Chapter 1.8

EVALUATION OF CURRENT STRATEGIES TO INHIBIT HIV ENTRY, INTEGRATION AND MATURATION

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Abstract: Human immunodeficiency virus type 1 (HIV-1) infection continues to be a massive global health crisis, particularly in developing countries. With no effective vaccine and no prospect for a cure in the foreseeable future, antiretroviral treatment is the only option at hand to combat HIV infection. Current HIV therapeutics target the viral enzymes reverse transcriptase and protease. The use of a combination of these drugs, termed highly active antiretroviral therapy (HAART), can efficiently reduce viral load in infected patients. Despite the success of HAART in reducing HIV related morbidity and mortality, HAART cannot eradicate virus in infected patients and might not confer life long suppression of HIV replication. In fact, due to ongoing HIV replication, drug resistant viruses frequently arise in treated patients and such viruses are increasingly transmitted between individuals. These observations, together with the considerable side effects of some HAART regimens, underline that current therapeutics need to be improved and that new antiviral agents with novel modes of action that are effective against current drug resistant viruses need to be sought. The replicative cycle of HIV affords multiple opportunities for therapeutic intervention. Entry of HIV into a cell, integration of the viral genome into the host cell chromosome and the generation of mature infectious progeny virions (“maturation”) are promising targets for inhibitors. Here, we will discuss how HIV accomplishes entry, integration and maturation and which strategies are being pursued to inhibit these processes.

1. INTRODUCTION

Human immunodeficiency virus (HIV) continues to be a significant global cause of mortality. More than 3 million deaths were attributed to acquired immune deficiency syndrome (AIDS) in 2003 alone (UNAIDS, 2004). In the same year, there was a record number of new HIV infections, estimated at around 5 million (UNAIDS, 2004). With no prospects for a vaccine or a cure in the foreseeable future, current therapy for HIV infection relies on the use of antiretroviral agents to reduce viral load, provide immunological benefit, delay disease progression and thus extend the life expectancy of infected individuals.

1.1 Current antiretroviral Therapy

There are currently twenty antiretroviral drugs approved for HIV therapy. These include agents that inhibit the activity of the viral enzymes reverse transcriptase (RT) and protease (Figure 1) or prevent the entry of HIV into cells (Figure 1 & 2; these are also discussed in chapter 1.7).

The first antiretroviral agent to be approved was the nucleoside analog zidovudine (AZT) that is incorporated by RT into the nascent polynucleotide chain during reverse transcription of viral RNA into a DNA copy (Figure 1). Subsequently, six other nucleoside reverse transcriptase inhibitors (NRTIs) and one nucleotide reverse transcriptase inhibitor (NtRTI) have been approved, along with three non-nucleoside reverse transcriptase inhibitors (nNRTIs), that bind and inactivate RT (reviewed in Balzarini, 2004; Ruane and DeJesus, 2004; Sharma *et al.*, 2004). These compounds are collectively referred to as RT inhibitors.

The second major class of antiretroviral agents target the protease enzyme directly to inhibit protease cleavage of viral Gag-Pol (Pr160^{GagPol}) and Gag (Pr55^{Gag}) polypeptide substrates and thus disrupt a late stage of the viral life cycle referred to as maturation (see Figure 1 and below). There are currently eight approved protease inhibitors (PIs) for antiretroviral therapy (reviewed in (Rodriguez-Barrios and Gago, 2004; Wynn *et al.*, 2004).

Enfuvirtide (Fuzeon/T-20) is presently the only approved member of a new class of antiretrovirals agents referred to as entry inhibitors (EIs) (reviewed in Kilby and Eron, 2003; Pöhlmann and Reeves, 2005; Reeves and Piefer, 2005). Enfuvirtide specifically targets the transmembrane subunit (gp41) of the envelope protein (Env) of HIV to block fusion of viral and cellular membranes, thus preventing entry of HIV into target cells (Greenberg *et al.*, 2004; Pöhlmann and Reeves, 2005; Reeves and Piefer, 2005).

Figure 1: The life cycle of HIV and targets for inhibition. Steps of the HIV life cycle targeted by approved or investigational antiretrovirals are labeled in bold text. Classes of antiretroviral agents targeting these steps are boxed. Figure adapted from Reeves and Piefer (Reeves and Piefer, 2005). RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor.

Antiretroviral therapy (ART) typically employs a combination of protease and/or RT inhibitors. Modern ART regimens, referred to as “highly active antiretroviral therapy” (HAART), can successfully reduce viral load in infected individuals to below the level of detection for a number of years (Gulick *et al.*, 1997; Hammer *et al.*, 1997; Hicks *et al.*, 2004) and the advent of HAART has considerably improved the prognosis for HIV infected individuals. However, HAART may ultimately not be “highly active” and failing regimens are associated with the development of drug resistant virus,

often contributed to by lack of compliance, due to difficult regimens and significant side effects associated with some inhibitors (Hammer and Pedneault, 2000). Furthermore, although there are nineteen approved RT and protease inhibitors, resistance to some inhibitors can confer cross-resistance to other members within the same class reducing the number of effective treatment options for some individuals.

Individuals failing current RT and protease inhibitor based regimens have the option of adding the fusion inhibitor enfuvirtide to an optimized background combination of RT and protease inhibitors. Enfuvirtide can result in a sustained reduction in viral load for a prolonged period (Lalezari *et al.*, 2003a; Lalezari *et al.*, 2003b; Lazzarin *et al.*, 2003). However, the requirement for administration of enfuvirtide by twice-daily subcutaneous injection, combined with the cost of this inhibitor, has resulted in limited acceptance. Additionally, as with all other antiretrovirals, resistant viral variants can be selected (reviewed in Greenberg and Cammack, 2004; Miller and Hazuda, 2004).

1.2 Prospects for Antiretroviral Therapy

Viruses resistant to current antiretrovirals are becoming increasingly common and drug resistant variants account for an increasing number of new infections (Little *et al.*, 2002; Wegner *et al.*, 2000; Weinstock *et al.*, 2004). Thus some individuals are left with a limited number of effective treatment options. These issues, along with the significant toxicities associated with some antiretrovirals, underscore the requirement for new antiretroviral agents that are active against current drug resistant strains as well as the necessity for new agents with reduced toxicities. Indeed, a number of investigational agents with novel mechanisms of action against current drug targets, or that block distinct stages of the viral life cycle, are under evaluation (Reeves and Piefer, 2005).

Antiretrovirals with novel mechanisms of action that are currently in clinical trials include inhibitors of viral entry, integration and maturation, as discussed below. The long-term side effects of these agents remain to be established, and experience with approved antiretrovirals would indicate that monotherapy directed against any target is likely to rapidly select for resistant viruses. However, addition of these investigational agents to an optimized background antiretroviral regimen may provide clinical benefit in patients with multi-drug-resistant viruses. Furthermore, combination therapy employing inhibitors directed against distinct targets, or with distinct modes of action against a single target, together with current antiretrovirals, will

likely offer improved prospects for containment of viral replication in first line therapy.

Figure 2: HIV entry and its inhibition. Binding of gp120 to heparan sulfates or lectins on the cell surface promotes HIV attachment to target cells. These interactions can augment infection efficiency but are dispensable for entry. The interaction of gp120 with CD4 initiates a series of interactions which are indispensable for viral entry. Binding of gp120 to CD4 induces conformational changes in Env that lead to the formation/exposure of a coreceptor binding site and trigger the exposure of structures in gp41 involved in membrane fusion. Subsequent binding of gp120 to coreceptor triggers further conformational changes in gp41 that promote the merger between the viral and the cellular membrane. Polyanions inhibit gp120 engagement of heparan sulfates, while antibodies prevent gp120 binding to lectins. CD4M33 and PRO-542 target the CD4 binding site of gp120 and block binding to cellular CD4. CV-N recognizes high-mannose carbohydrates on gp120 and inhibits gp120 engagement of CD4 and coreceptor, however, the precise mechanism of action of this agent remains to be determined. BMS-378806 targets the CD4 binding site in gp120 and arrests gp120 in a rigid conformation, thereby preventing the exposure of epitopes in gp41 involved in membrane fusion. Binding of gp120 to CCR5 can be inhibited by the CCR5 antagonists SCH-D, UK-427,857 and GSK873140. The peptide inhibitors T-20 (enfuvirtide/fuzeon) and T1249 mimic the HR2 region in gp41 and bind to HR1, thereby preventing the back-folding of HR2 onto HR1, which is required for membrane fusion. HR, helical region.

