# Targeting Human Immunodeficiency Virus Type 1 Assembly, Maturation and Budding

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**Abstract:** The targets for licensed drugs used for the treatment of human immunodeficiency virus type 1 (HIV-1) are confined to the viral reverse transcriptase (RT), protease (PR), and the gp41 transmembrane protein (TM). While currently approved drugs are effective in controlling HIV-1 infections, new drug targets and agents are needed due to the eventual emergence of drug resistant strains and drug toxicity. Our increased understanding of the virus life-cycle and how the virus interacts with the host cell has unveiled novel mechanisms for blocking HIV-1 replication. This review focuses on inhibitors that target the late stages of virus replication including the synthesis and trafficking of the viral polyproteins, viral assembly, maturation and budding. Novel approaches to blocking the oligomerization of viral enzymes and the interactions between viral proteins and host cell factors, including their feasibility as drug targets, are discussed.

**Keywords:** HIV-1, antiretroviral drugs, drug targets, assembly, maturation, budding, protease dimerization, reverse transcriptase dimerization.

## Introduction

HIV-1 is a major public health problem affecting an estimated 40 million individuals worldwide (www.unaids.org). Although it has been over 20 years since HIV-1 was identified as the etiologic cause of acquired immune deficiency syndrome (AIDS) an effective vaccine is not available. Thus, apart from public health measures that aim at HIV-1 prevention, the only effective strategy for controlling HIV-1 infections and lowering HIV-1 transmission is the use of antiretroviral drugs either for the treatment or prevention of infections.

Current antiretroviral drugs belong to four classes, the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease (PR) inhibitors (PI) and fusion inhibitors (Vivet-Boudou et al. 2006; De Clercq, 1998; Abdel-Rahman et al. 2002; Manfredi and Sabbatani, 2006). NRTIs and NNRTIs are respectively, competitive and allosteric inhibitors of the HIV-1 reverse transcriptase (RT) and act early in the viral life-cycle by blocking the conversion of the viral RNA genome into a double stranded proviral DNA precursor (Shehu-Xhilaga et al. 2005). Fuzeon (enfuvirtide or T20) is a peptide that also acts early in the virus life-cycle by preventing viral entry through interaction with the gp41 transmembrane protein (Shehu-Xhilaga et al. 2005). In contrast, PIs inhibit the late stage of virus replication by blocking the specific cleavage of Gag and Gag-Pol polyproteins to mature structural proteins and enzymes (Shehu-Xhilaga et al. 2005). Early antiretroviral regimens consisted of one or two RTIs, which were delivered as sequential monotherapy and led to treatment failure (Piacenti, 2006). The advent of combination therapy, or highly active antiretroviral therapy (HAART) since 1996 has been responsible for a dramatic decrease in AIDS mortality (Palella et al. 1998). Current HAART regimens generally comprise three antiretroviral drugs, usually two NRTIs and either a PI or an NNRTI (Yeni et al. 2002). While an armoury of agents is available for the treatment of HIV-1 patients, new drugs and drug targets need to be identified due to drug toxicity and the eventual emergence of drug resistant strains to current antiretroviral inhibitors (Clavel and Hance, 2004). Moreover, resistance to one drug normally results

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in cross-resistance to inhibitors of the same class, rendering a large number of agents to limited clinical use (Clavel and Hance, 2004). Therefore, the development or availability of new drugs such as Fuzeon, the HIV-1 integrase inhibitor raltegravir (MK-0518) (Grinsztejn et al. 2007) and the CCR5 antagonist maraviroc (Stephenson, 2007) that remain active against drug resistant virus is essential for the continuing success of HAART (Yeni, 2006).

The increased understanding of how HIV-1 reproduces and interacts with the host cell machinery has resulted in the identification of potential drug targets, which can be exploited for the development of new classes of inhibitors. Here we describe strategies and agents that block the late stages of HIV-1 replication including the synthesis and trafficking of viral polyproteins, viral assembly, maturation and budding. Novel approaches to blocking the oligomerization of viral enzymes and the interactions between viral proteins and host cell factors are discussed including their feasibility as drug targets. While peptidomimetic PIs act at the late stage of HIV-1 replication to block viral maturation, this review will deal with agents that inhibit HIV-1 PR by novel mechanisms that are distinct to these transition state mimetics that are competitive inhibitors of the HIV-1 PR.

