of gp41 to insert into the target cell membrane. Following insertion of the fusion peptides, the heptad repeat regions of gp41, HR1 and HR2, undergo an energetically favorable structural reorganization that results in the formation of a thermostable, six-helix bundle structure that is essential for membrane fusion (Fig. 1D). In the six-helix bundle, three HR2 regions wrap in an antiparallel direction around

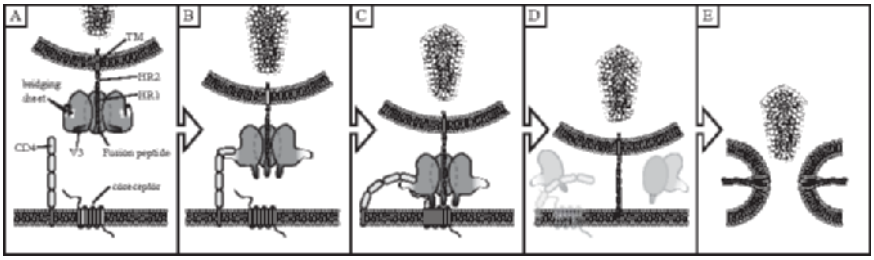

Figure 3. Model of the multi-step entry process that enables HIV to gain access to target cells. (A) The CD4 and coreceptor molecules are embedded in the host membrane (bottom), while the gp120 and gp41 proteins are associated with the viral membrane (curved, top). The V3 loop and bridging sheet domain of gp120 are identified. The gp41 fusion peptide (FP), heptad-repeat (HR1 and HR2), and TM regions are also labeled. (B) The attachment of gp120 to CD4 is associated with conformational changes in gp120 that result in the formation of the bridging sheet domain (white) and the extension of the V3 loop which prior to CD4 binding partially occludes the coreceptor binding site. (C) Coreceptor binding relies on interactions between the bridging sheet and CD4-induced (CD4i) epitopes and the extracellular N' terminal peptide on the coreceptor as well as on interactions between the V3 loop of gp120 and the extracellular loops on the coreceptor. (D) Interactions between gp120, CD4, and coreceptor are believed to result in a conformational change in gp120 that results in dissociation of the envelope trimeric spike, releasing the fusion peptide of gp41, which then inserts into the host membrane. (E) Interaction of the HR1 and HR2 domains of gp41 result in the formation of a six-helix bundle that brings host and viral membranes into close proximity and creates a fusion pore, allowing entry of the HIV capsid into the target cell.

the central coiled-coil of HR1 domains, bringing the N-terminal fusion peptides of the gp41 trimer, which have inserted into the cellular membrane, into close proximity to the TM regions, which traverse the viral membrane [43, 44]. This juxtaposition of the viral and cellular membranes results in the formation of a fusion pore. A schematic model of the multi-step fusion process is presented in Figure 3.

Entry inhibitors

Blocking the interaction between CD4 and gp120 is a logical strategy for preventing HIV infection, although targeting CD4 itself is complicated by side effects due to disruption of CD4 function in immune processes. In contrast, agents that interact with the CD4 binding site on gp120 hold greater promise. One such antiretroviral agent is the small-molecule inhibitor BMS-806, that appears to bind in a pocket in gp120 and either prevents CD4 binding, or prevents CD4-induced conformational changes [45, 46]. However, a major challenge with this class of compounds is the highly variable nature of gp120. It is not uncommon to identify virus strains that are resistant to BMS-806, and those that are sensitive can easily acquire resistance via mutations [47]. More potent, broadly cross-reactive agents are needed if this is to prove to be a viable antiviral strategy. To do this, a structure of unliganded gp120, preferably

with the bound drug, may be needed to assist in drug design. A compound with strategic flexibility at specific bonds may be required to enable the inhibitor to adapt to a somewhat variable drug-binding pocket. Until more potent and broadly cross-reactive inhibitors of gp120-CD4 binding are produced, clinical development of this inhibitor class is unlikely to proceed.