2. ENTRY

The entry of a virion into a target cell represents the first step in the life cycle of HIV (Figure 1 and 2). HIV enters cells via a multi-step process whereby a virion first attaches to the surface of a cell, interacts with a cell surface receptor, and then a coreceptor molecule, which brings about the merger of viral and cellular membranes and thus entry into a target cell.

The entry process is mediated by the viral envelope protein (Env), which is comprised of a surface subunit, gp120, and a transmembrane subunit, gp41, which assemble as trimers of heterodimers on the virion surface. Virions can first attach to target cells via non-specific interactions that include electrostatic attraction between gp120 and cell surface molecules such as heparan sulfate (reviewed in Ugolini *et al.*, 1999), or by the interaction of carbohydrate moieties on gp120 with cell surface lectins such as DC-SIGN (reviewed in Baribaud *et al.*, 2001a).

The entry process proper is initiated by gp120 binding to the cell surface receptor CD4. The requirement of CD4 binding for entry largely governs the tropism of HIV for CD4-positive T-cells and macrophages. CD4 binding induces structural rearrangements within Env, that include the repositioning of variable loop structures (V1/V2 and V3) within gp120, to expose/form a coreceptor-binding site within gp120 (Kwong *et al.*, 1998; Wyatt *et al.*, 1998). Env can then bind to a cell surface coreceptor molecule, typically the seven transmembrane chemokine receptor CCR5 or CXCR4 (reviewed in Berger *et al.*, 1999; Reeves and Doms, 2003). Some primary HIV-2 isolates can interact directly with a coreceptor molecule to infect CD4-negative cells, whereas primary HIV-1 isolates are usually strictly CD4-dependent (Bhattacharya *et al.*, 2003; Reeves *et al.*, 1999).

Amino acid residues within the V3 loop of gp120 can determine whether an Env interacts with CCR5 or CXCR4, thus, in addition to CD4, V3 also governs cell tropism (Choe *et al.*, 1996; Cocchi *et al.*, 1996; Hartley *et al.*, 2005; Speck *et al.*, 1997; Wu *et al.*, 1996). Furthermore, the coreceptor specificity of Env influences HIV transmission as well as pathogenesis. Viruses that use CCR5 as a coreceptor (R5 tropic) are transmitted between individuals and those who fail to express functional CCR5 molecules, due to a deletion in both CCR5 alleles ($\Delta 32ccr5$ homozygotes), are highly resistant to infection (Dean *et al.*, 1996; Huang *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). In addition, the acquisition of viruses that use CXCR4 as a coreceptor (X4 tropic), either in addition to CCR5 (R5/X4 tropic) or instead of CCR5, is associated with, but is not a prerequisite for, disease progression (Connor *et al.*, 1997; Moyle *et al.*, 2005).

Coreceptor binding induces considerable conformational changes within gp41. The ectodomain of gp41 contains an amino-terminal hydrophobic fusion peptide and two heptad repeat regions, HR1 and HR2. Coreceptor binding is thought to result in insertion of fusion peptides into the membrane of the target cell. Then three HR2 regions, from individual gp41 subunits, are thought to fold to interact with grooves formed between three associated HR1 subunits, forming a highly stable six-helix bundle structure (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). This brings the amino and carboxy-terminal regions of the ectodomain of gp41 into proximity and, in consequence, the target cell membrane and viral membrane are brought into apposition. Six-helix bundle formation also pulls a fusion pore in the target cell membrane, and pore formation by a number of Env trimers is thought to cooperatively bring about fusion of viral and cellular lipid membranes (Kuhmann *et al.*, 2000; Melikyan *et al.*, 2000).

Each step of the HIV entry pathway presents a viable target for antiretroviral intervention. Indeed, inhibitors targeting attachment, CD4 binding, coreceptor binding, as well as fusion are currently in development, as described below.

2.1 Entry Inhibitors

HIV entry into target cells involves a variety of sequential interactions between the viral envelope glycoprotein and cellular factors. Some of these interactions are essential for entry, others boost entry efficiency but are ultimately dispensable for infection, however, all are potential targets for therapeutic intervention. While most inhibitors directed against HIV Env face the same problems as the antiretrovirals used in current HAART – i.e. high variability of HIV and rapid outgrowth of resistant viruses – agents directed against invariant cellular factors involved in HIV entry might have a more sustained effect. Since HIV Env mediated membrane fusion is initiated at the cell surface (Smith and Helenius, 2004), this process can be efficiently targeted by non-membrane permeable inhibitors like antibodies or certain peptidic agents directed against structures intimately involved in the merger between viral and cellular membranes. In contrast, similar compounds might be less or non-effective against viruses which fuse with the membrane of endosomal vesicles, like influenza or vesicular stomatitis virus (Smith and Helenius, 2004). Thus, HIV entry is vulnerable to compounds with different chemical properties and modes of action. However, only inhibitors that are effective in the low nanomolar range *in vitro* and that are ideally orally bioavailable and reach effective concentrations systemically, with a

reasonable half-life and minimal side effects, are likely to be successful in the clinic. Nevertheless, compounds that do not fit some of these criteria, for example those lacking oral bioavailability or with unwanted side effects upon systemic administration, might still be beneficial when applied topically within a microbicide formulation, aimed at preventing HIV transmission at mucosal sites (Shattock and Solomon, 2004; Shattock and Moore, 2003).

Current strategies to inhibit HIV entry target viral attachment to cells, Env binding to the cellular receptor CD4 and the coreceptors CCR5 and CXCR4, and transient structures in Env involved in membrane fusion. All of these approaches hold promise and one has already resulted in a drug approved for use in humans (enfuvirtide) (Greenberg and Cammack, 2004; Kilby and Eron, 2003). Inhibitors targeting the viral coreceptor CCR5 are currently in advanced stages of clinical development and might within the foreseeable future complement current HAART. Another promising potential for entry inhibitors is that a combination regimen of inhibitors to different entry targets might allow for sustained containment of viruses resistant to current inhibitors. Synergistic HIV inhibition *in vitro* by agents blocking different stages of the entry process indicates that this goal might indeed be attainable (Tremblay, 2004). Here, we will introduce compounds that target major steps of the entry process and will discuss their prospects as therapeutics – and the prospect of HIV to acquire resistance against these agents.

2.1.1 Inhibitors of viral attachment to cells

For the purpose of this review we will define viral attachment to cells as interactions between proteins inserted in the viral membrane and cellular factors other than CD4 and coreceptor, which mediate tethering of viral particles to the cell surface. Although these interactions are not essential for viral entry, they can dramatically increase infection efficiency and are thus targets for inhibitors (Ugolini *et al.*, 1999).