## Late Stages of the HIV-1 Life Cycle

Following virus attachment, fusion and uncoating the single stranded positive sense RNA genome of HIV-1 is reverse transcribed by the viral RT into a proviral DNA precursor in a reverse transcription complex (RTC) containing viral and possibly host cell factors (Fig. 1). The RTC matures into a preintegration complex (PIC) and traffics to the nucleus where the viral cDNA is inserted into the host cell chromosome by the HIV-1 integrase (IN) (Telesnitsky A. and Goff, 1997). The processes from entry up to and including integration are defined as the early steps in the viral life cycle. The late stage of virus replication begins with transcription of the viral mRNAs from the integrated provirus (Fig.1). Singly and multiply spliced mRNAs encode the HIV-1 envelope proteins and regulatory/accessory proteins, respectively (Rabson and Graves, 1997). $Pr55^{gag}$ (Gag) and $Pr160^{gag\text{-}pol}$ (Gag-Pol) polyproteins are translated from unspliced mRNAs (Swanstrom, 1997). Formation of two types of polyproteins from the same unspliced mRNA is mediated by a ribosomal frameshifting mechanism that brings the *pol* sequence in the same reading frame as *gag*. Perturbation of ribosomal frameshifting leads to changes in the Gag and Gag-Pol ratio that is detrimental to virus assembly, morphogenesis and release (Swanstrom, 1997).

Gag encodes the viral structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), p6 and two spacer peptides, p1 and p2. Gag-Pol also encodes MA, CA and NC in addition to the three viral enzymes, PR, RT and IN. After translation, Gag and Gag-Pol are targeted to the host cell plasma membrane, a process that is dependent on the myristoylation of the N-terminus of Gag (Fig. 1) (Swanstrom, 1997). Inhibition of myristoylation disrupts the proper targeting of Gag and Gag-Pol to the plasma membrane (Swanstrom, 1997).

Gag-Gag, Gag/Gag-Pol and Gag-RNA interactions are also essential for the proper assembly and maturation of infectious virions. Gag and Gag-Pol assemble at the plasma membrane along with viral envelope glycoproteins gp120 and gp41 to form immature viral particles (Fig. 1). Gag is necessary and sufficient for virus particle formation (Freed, 1998; Swanstrom, 1997). The viral genomic RNA is also packaged into virions through interactions with the NC of Gag and a psi packaging signal in the genome (Swanstrom, 1997). As the newly assembled virions bud from the cell it is believed that Gag-Pol polyproteins oligomerize in order to activate the HIV-1 PR by forming an active PR homodimer. This results in the sequential cleavage of Gag and Gag-Pol into the mature structural proteins and enzymes (Kaplan et al. 1994; Pettit et al. 1998). Agents that bind to domains in Gag or Gag-Pol and modulate their oligomerization are likely to have a negative effect on virus assembly, maturation and budding (Fig. 1). Agents that interfere with HIV-1 PR mediated cleavage of Gag and Gag-Pol result in the production of immature viral particles that are non-infectious (Kohl et al. 1988).

Virus particle budding and egress is mediated by interactions of viral proteins such as the p6 late domain with components of the endosomal sorting machinery. Ion channels formed by viral protein U (Vpu) also facilitate viral particle egress from the host cell. Below we describe in more detail the