Targeting the interaction between virus and the coreceptor molecules is perhaps a more viable strategy for preventing HIV-1 infection of host cells. The CCR5 coreceptor is particularly important for HIV transmission and pathogenesis: the vast majority of virus strains that establish infections in new hosts are those that use CCR5 (R5 strains) [48–53]; the genetic absence of CCR5 results in a high level of protection from HIV infection without significant side effects due to loss of CCR5 function [54–56]; heterozygosity for the inactivating Δ32-ccr5 polymorphism confers a survival advantage upon HIV infection [55–59], indicating that CCR5 levels are rate-limiting for HIV infection *in vivo*; and seven-TM domain receptors are good pharmacological targets. In fact, several CCR5 inhibitors under clinical development and have been shown to reduce viral loads in infected humans [60–62]. Nonetheless, the development of CCR5 inhibitors is not without challenges. Slight variation in the conformation of the helices and extracellular loops of chemokine receptors may result in significant differences in sensitivity to coreceptor inhibitors *in vivo*. Viral resistance to this class of entry inhibitors may occur from either a coreceptor 'switch', either from CCR5 to CXCR4 or *vice versa*, or from altered utilization of the same coreceptor [63–65]. Evidence for both resistance pathways have been found in patients treated with these compounds.

Finally, entry inhibitors targeting the gp41-mediated fusion stage of the entry process have been developed. One of these agents, enfuvirtide, is the only currently approved member of the entry inhibitor class of antiretroviral agents, and is a peptide with an amino acid sequence identical to the HR2 region of gp41. This agent has been demonstrated to potently inhibit HIV infection *in vitro* and *in vivo*, but viral resistance to these compounds has also been identified [66, 67]. Mutations in the HR1 region of gp41 result in decreased sensitivity to enfuvirtide but also result in slower fusion kinetics [68]. *In vivo*, compensatory mutations occur in the HR2 region that improve the kinetics of viral fusion, while maintaining resistance to enfuvirtide [69, 70].

Challenges in the development and use of entry inhibitors

The emerging class of entry inhibitors holds considerable potential for the treatment of patients with HIV infection, particularly those harboring viruses that have resistance to RT and protease inhibitors. However, while progress has been made in understanding the HIV-1 entry process, a number of critical gaps remain. As noted previously, structures of gp120 in an unliganded state and bound to CD4 have been solved, as has the structure of the core of gp41 in the post-fusion state. However, determination of the structures of the gp120-core-

ceptor interaction, the conformation of gp41 prior to fusion, and the structure of the native trimer remain elusive. Additionally, structures of CD4 inhibitors bound to gp120 and of coreceptor inhibitors bound to CCR5 or CXCR4 are also unavailable. A better understanding of how entry inhibitors bind to Env or coreceptors should make it possible to develop more potent and broadly cross-reactive inhibitors, as well as to design drugs with 'strategic flexibility' that might enable them to bind to a somewhat variable target, such as HIV gp120.

Other challenges in the use of the entry inhibitors are viral and host factors that may alter drug effectiveness *in vivo*. The diversity of the viral envelope proteins suggests that not all viral isolates interact with CD4 and coreceptor in exactly the same way. As a result, there are likely to be some viral isolates that are more sensitive to entry inhibitors and others that are more resistant. Host diversity may also have a role. As indicated by the slower rate of disease progression in patients with the heterozygous Δ32-ccr5 mutation [55, 57–59], the amount of CCR5 expressed on the cell surface is a critical factor in viral pathogenesis. Differences between patients in CCR5 structure or expression levels may also modulate their susceptibility to entry inhibitors [71]. Together, these viral and host factors have a potent effect: viral isolates from patients have differed in susceptibility to enfuvirtide by several orders or magnitude, a much larger range than has been seen with other classes of antiretrovirals [72, 73]. Whether this diversity will affect the clinical outcomes of these patients remains unclear, but must be monitored as use of these agents becomes more established.

Resistance pathways

There are a number of fundamental clinical questions regarding the use of entry inhibitors in the treatment of patients *in vivo*. One of the principal concerns with all antiretroviral agents is the development of viral resistance, and resistance mechanisms to entry inhibitors may not only be complex and variable, but might have the potential to alter viral tropism and pathogenesis by altering the way in which Env binds coreceptors.