Polyanions inhibit binding of HIV Env to cellular heparan sulfates

Binding of HIV to cells, via electrostatic interactions or engagement of cellular lectins (see below), can not only increase infection of the cells to which the virus particles are attached (infection in *cis*), but in some cases can also augment entry into adjacent cells, in a process termed infection in *trans*. Both *cis* and *trans* infection can be mediated by interactions of positively charged residues in gp120 with negatively charged heparan sulfates on the surface of various cell types, especially the endothelium of blood vessels (Bobardt *et al.*, 2003; Gallay, 2004). Thus, the interaction of viral particles with endothelial cells has the potential to modulate infection within the

circulatory system. Furthermore, binding of virus to heparan sulfates on brain microvascular endothelial cells followed by transcytosis of viral particles might also promote HIV infection of the brain (Argyris *et al.*, 2003; Bobardt *et al.*, 2004b; Liu *et al.*, 2002). The interaction of HIV with cellular heparan sulfates can be inhibited by anionic polymers that are negatively charged at neutral pH (Shattock and Moore, 2003). These compounds can be employed within a microbicide formulation to prevent HIV transmission (Shattock and Moore, 2003). The precise mechanism of action of polyanionic inhibitors is unclear, however, it has been suggested that they mainly target the positively charged V3 loop in Env and inhibit binding to coreceptor (Vives *et al.*, 2005), which requires V3 function (Hartley *et al.*, 2005). In this regard it is of note that V3 regions of R5-tropic viruses usually exhibit a lower positive charge than V3 regions of X4-tropic viruses and consequently polyanions inhibit the latter viruses more potently (Hartley *et al.*, 2005). Interestingly, HIV can acquire resistance to polyanionic inhibitors and resistant viruses exhibit multiple changes in gp120 (Bobardt *et al.*, 2004a), confirming that Env is indeed the major target of these compounds. Resistant variants are still capable of infecting cells that do not express heparan sulfate containing proteoglycans with high efficiency, but exhibit markedly reduced ability to infect cells that express these structures (Bobardt *et al.*, 2004a). Engagement of heparan sulfate harboring structures can thus impact HIV cell tropism and infectivity, validating this interaction as a target for inhibition.

Carbohydrate dependent binding of HIV Env to DC-SIGN on dendritic cells: A target for microbicides?

In addition to heparan sulfates, cellular lectins can also augment the infectivity of HIV. The lectins dendritic cell specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN) and DC-SIGN related (DC-SIGNR, also termed L-SIGN, for liver SIGN) promote HIV-1, HIV-2 and SIV infection and might play an important role in viral dissemination (reviewed in Baribaud *et al.*, 2002a; van Kooyk and Geijtenbeek, 2003). Thus, it has been postulated that DC-SIGN on submucosal dendritic cells can capture sexually transmitted HIV and that virus loaded cells could then migrate into lymphoid tissue where the virus could be transferred to adjoining T-cells – a process that might boost HIV spread in and between infected individuals (Geijtenbeek *et al.*, 2000). In contrast, expression of DC-SIGNR on endothelial cells lining liver and lymph node sinusoids as well as in placenta (Bashirova *et al.*, 2001; Pöhlmann *et al.*, 2001) might endow these cells with the ability to capture blood borne HIV and transfer it to transmigrating T-cells or adjacent macrophages. Moreover, DC-SIGNR might promote HIV infection of liver sinusoidal endothelial cells (Steffan *et al.*, 1992) and these cells might constantly release new virions into the blood

stream. Both DC-SIGN and DC-SIGNR bind to high-mannose glycans in Env (Feinberg *et al.*, 2001; Guo *et al.*, 2004; Lin *et al.*, 2003a) and, upon expression on certain cell lines, facilitate infection in cis and in trans (Trumpfheller *et al.*, 2003; Wu *et al.*, 2004). Augmentation of HIV infectivity by DC-SIGN expressing cell lines might involve internalization and intracellular transport of virus particles (Kwon *et al.*, 2002; McDonald *et al.*, 2003). However, recent studies on a widely used cell culture system for DC-SIGN function revealed that these observations might have been confounded by productive infection of the lectin expressing cells in a CD4 and coreceptor dependent manner (Nobile *et al.*, 2005). Similarly, dendritic cell mediated HIV infection in trans, which has been mainly attributed to DC-SIGN function by some laboratories, probably involves direct infection of these cells and might also be promoted by HIV engagement of other lectins (Nobile *et al.*, 2005; Turville *et al.*, 2002). While these studies call a major role of DC-SIGN in HIV infection into question, two recent observations underline that DC-SIGN is likely important for HIV spread. First, inhibitors of DC-SIGN reduce HIV transmission in a mucosal explant model (Hu *et al.*, 2004), suggesting that a microbicide formulation should contain DC-SIGN inhibitors to be effective. Second, and perhaps more relevant, polymorphisms in the DC-SIGN gene can modulate the risk of HIV-1 infection (Liu *et al.*, 2004), at least upon certain routes of transmission (Martin *et al.*, 2004b) and it has been suggested that reduced DC-SIGN expression might hamper viral dissemination. Therefore, inhibition of DC-SIGN in HIV infected individuals might also be associated with a therapeutic benefit. Moreover, DC-SIGN and DC-SIGNR interact with a variety of viral and non viral pathogens (van Kooyk and Geijtenbeek, 2003), among them Ebolavirus (Alvarez *et al.*, 2002; Simmons *et al.*, 2003), hepatitis C virus (Gardner *et al.*, 2003; Lozach *et al.*, 2003), SARS associated coronavirus (Jeffers *et al.*, 2004; Marzi *et al.*, 2004; Yang *et al.*, 2004), Mycobacterium (Geijtenbeek *et al.*, 2003; Maeda *et al.*, 2003) and Leishmania (Colmenares *et al.*, 2002), suggesting that inhibitors of these lectins might be useful for treatment of a variety of infectious diseases.

Several antibodies have been described that potently block binding of ligands to DC-SIGN and DC-SIGNR (Baribaud *et al.*, 2002b; Wu *et al.*, 2002) and these reagents could be employed to inhibit sexual transmission of HIV. However, the *in vivo* potency of these antibodies still needs to be assessed in an experimental model such as SIV infection of macaques. Macaque and human DC-SIGN are equally adept in transmitting immunodeficiency viruses (Baribaud *et al.*, 2001b; Geijtenbeek *et al.*, 2001) and both are expressed in the genital mucosa (Jameson *et al.*, 2002), underlining that experiments to block DC-SIGN in macaques would yield valuable information on the validity of this target in humans. Since DC-SIGN and DC-SIGNR recognize carbohydrates on the surface of ligands, it will be difficult to generate conventional small molecule inhibitors. However, glycodendritic structures that present multiple mannose residues

prevent ligand binding to DC-SIGN and could be employed as microbicides (Lasala *et al.*, 2003; Rojo and Delgado, 2004). Finally, HIV engagement of DC-SIGN could be inhibited by compounds that bind glycans in the HIV-Env and such inhibitors are described below.

Cyanovirin-N binds mannose residues on HIV Env and inhibits infection.

Cyanovirin, a 101 amino acid protein derived from the bacterium *Nostoc ellipsosporum*, recognizes terminal di- and tri-mannose residues on high-mannose glycans (Barrientos and Gronenborn, 2005; Shenoy *et al.*, 2002) present on the HIV Env and on the glycoproteins of other viruses and can inhibit infection (Dey *et al.*, 2000). CV-N can interfere with gp120 binding to both CD4 and coreceptor (Dey *et al.*, 2000), however, the precise mechanism of inhibition needs to be determined. The structure of CV-N has been solved (Bewley *et al.*, 1998) and two carbohydrate binding sites with different affinities have been identified (Shenoy *et al.*, 2002), however, only the high affinity binding site is required for inhibition of HIV infection (Chang and Bewley, 2002). CV-N is present in solution as a monomer or a domain swapped dimer and both exhibit comparable antiviral activity (Barrientos *et al.*, 2004). When applied topically as a microbicide, CV-N can inhibit infection of macaques by a simian/human immunodeficiency virus hybrid (SHIV) upon vaginal (Tsai *et al.*, 2004) and rectal (Tsai *et al.*, 2003) challenge without eliciting major side effects, indicating the CV-N is a promising candidate compound for a microbicide formulation targeting HIV. Compounds with a similar mode of action might, at least in theory, be suitable for HIV-therapy. However, HIV can acquire resistance against such inhibitors by partially removing its carbohydrate shield (Balzarini *et al.*, 2004). Such variants are at least partially resistant to compounds that target glycans, like CV-N or the antibody 2G12 (directed against a carbohydrate epitope in HIV Env) (Balzarini *et al.*, 2004), and these variants are likely to exhibit enhanced neutralization sensitivity.

2.1.2 Inhibitors of gp120 binding to the CD4 receptor

The interaction of gp120 with cellular attachment factors can promote infection but is ultimately dispensable for viral entry. In contrast, binding of gp120 to CD4 is essential for HIV-1 entry into target cells. The structures of CD4 (Ryu *et al.*, 1990; Wang *et al.*, 1990; Wu *et al.*, 1997), gp120 (Chen *et al.*, 2005a; Chen *et al.*, 2005b), and gp120 bound to CD4 and a neutralizing antibody fragment (Kwong *et al.*, 1998) have been determined on the atomic level and the interface between CD4 and gp120 is therefore well characterized. Thus, 22 amino acids in domain 1 (D1) of CD4, particularly

those located in the CDR2-like loop, contact 26 residues in gp120, located in a cavity formed at the interface between the inner and outer domain and the bridging sheet of gp120 (Kwong *et al.*, 1998). Despite, the wealth of information on the structures involved in gp120 binding to CD4, the screening for small molecule inhibitors targeting this interaction has thus far been unsuccessful. Antibodies that target the gp120 binding site in CD4 and block HIV infection are available, however, concerns have been raised that systemic administration of such antibodies might cause immunodeficiency (Shattock and Moore, 2003). Nevertheless, a single dose of a humanized antibody, that targets a different domain in CD4 (domain 2 (D2)) to block HIV entry, reduced viral load in infected individuals without eliciting major side effects (Kuritzkes *et al.*, 2004), indicating that this agent warrants further evaluation. Structure based approaches have been employed to inhibit the interaction between CD4 and gp120 and are discussed below.

CD4M33: A mini-CD4 protein that inhibits HIV infection

Several peptides that mimic portions of the CD4-Env interaction site have been generated and shown to inhibit HIV infection (Choi *et al.*, 2001; Ferrer and Harrison, 1999). Moreover, small proteins, that harbor the gp120 binding site of CD4 have also been generated and shown to have potent antiviral effects. Thus, 31 amino acids comprising the CRD2-like loop of CD4 were originally transferred onto a structural scaffold derived from the scorpion toxin charybdotoxin (Vita *et al.*, 1999), and the protein was subsequently optimized for efficient gp120 binding and antiviral activity and its structure determined (Huang *et al.*, 2005; Martin *et al.*, 2003). The most active compound generated, CD4M33, inhibited primary HIV isolates in the nanomolar range and induced structural changes in Env comparable to those triggered by binding to cellular CD4 (Huang *et al.*, 2005; Martin *et al.*, 2003). Although this compound has the general disadvantages of peptide inhibitors, such as low bioavailability and antigenicity, CD4M33 and derivatives thereof could be used as microbicides and, in combination with gp120, within vaccine formulations, since CD4M33 bound gp120 exposes otherwise hidden epitopes which may elicit broadly neutralizing antibodies (Huang *et al.*, 2005; Martin *et al.*, 2003).

PRO 542: A tetravalent CD4-immunoglobulin fusion protein with potent antiviral effects

Expression of the gp120 binding site of CD4 in a heterologous context is also the basis of the inhibitory activity of PRO 542, an immunoglobulin fusion protein, in which the variable regions of both the heavy and light chains are replaced by the D1D2 regions of human CD4 (Allaway *et al.*, 1995). PRO 542 is tetravalent and is most likely capable of cross linking Env

timers on the surface of virions or infected cells (Zhu *et al.*, 2001), explaining its superior antiviral activity compared to previously investigated divalent compounds. The compound neutralizes primary and laboratory adapted HIV-1 strains independently of coreceptor usage (Gauduin *et al.*, 1996; Trkola *et al.*, 1998; Trkola *et al.*, 1995), protects SCID (severe combined immunodeficiency) mice harboring human peripheral blood mononuclear cells against challenge with primary HIV-1 isolates (Gauduin *et al.*, 1998), inhibits HIV-1 infection in a mucosal explant model (Hu *et al.*, 2004) and reduces viral load in HIV-1 infected individuals upon a single administration (Jacobson *et al.*, 2004; Jacobson *et al.*, 2000). Since no appreciable side effects were observed in PRO 542 treated individuals the compound merits further testing and could ultimately be employed for salvage therapy of patients with late stage AIDS or within a microbicide formulation.

BMS-378806 binds to the CD4 binding cavity in gp120 and blocks HIV infection

BMS-378806 (BMS-806) is a small molecule (407 Da) that potently inhibits subtype B HIV-1 strains but has no effect against HIV-2 and SIV (Lin *et al.*, 2003b). BMS-806 binds to gp120 and was originally shown to inhibit the interaction with CD4, suggesting that BMS-806 might target the CD4 binding site in gp120 (Lin *et al.*, 2003b). Two further observations supported such a mode of action. First, BMS-806 was shown to inhibit CD4-independent viruses (viral variants that do not require CD4 binding for infection but usually infect cells with higher efficiency in the presence of CD4) only when CD4 was expressed on target cells. Second, viruses that acquired resistance against the compound exhibited alterations in the CD4 binding site in gp120 (Guo *et al.*, 2003; Lin *et al.*, 2003b). Thus, it was suggested that BMS-806 blocks HIV-1 entry by preventing gp120 binding to CD4. However, subsequent studies indicate that BMS-806 might exert its inhibitory activity in a different fashion (Madani *et al.*, 2004; Si *et al.*, 2004). Thus, no inhibitory effect of BMS-806 on gp120 binding to CD4 (or coreceptor) was observed and CD4-independent viruses were equally sensitive to BMS-806 inhibition in the context of target cells expressing receptor and coreceptor or coreceptor alone (Madani *et al.*, 2004; Si *et al.*, 2004). These reports proposed that BMS-806 targets the recessed CD4 binding site and, instead of directly inhibiting CD4 binding to gp120, blocks structural rearrangements in Env that are normally induced by CD4 binding, as BMS-806 prevented the exposure of a structure in gp41 involved in the membrane fusion reaction (Si *et al.*, 2004). Structural analysis of gp120 in the absence of ligand indicates that BMS-806 might stabilize the unliganded

conformation of Env and supports the finding that BMS-806 might act by inhibiting conformational changes required for fusion (Chen *et al.*, 2005b). In either case, BMS-806 is orally bioavailable and is highly effective at least against subtype B viruses (Lin *et al.*, 2003b), therefore compounds such as BMS-806 warrant further evaluation.

2.1.3 Inhibitors of gp120 binding to the CCR5 and CXCR4 coreceptors

CCR5 is currently the most promising new target for inhibitors of HIV entry and is an attractive target for several reasons. First, viruses transmitted between individuals via the sexual route employ CCR5 (Connor *et al.*, 1997). Second, individuals with two defective copies of the *ccr5* gene ($\Delta 32$ *ccr5* homozygotes) are healthy and third, these individuals are highly resistant to HIV-1 infection (Garred *et al.*, 1997; Huang *et al.*, 1996; Meyer *et al.*, 1997; Michael *et al.*, 1997). Several efforts are therefore underway to block HIV usage of CCR5 and some of these inhibitors are currently being tested in phase II/III clinical trials and might complement current HAART in the nearer future.

In a considerable percentage of infected individuals, viral variants arise that can use CXCR4 alone or in conjunction with CCR5 to infect cells, and the emergence of such variants is associated with progression to immunodeficiency (Connor *et al.*, 1997). Thus, compounds that block CXCR4 use by HIV are also under evaluation (reviewed in detail in (De Clercq, 2003; Schols, 2004)). Several such agents, usually positively charged compounds that specifically interact with the negatively charged surface of CXCR4, have been identified and shown to inhibit the spread of X4-tropic viruses in culture. However, CXCR4 and its natural ligand SDF are critical for hematopoiesis, cardiac function and cerebellar development (Nagasawa *et al.*, 1996; Zou *et al.*, 1998), therefore inhibition of CXCR4 may be associated with unwanted side effects. Indeed, development of the CXCR4 antagonist AMD3100 was halted due to side effects, as well as the lack of oral absorption. AMD070, a follow up CXCR4 antagonist with oral bioavailability, is currently in clinical development and was generally well tolerated in Phase I trials (Schols *et al.*, 2003; Stone *et al.*, 2004). As with AMD3100, CXCR4 antagonism by AMD070 results in a mobilization of white blood cells from the bone marrow (Stone *et al.*, 2004), for which the long-term consequences are unknown. Therefore, agents that block CXCR4 usage by HIV without interfering with the natural function of CXCR4 would be desirable.