In contrast to CD4-binding inhibitors and fusion inhibitors targeting gp41, the coreceptor inhibitors will theoretically be less susceptible to viral resistance mechanisms since they target host proteins rather than the viral envelope. However, the coreceptor inhibitors present some unique challenges also based on the relationship between coreceptor usage, cell tropism, and viral pathogenicity. Coreceptor usage is a principal factor in determining the cellular targets of HIV, with R5-tropic viruses infecting primarily cells of the monocyte and macrophage lineage and memory $CD4^+$ T cells, while X4-tropic viruses predominantly infect naïve $CD4^+$ T cells [74, 75]. X4-tropic viruses are also more infectious and more pathogenic for developing thymocytes than are R5 isolates [76–81]. Viral isolates from patients in early stages of disease are almost universally R5-tropic, regardless of the route of transmission [48–53,

82–85]. Since it appears that a mix of R5 and X4 viruses are transmitted in certain cases [86, 87], and the number of mutations needed to switch coreceptor usage are minimal [88–90], it seems evident that a selection pressure is acting to maintain R5 dominance in early disease. A coreceptor switch from R5- to X4-tropic viruses has been observed in patients during late-stage HIV disease and has been associated with rapid depletion of $CD4^+$ T cells and progression to AIDS [49, 50, 91–95]. However, it remains to be determined whether the emergence of X4-tropic strains is a cause or a consequence of deteriorating immune function.

Viral resistance to coreceptor inhibitors in patients has been seen with two distinct mechanisms. In several patients treated with the R5 inhibitor maraviroc, viral resistance to coreceptor inhibitors has been the result of a 'shift' in viral coreceptor usage from CCR5 to CXCR4 [63]. Notably, the X4-tropic strains that emerged were found to be pre-existing in the patients' viral reservoirs. A second mechanism has been observed in patients treated with other R5 inhibitors, including AD101 and SchD, in which virus continued to utilize the same chemokine receptor but in a drug-insensitive manner [64, 65]. Both of these resistance mechanisms may have profound effects of HIV cell tropism and pathogenicity. Whether a treatment-induced shift from R5- to X4-tropism will accelerate disease progression in patients with preserved immune function is unclear, and will need to be closely monitored during the clinical trials of these agents. The alternative pathway of resistance to coreceptor inhibitors – altered utilization of the same chemokine receptor – may also influence the cellular tropism of HIV. Studies of chemokine receptor mutations that influence sensitivity to AD101 and SchC have suggested that chemokine receptors can exist in several possible conformations on the cell surface [96]. This raises the possibility that altered chemokine receptor usage may influence the subsets of R5- and X4-expressing cells that HIV can infect, potentially changing the pathogenicity of the virus. Future studies of patients developing resistance to coreceptor inhibitors will be important to dissect the mechanisms of viral resistance and their effects on viral pathogenicity and clinical outcome.

Clinical use of HIV entry inhibitors

Although the fusion inhibitor enfuvirtide is the only entry inhibitor currently approved for the treatment of patients, CD4 and coreceptor inhibitors are in various phases of testing (Chapter 10). The varied mechanisms of actions of these agents, acting at different stages of the multi-step entry process, combined with the complications of targeting a highly diverse viral protein with complex resistance pathways, indicates that the effective use of entry inhibitors will require a high degree of clinical acumen. Phenotypic or genotypic tests that predict sensitivity to entry inhibitors – as are available for RT and protease inhibitors – will likely be possible with a better understanding of the mechanisms of viral resistance, and would be useful in selecting agents

when initiating therapy or if a change in therapy is required. Similarly, studies will need to be done to address whether combination therapy with several entry inhibitors targeting multiple stages of the entry process may have synergistic effects that may improve viral suppression and reduce side effects. The use of entry inhibitors along with other classes of antiretroviral agents will also have to be investigated. Collectively, the entry inhibitors are a complex but exciting new class of antiretroviral agents that provides significant opportunities and challenges for the treatment of HIV infection and the understanding of HIV pathogenesis.

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