Early strategies to inhibit HIV Env engagement of CCR5 relied on modifications of natural CCR5 ligands. Some of these compounds exhibited robust HIV inhibitory activity, but their poor oral bioavailability coupled with their capacity to induce receptor signaling likely impedes their usage as therapeutics (Pierson *et al.*, 2004; Pöhlmann and Reeves, 2005). In contrast, several small molecule CCR5 inhibitors have been generated that block Env-CCR5 interactions to inhibit infection and some of these exhibit favorable pharmacokinetic profiles. Three of these are currently poised to enter into Phase III clinical trials and are discussed below.

UK-427,857, SCH-D and GW873140

UK-427,857 (Maraviroc) is a small molecule CCR5 antagonist that inhibits primary HIV isolates and recombinant viruses harboring *env* genes from RT- and protease-inhibitor susceptible and resistant viruses in the low nanomolar range (MacCartney *et al.*, 2003; Westby *et al.*, 2003). This compound is orally bioavailable and no serious side effects were observed in treated patients following short-term administration (Russell *et al.*, 2003). Monotherapy with UK-427,857 significantly reduced viral load in infected individuals (average 1.6 log₁₀ decline) (van der Ryst *et al.*, 2004). Similarly, SCH-D is an orally bioavailable, potent inhibitor of R5-tropic viruses that is well tolerated and upon monotherapy can also significantly reduce viral load in infected patients (average 1.62 log₁₀ decline) (Schurmann *et al.*, 2004).

The spirodiketopiperazine GW873140 inhibits infection of R5-tropic HIV-1 primary isolates of different clades in the subnanomolar range and acts synergistic in conjunction with approved RT- and protease-inhibitors (Demarest *et al.*, 2004b). GW873140 is orally bioavailable, generally well tolerated and monotherapy with GW873140 can reduce viral load (average of 1.66 log₁₀ decline) (Demarest *et al.*, 2004a; Lalezari *et al.*, 2004). Furthermore, GW873140 exhibits prolonged CCR5 occupancy *in vitro* and *in vivo* explaining sustained antiviral effects days following treatment cessation (Demarest *et al.*, 2004a; Demarest *et al.*, 2004c; Sparks *et al.*, 2005; Watson *et al.*, 2005).

GW873140 functions as a receptor antagonist (Watson *et al.*, 2005), similar to other well characterized small molecule CCR5 inhibitors, including SCH-D and UK-427,857. In contrast to these compounds however, GW873140 does not interfere with binding of the natural CCR5 ligands MIP-1 α and RANTES (Watson *et al.*, 2005), indicating that GW873140 interacts with CCR5 differentially compared to SCH-D, UK-427,857 and other CCR5 antagonist, including TAK-779 and SCH-C. All of these compounds are believed to inhibit HIV use of CCR5 by exerting allosteric effects, rather than steric hindrance, on the receptor (Watson *et al.*, 2005).

Thus, binding of these compounds likely induces conformational changes in CCR5 that are incompatible with gp120 recognition of CCR5.

In the light of encouraging results from Phase I/II trials with UK-427,857, SCH-D and GW873140, all three are now poised to enter Phase III trials. The potential consequences of *in vivo* resistance to these inhibitors however, raise novel concerns, as discussed below.

Resistance against small molecule coreceptor inhibitors

As with other antiretroviral agents, viruses can acquire resistance to coreceptor inhibitors. Several resistance mechanisms are possible, however the virus might not always escape by the most obvious route. Thus, a switch from CCR5 to CXCR4 usage and vice versa has been observed when one coreceptor was blocked (Este *et al.*, 1999; Mosier *et al.*, 1999). Also, culture of viruses in cells that express CXCR4 in the presence of a CXCR4 inhibitor can lead to selection of resistant viruses that use this coreceptor in the presence of inhibitor (de Vreese *et al.*, 1996; Kanbara *et al.*, 2001; Schols *et al.*, 1998). Both findings are not unexpected given the variability in HIV Env and a certain degree of flexibility in gp120 interactions with coreceptor. Strikingly, however, in an experimental setting in which both CCR5 and CXCR4 were available, a R5-tropic HIV-1 isolate chose to adapt to CCR5 usage in the presence of drug rather than adapting to utilize CXCR4 (Trkola *et al.*, 2002). Resistance was associated with changes in Env that reduced affinity for CCR5 and diminished entry into CCR5 expressing cell lines, but presumably allowed CCR5 engagement in the presence of drug (Kuhmann *et al.*, 2004; Trkola *et al.*, 2002). These observations indicate that a coreceptor switch, for which as little as one to three substitutions in the V3 loop can be sufficient (Pastore *et al.*, 2004; Pöhlmann *et al.*, 2004; Shimizu *et al.*, 1999; Shioda *et al.*, 1994), might, at least under some circumstances, be associated with a considerable disadvantage to the virus. In such cases, the emergence of viruses that engage coreceptor in the presence of drug can be the consequence. If these results reflect the situation in HIV-1 infected patients treated with CCR5 inhibitors, the emergence of X4-tropic viruses might not be a general phenomenon. Nevertheless, HIV escape from CCR5 inhibitors by adaptation to CXCR4 usage, which is associated with disease progression, remains a major concern. The use of CCR5 inhibitors will therefore require careful evaluation of relevant clinical parameters, including determination of the coreceptor tropism of patient derived viruses in order to maximize the potential therapeutic benefit of these inhibitors.

2.1.4 Peptide inhibitors targeting transient structures in gp41

Binding of HIV-1 Env to CD4 is thought to induce conformational changes in gp120 that allow engagement of a coreceptor and also lead to the exposure of epitopes in gp41 which are involved in driving membrane fusion. Coreceptor binding then induces further conformational rearrangements in gp41 that facilitate fusion of the viral and the host cell membrane. As described above, the latter conformational changes in gp41 involves association of two helical regions, HR1 and HR2, which fold back onto each other to drive the formation of the six helix bundle, a structure intimately associated with membrane fusion. This process is conserved between class I fusion proteins of different viruses and can sometimes be inhibited by peptides that mimic either HR1 or HR2, with HR2 derived peptides often being more effective (Eckert and Kim, 2001). Enfuvirtide (T-20/Fuzeon) is a 36 amino acid peptide comprising sequences derived from HR2 and can efficiently block HIV-1 infection *in vitro* and *in vivo* (Kilby *et al.*, 1998). Use of enfuvirtide for therapy of individuals with multi drug resistant virus was approved after the demonstration that enfuvirtide treatment does not elicit major unwanted side effects and that administration of the compound in combination with an optimized RT- and protease-inhibitor regimen is more effective than treatment with RT- and protease-inhibitors alone. Enfuvirtide is the first entry inhibitor approved for therapy of HIV-1 infection and serves as proof of principle that inhibition of HIV entry is a promising new avenue for HIV drug development.

Despite the efficacy of enfuvirtide, HIV-1 variants resistant to enfuvirtide can develop readily in cell culture as well as in treated HIV patients, resulting in treatment failure (Greenberg and Cammack, 2004; Miller and Hazuda, 2004; Rimsky *et al.*, 1998; Wei *et al.*, 2002). Mutations are mainly localized to HR1 (Marcelin *et al.*, 2004; Rimsky *et al.*, 1998; Wei *et al.*, 2002), the target of enfuvirtide, and often alter a conserved GIV motif, required for optimal fusogenic activity of gp41 (Kinomoto *et al.*, 2005; Reeves *et al.*, 2005). Enfuvirtide resistance can result in slower Env fusion rates which can increase Env susceptibility to a subset of neutralizing antibodies (Reeves *et al.*, 2005) and, in general, resistant viruses exhibit reduced fitness *in vitro* (Lu *et al.*, 2004; Reeves *et al.*, 2005). It remains to be determined, however, if enfuvirtide resistant viruses are less pathogenic. One case of evolution of an enfuvirtide dependent virus has also been reported (Baldwin *et al.*, 2004), highlighting the complex interplay of this compound with the fusion machinery in gp41.

Enfuvirtide resistant viruses can be inhibited by T-1249, a related second generation compound that exhibits more potent antiviral activity and is

effective against enfuvirtide resistant viruses *in vitro* and *in vivo* (Eron *et al.*, 2004; Lalezari *et al.*, 2005; Menzo *et al.*, 2004; Reeves *et al.*, 2005). Despite, the encouraging antiviral activity of T-1249, clinical trials to further evaluate the compound have been halted (Martin-Carbonero, 2004). A major disadvantage of enfuvirtide and T-1249 is the requirement for administration by twice daily intramuscular injection. This issue could be improved or resolved by methods to increase the circulatory half-life of these peptides or by devising improved delivery strategies for these drugs. Furthermore, the search for orally bioavailable small molecules that inhibit the membrane fusion machinery in gp41 is underway. Two such compounds have recently been described as potential leads (Jiang *et al.*, 2004).

2.2 Considerations for Antiretrovirals Targeting Entry

The inhibition of HIV entry into cells raises novel considerations specific to this class of antiretrovirals. All entry inhibitors target the Env protein, either directly or indirectly, and Env is the most variable HIV protein. Thus, it is perhaps not surprising that drug naïve viruses with divergent Env proteins can exhibit considerable variation in susceptibility to certain entry inhibitors *in vitro* (Derdeyn *et al.*, 2000; Derdeyn *et al.*, 2001; Labrosse *et al.*, 2003), whereas differences in susceptibility to RT and protease inhibitors are comparatively modest (Parkin *et al.*, 2004).

Factors that can contribute to marked differences in coreceptor antagonist susceptibility include variation in the affinity with which an Env protein binds a coreceptor molecule and differences in cell surface coreceptor expression levels (Reeves *et al.*, 2002; Reeves *et al.*, 2004), which can vary between targets cells and individuals (Lee *et al.*, 1999). Thus, low Env-coreceptor affinity or low coreceptor expression is associated with enhanced susceptibility to coreceptor antagonists (Reeves *et al.*, 2002; Reeves *et al.*, 2004). Less intuitively perhaps, these factors also affect susceptibility to the fusion inhibitor enfuvirtide (Reeves *et al.*, 2002; Reeves *et al.*, 2004). Mechanistically, a reduction in Env-coreceptor affinity or coreceptor levels can result in a slower rate of membrane fusion, which in consequence extends exposure of the temporal target for enfuvirtide (Reeves *et al.*, 2002; Reeves *et al.*, 2004). These factors likely explain synergistic inhibition of HIV by coreceptor antagonists and enfuvirtide *in vitro* (Tremblay, 2004; Tremblay *et al.*, 2002; Tremblay *et al.*, 2000). Thus, coreceptor antagonists will act to block Env-coreceptor binding and also, in effect, reduce the number of cell surface coreceptors available for Env interaction, thereby delaying fusion and enhancing enfuvirtide susceptibility. The use of a combination of these entry inhibitors for antiretroviral therapy, with or without RT and/or protease inhibitors, remains to be evaluated, as does the

impact of Env-coreceptor affinity and coreceptor levels on entry inhibitor potency *in vivo*.

In addition to extensive variability, HIV Env exhibits considerable plasticity and is able to accommodate mutations that allow rapid adaptation to selective pressure. Thus the potential for HIV to readily acquire resistance to entry inhibitors was a concern. Indeed, HIV can rapidly escape from enfuvirtide inhibition both *in vitro* and *in vivo* (Greenberg and Cammack, 2004; Miller and Hazuda, 2004; Rimsky *et al.*, 1998; Wei *et al.*, 2002). Nevertheless, enfuvirtide therapy can remain effective for a prolonged time (Greenberg *et al.*, 2004; Lazzarin *et al.*, 2003), validating entry as a target for antiretrovirals. Furthermore, a number of reports indicate that it has been surprisingly difficult to generate viruses resistant to certain CCR5 antagonists *in vitro* (Trkola *et al.*, 2002; Westby *et al.*, 2004), further supporting a role for entry inhibitors in antiretroviral regimens. Mutations conferring relative resistance to enfuvirtide as well as CCR5 antagonists (in the absence of a coreceptor switch) can result in reduced viral infectivity and fitness *in vitro* (Lu *et al.*, 2004; Reeves *et al.*, 2005; Trkola *et al.*, 2002), and some enfuvirtide resistance mutations can confer enhanced susceptibility to a subset of neutralizing antibodies (Reeves *et al.*, 2005), thus drug resistance viruses might be less pathogenic and their emergence might still be associated with a clinical benefit.

A potential mechanism of escape from inhibitors that target CD4 binding is for viruses to adapt to utilize CCR5 or CXCR4 directly for infection. CD4-independent infection is usually less efficient than infection via CD4 and CD4-independent viruses are usually more susceptible to neutralizing antibodies (Bhattacharya *et al.*, 2003; Edwards *et al.*, 2001; Hoffman *et al.*, 1999; Reeves *et al.*, 1999), thus this mechanism of escape may also be associated with reduced fitness *in vivo*. However, CD4-independence does have the potential to broaden HIV cell tropism (Bhattacharya *et al.*, 2003; Reeves *et al.*, 1999; Willey *et al.*, 2003).

As discussed above, a poignant concern for antiretroviral therapy with CCR5 antagonists is the potential for virus to escape inhibition by switching to use CXCR4 as a coreceptor. While CXCR4 utilizing viruses are associated with pathogenesis, it still remains to be established whether their emergence is a cause or effect of disease progression. Thus, the clinical efficacy of CCR5 antagonists will require careful evaluation. Another factor specific to the utilization of coreceptor inhibitors is the requirement for determination of viral coreceptor specificity prior to treatment. Thus, a CCR5 antagonist will not be active against CXCR4 utilizing viruses and vice versa. Furthermore, the utilization of a CCR5 antagonist in individuals harboring a mixed population of R5, X4 and dual-tropic viruses might select

for outgrowth of X4 and/or dual-tropic viruses. Again, any potential impact of this scenario on disease progression remains to be determined. The use of a combination of coreceptor antagonist that target both CCR5 and CXCR4 would alleviate these concerns, however there remains the potential for virus to escape these inhibitors by adapting to utilize drug bound coreceptor (as discussed above) or by utilizing one of a number of alternative coreceptors that can mediate infection *in vitro* (Berger *et al.*, 1999).

3. INTEGRATION

3.1 The HIV Integration Reaction

Following entry of HIV into its target cell the viral RNA genome is converted into a double-stranded DNA molecule. In the cytoplasm, the DNA molecule associates with several viral and host proteins to form a multimeric complex that bridges both ends of the linear DNA molecule in what is referred to as a “preintegration complex” (PIC). Soon after completion of DNA synthesis, the viral DNA ends are primed by the enzyme integrase in a process called 3' processing. 3' processing involves cleavage of the terminal two nucleotides immediately 3' of a conserved CA dinucleotide motif of each LTR, resulting in two recessed 3'-hydroxyl groups (Bushman *et al.*, 1990; Craigie *et al.*, 1990; Katz *et al.*, 1990; Katzman *et al.*, 1989; Sherman and Fyfe, 1990). After docking with a host chromosome, the viral DNA undergoes a strand transfer reaction that involves a nucleophilic attack by each of the two recessed 3'-hydroxyl groups on a 5'-phosphate of the target DNA. For HIV, the points of joining of each end of the viral DNA are staggered by five base pairs across the major groove of the target DNA helix. After joining, the intervening five base pairs are melted, yielding gaps at the junctions of proviral and target DNA. The integration reaction is completed when the two protruding 5' proviral nucleotides are trimmed and the gaps repaired, most likely by host repair enzymes (Daniel *et al.*, 2004; Yoder and Bushman, 2000).

3.2 Integrase Structure and Function

HIV integrase is a critical component for the integration reaction. Integrase is encoded by the 3' end of the pol gene and is produced as a result of protease-mediated cleavage of the gag-pol precursor. The integrase enzyme is 288 amino acids long with a molecular weight of 32kDa. Residues 1-50 comprise the amino-terminal domain (NTD), 50-212 the catalytic core

domain (CCD) and 212-288 the carboxy-terminal domain (CTD). The full-length structure of integrase complexed with viral DNA has eluded scientists for many years due to solubility difficulties with the complex. In the interim, many groups have used X-ray diffraction or solution NMR and solved the structures of individual integrase domains, the CCD complexed with the NTD and the CCD complexed with the CTD and two domain fragments (Bujacz *et al.*, 1995; Cai *et al.*, 1997; Chen *et al.*, 2000a; Chen *et al.*, 2000b; Dyda *et al.*, 1994; Eijkelenboom *et al.*, 1997; Goldgur *et al.*, 1998; Greenwald *et al.*, 1999; Lodi *et al.*, 1995; Maignan *et al.*, 1998; Reeves *et al.*, 2005; Wang *et al.*, 2001).

The NTD is characterized by a conserved two histidine and two cysteine motif, HX₃₋₇HX₂₃₋₃₂CX₂C (referred to as the HHCC motif), that binds zinc (Burke *et al.*, 1992; Bushman *et al.*, 1993 1993; Cai *et al.*, 1997; Eijkelenboom *et al.*, 1997). This HHCC motif is essential for 3' processing and strand transfer activity *in vitro* and has been shown to promote tetramerization of integrase protomers and to enhance activity (Cannon *et al.*, 1994; Ellison *et al.*, 1995; Engelman *et al.*, 1995 1995; Zheng *et al.*, 1996). The CCD contains a D₆₄DX₃₅E motif formed by the catalytic triad D64, D116 and E152 embedded in a protein fold that is highly conserved among polynucleotide phosphotransferase enzymes (Bujacz *et al.*, 1995; Dyda *et al.*, 1994; Goldgur *et al.*, 1998; Greenwald *et al.*, 1999; Maignan *et al.*, 1998). Two monomeric core domains associate to form a dimer in solution, with each monomer being structurally similar to RNaseH, MuA transposase and RuvC resolvase, (Chiu and Davies, 2004). The D64 and the D116 residues form a coordination complex with a divalent metal (Mg²⁺ or Mn²⁺) once the integrase binds to its DNA substrate (Gao *et al.*, 2004; Grobler *et al.*, 2002; Marchand *et al.*, 2003). The CCD requires the NTD and the CTD in a dimeric complex in order to maintain its 3' processing and strand transfer activities and mutation in any one of the three conserved D64, D116 or E152 residues abolishes this activity. The CTD sequence is less conserved and the overall structure resembles the Src-homology 3 (SH3) domain (Eijkelenboom *et al.*, 1995; Lodi *et al.*, 1995). The CTD binds DNA non-specifically and is required for 3' processing and strand transfer activities.

3.3 Integrase as a Drug Target

It is well known that HIV therapy is much more effective when combinations of drugs are used instead of single drug regimens, which has accelerated the search for additional anti-HIV drug targets. Currently there are no known human homologs of HIV integrase, making this enzyme a very

attractive drug target. There are a number of criteria that are adhered to in screening integrase inhibitors. The inhibitor must have low cell toxicity and be specific for the integration step and not any of the other steps in the HIV lifecycle. Cells treated with the inhibitor should show an accumulation of 2-LTR circles, which is a by-product of unproductive integration, and cells should have a decreased number of integrated proviruses. Selection of drug-resistant viruses should also be shown to be a result of mutations solely in the integrase enzyme. This is usually verified by testing the inhibitor against recombinant integrase bearing the same mutations identified in the drug-resistant virus. Unfortunately, numerous drugs that inhibit integrase fail these criteria, especially in cell culture (see (Li *et al.*, 2004; Pommier *et al.*, 2000; Pommier and Neamati, 1999) for examples). Several assays exist for assessing integrase inhibition (reviewed in (Butler *et al.*, 2001; Hansen *et al.*, 1999; Witvrouw *et al.*, 2004)). The classical assays use LTR mimics to evaluate the 3' processing and strand transfer activities of integrase in the presence of inhibitors (Craigie *et al.*, 1991; Sherman and Fyfe, 1990). To date, the only class of integrase inhibitors that meet all of the above criteria is the diketo acids and their related naphthyridines. Another class of inhibitors called pyranodiprimidines is currently under study and is hoped to provide an alternative to the diketo acid inhibitor family. Several other integrase inhibitors are currently under investigation and will advance our knowledge of integrase function and will ultimately help to define far superior integrase inhibitors in the future. These inhibitors are far too numerous to discuss here and so readers are referred to several reviews on these inhibitors (Johnson *et al.*, 2004; Pommier *et al.*, 2005; Pommier *et al.*, 2000; Pommier and Neamati, 1999; Pommier *et al.*, 1997).

3.4 Integrase Inhibitors

Diketo Acids

The most advanced integrase inhibitors reported to date are the diketo acids and their derivatives discovered by Merck Research Laboratories (Hazuda *et al.*, 2000) and Shionogi & Co. Ltd (Goldgur *et al.*, 1999). The diketo acids were the first reported integrase inhibitors that showed high specificity for the integration reaction and antiviral activity in cells (Goldgur *et al.*, 1999; Hazuda *et al.*, 2000). The diketo moiety is usually flanked by an acidic group or an equivalent group such as a carboxyl, tetrazole or triazole group, and an aromatic group. Several substitutions of the aromatic group have been studied and shown to be critical for activity (Marchand *et al.*, 2003; Marchand *et al.*, 2002; Pais *et al.*, 2002; Wai and al., 2000). The diketo acids selectively recognize a particular conformation of the integrase active site only after it assembles on the viral DNA ends. Once bound, the

diketo acids compete with the target DNA and inhibit strand transfer, most likely by sequestering the active site metal (Mg^{2+}) (Espeseth *et al.*, 2000; Grobler *et al.*, 2002; Pommier *et al.*, 2005). Merck increased enthusiasm in the field when they showed that naphthyridines (derivatives of diketo acids) have potent antiviral activity against HIV-1, HIV-2 and simian immunodeficiency virus (SIV) with no cross-resistance to drugs that target other aspects of the viral lifecycle (Hazuda *et al.*, 2000; Pluymers *et al.*, 2002). At that time, L-870,810 was the most promising diketo acid, having the most potent anti-viral activity and entered clinical trials. However, L-870,810 studies were recently halted due to liver and kidney toxicity observed in dogs. Currently, Merck is developing the integrase inhibitors L-870,812 and Compound B. L-870,812 has been tested in rhesus macaques and has been shown to suppress viremia and chronic infections caused by SIV (Hazuda *et al.*, 2004).

Detailed analyses of the integrase gene of viruses that have become resistant to the Merck diketo acids (L-708,906 and L731,988) have revealed several mutations located near the metal coordinating residues of the D,DX35E motif in the CCD subunit of the enzyme. Specificity of these drugs was further supported by the finding that the resistant viruses still maintained sensitivity to inhibitors of reverse transcriptase, protease and viral entry (Fikkert *et al.*, 2003; Hazuda *et al.*, 2000). L-708,906-resistant viruses were also shown to exhibit cross-resistance to the diketo analogue S-1360, but they remained fully susceptible to the pyranodipyrimidine inhibitor V-165 (discussed later).

The diketo acid 1-(5-chloroindole-3-yl)-3-hydroxy-3(2H-tetrazol-5-yl)-propanone, otherwise known as 5CITEP, from Shionogi & Co. Ltd made a breakthrough when they co-crystallized 5CITEP with the CCD of HIV integrase and showed it to be in close association with the conserved D,DX35E motif (Goldgur *et al.*, 1999). 5CITEP is active against both 3' processing and strand transfer, thereby distinguishing this diketo derivative from the Merck derivatives (Marchand *et al.*, 2003; Marchand *et al.*, 2002; Pais *et al.*, 2002). In addition, molecular docking and dynamics simulation studies suggest that the Merck inhibitor L-731,988 and 5CITEP bind to integrase in a different way than do the diketo acids, likely involving metal chelation differences contributed by the aromatic and acidic groups of the diketo moiety (Keseru and Kolossvary, 2001; Marchand *et al.*, 2003).

Shionogi & Co. Ltd developed a more potent derivative of 5CITEP called S-1360 (Billich, 2003). S-1360 retains the diketo functionality but contains a triazole instead of the tetrazole group of 5CITEP. Numerous mutations arising from the selection of resistant virus in the presence of S-1360 appear to be in the vicinity of the highly conserved D,DX35E motif of the CCD.

Cross-resistance to the diketo acid L-708,906 was observed with these integrase mutants, but they remained fully susceptible to V-165 (Fikkert *et al.*, 2004). S-1360 has recently entered phase II clinical trials.

Pyranodipyrimidine

5H-pyrano[2,3-d:-6,5-d']dipyrimidines (PDPs) is a second class of inhibitors showing promise as a new integrase inhibitor. 5-(4-nitrophenyl)-2,8-dithiol-4,6-dihydroxy-5H-pyrano[2,3-d:-6,5-d']dipyrimidine (referred to as V-165) has been shown to inhibit integrase activities in enzymatic assays, although inhibition of reverse transcriptase activities has also been observed (Pannecouque *et al.*, 2002). In contrast to the diketo acids, V-165 was shown to have inhibitory effects against both the 3' processing and strand transfer activities in enzymatic assays, however in cell culture the anti-HIV activity of V-165 appears to correlate with inhibition of the strand transfer activity during integration. The mechanism of this inhibition is likely attributed to inhibition of integrase-DNA complex formation (Pannecouque *et al.*, 2002). Interestingly, V-165 remained fully effective against viruses resistant to the diketo inhibitors and inhibitors of viral entry and reverse transcription (Fikkert *et al.*, 2004; Fikkert *et al.*, 2003; Pannecouque *et al.*, 2002). Recently some mutations in the *RT* and *env* genes of resistant viruses that altered viral phenotype was reported (Cold Spring Harbor Retrovirus Meeting May 2005) and therefore further studies on the characterization of V-165-resistant HIV strains are required to verify that V-165 specifically targets integrase.

3.5 Considerations for Antiretrovirals Targeting Integration

The absence of a host equivalent to integrase greatly increases the therapeutic index of integrase inhibitors. However, caution must be taken since integrase shares mechanistic and structural similarities with various recombinases, RNases and integrases (Chiu and Davies, 2004; Rice and Baker, 2001; Shaw-Reid *et al.*, 2003). Such similarities help explain the finding that diketo acids inhibit the V(D)J RAG1/2 recombinases, albeit at about a 20 fold higher concentration than that needed to inhibit integrase (Melek *et al.*, 2002).

Other targets of the integration reaction that may lead to the discovery of new inhibitors include the 3' end processing of the viral DNA, multimerization of the integrase complex, assembly of the PIC and targeting of the PIC to chromosomes. Such studies will benefit tremendously once the crystal structure of full-length integrase complexed with DNA is solved.

4. MATURATION

The last step in the life cycle of HIV is referred to as “maturation” (Figure 1; reviewed in (Bukrinskaya, 2004; Vogt, 1996)). Immature HIV virions undergo morphologic changes that include condensation of the viral capsid protein (CA) to form a cone shaped core structure that is associated with mature, infectious virions. Maturation occurs during and following viral egress and is coordinated by the viral protease enzyme. Protease cleaves the Gag precursor polyprotein (Pr55^{Gag}) into the individual protein and peptide subunits, matrix (MA), CA, spacer peptide 1 (SP1), nucleocapsid (NC), spacer peptide 2 (SP2) and p6.

4.1 Maturation Inhibitors

Protease inhibitors, a core constituent of antiretroviral therapy, act by directly targeting the protease enzyme to block enzymatic activity and thus viral maturation. Issues that include resistance to current protease inhibitors and drug toxicity are driving the development of new inhibitors that target the protease enzyme either directly or indirectly (Rodriguez-Barrios and Gago, 2004; Wynn *et al.*, 2004). Indeed, a derivative of betulinic acid (3-O-betulinic acid, referred to as PA-457, DSB or YK-FH312) (Zhou *et al.*, 2004a), that is under clinical development by V. I. Technologies (Vitech; formally Panacos), acts to block HIV maturation via a novel mechanism of action and is a representative of a new class of antiretroviral agents referred to as maturation inhibitors (Kanamoto *et al.*, 2001; Li *et al.*, 2003; Zhou *et al.*, 2004b).

PA-457 is active against diverse primary HIV isolates *in vitro*, including viruses resistant to approved protease and RT inhibitors, with IC₅₀s in the low nanomolar range (Li *et al.*, 2003). PA-457 blocks virion maturation by inhibiting protease cleavage between the junction of CA and SP1, the last step in the processing of Pr55^{Gag} (Li *et al.*, 2003; Zhou *et al.*, 2004b). Processing of CA-SP1 into CA and SP1 is a prerequisite for condensation of CA into a mature viral core, thus virions produced in the presence of PA-457 have defective core structures and are not infectious. Passage of virus in the presence of PA-457 *in vitro* selects for mutations at the CA-SP1 junction (Li *et al.*, 2003). Mutations within this region are associated with resistance to PA-457, but also correlate with reduced viral fitness (Li *et al.*, 2003; Liang *et al.*, 2002; Zhou *et al.*, 2004a; Zhou *et al.*, 2004b). The precise mechanism of action of PA-457 is under investigation.

PA-457 is orally bioavailable, has favorable pharmacokinetics and was well tolerated in Phase I trials (Martin *et al.*, 2005a; Martin *et al.*, 2004a). Phase I/II trials of a single dose in HIV-infected individuals demonstrated that PA-457 could reduce viral load up to approximately 0.5 log (Martin *et al.*, 2005b). Phase II trials are underway and PA-457 has been granted fast-track review status by the FDA. A distinct mode of action from approved protease inhibitors means that PA-457 will likely be active against protease inhibitor resistant viruses *in vivo* as well as viruses resistant to other currently approved antiretroviral agents (Li *et al.*, 2003; Martin *et al.*, 2005b). Additionally, escape from PA-457 *in vivo* will likely come at a cost to viral fitness.

5. CONCLUSIONS

Despite significant advances in antiretroviral therapy over the past few years, an increasing number of individuals are harboring multi drug resistant viruses and have little options for effective therapy. Thus there is a pressing need for new antiretroviral agents that are active against viruses resistant to current drugs. Indeed, new inhibitors to current drug targets as well as inhibitors to new drug targets are in various stages of development, fueled by advances in our understanding of the viral life cycle. The life cycle of HIV presents numerous potential targets for intervention, and, as reviewed here, inhibitors to new targets that are furthest along in development include agents that interfere with various steps of the entry process, compounds that inhibit the integration of HIV into the host cell genome and an agent that prevents the formation of mature infectious virions. Distinct modes of action from approved antiretrovirals mean that these inhibitors will likely be effective against viruses resistant to current drugs. Furthermore, the use of a combination of inhibitors directed against distinct targets in first line therapy holds promise for enhanced viral containment. Entry inhibitors are also being developed as candidate microbicides and hold promise for the prevention of HIV transmission.

In summary, the development of novel entry, integration and maturation inhibitors will complement current antiretroviral therapy and future HAART regimens that attack HIV from various angles will likely offer better prospects for sustained inhibition of viral replication.

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