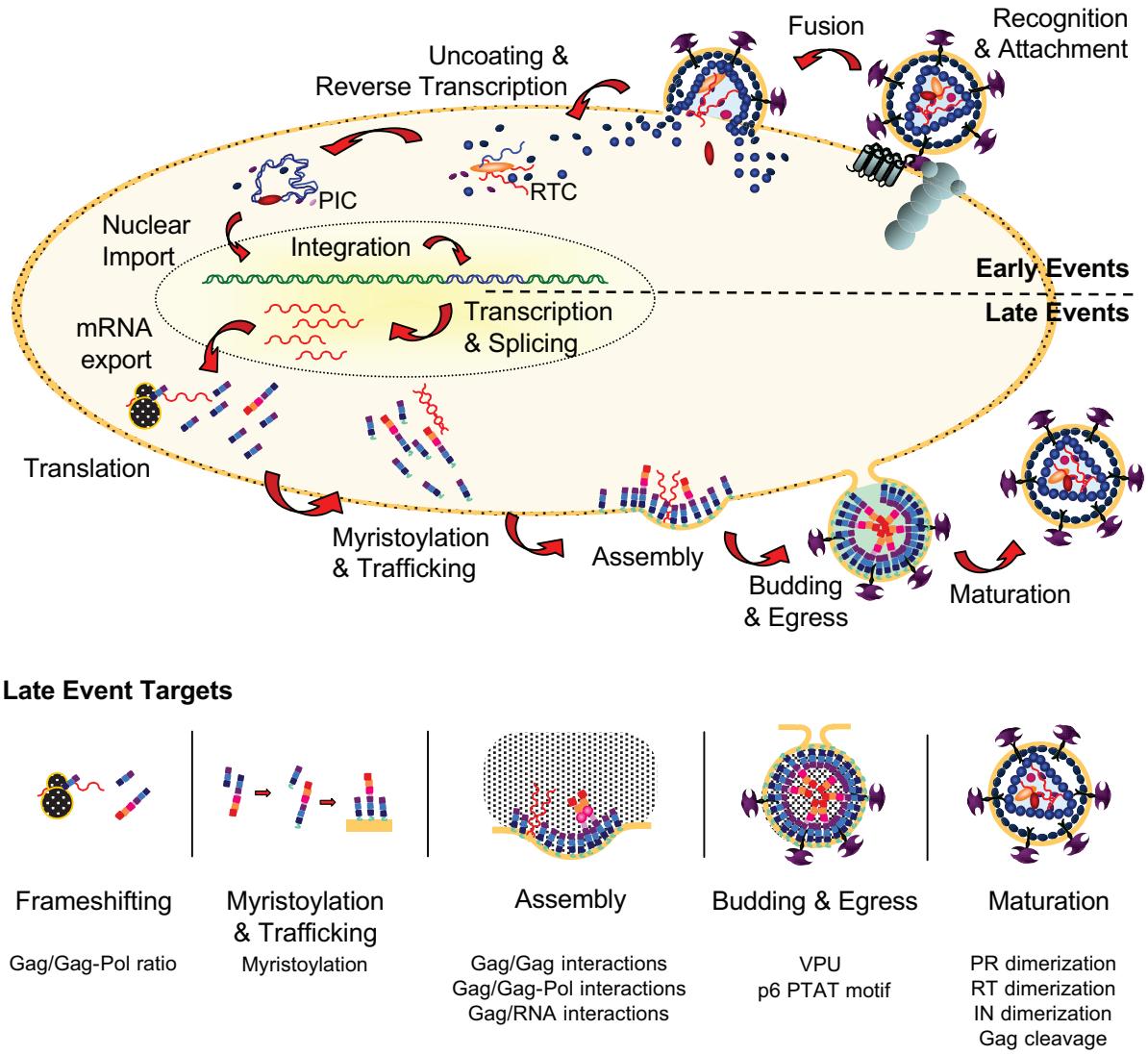


**Figure 1.** Overview of the HIV-1 life-cycle. Early events in virus replication include attachment, fusion and uncoating of the virus followed by reverse transcription in the cytoplasm of the cell, nuclear import of the preintegration complex and integration of the proviral DNA precursor into the host cell chromosome. Late events begin with transcription of unspliced and spliced RNA from the provirus and export of the mRNAs to the cytoplasm, resulting in the translation of Gag, Gag-Pol, Env and the accessory and regulatory proteins of HIV-1. Regulation of Gag-Pol synthesis is mediated by a ribosomal frameshifting mechanism from unspliced mRNA that also expresses Gag. Myristoylation of Gag is necessary for trafficking of Gag and Gag-Pol to the site of viral assembly. Assembly is driven by interactions between Gag-Gag, Gag/Gag-Pol, Gag-RNA. Viral budding and egress involves host cell factors. During or shortly after budding the HIV-1 PR cleaves the Gag and Gag-Pol polyproteins resulting in a mature and infectious viral particle.

specific processes required for viral assembly, maturation and budding and agents that have been described that block these steps.

## Inhibitors of Gag and Gag-Pol Expression: Targeting Ribosomal Frameshifting

HIV-1 Gag and Gag-Pol polyproteins are encoded by overlapping open reading frames on the same unspliced mRNA. During translation Gag-Pol is synthesized by a -1 ribosomal frameshifting mechanism that occurs at a frequency of 5 to 10% of Gag translation events (Jacks et al. 1988b). Similar frameshifting mechanisms are also used by other retroviruses including Rous sarcoma virus and Mouse mammary tumor virus in order to regulate expression of Gag-Pol (Jacks and Varmus, 1985; Jacks et al. 1987; Jacks et al. 1988a). The HIV-1 frameshift site is a heptanucleotide AU-rich sequence (UUUUUUA) found at the 3′ end of the NC coding sequence and is conserved amongst HIV-1 isolates. This slippery sequence and a downstream RNA stem loop structure stall the ribosome during the synthesis of Gag, allowing the ribosome to slip

back one nucleotide and enable synthesis of the Gag-Pol fusion protein (Jacks et al. 1988b). This sequence, the stem-loop structure and its stability and adjacent interacting sequences are believed to be the key components of the frameshifting signal (Jacks et al. 1988b; Kollmus et al. 1994; Hill et al. 2005). Details of a recently reported NMR structure and an analysis of current HIV-1 frameshifting models have recently been reviewed (Brierley and Dos Ramos, 2006).

Studies demonstrate that perturbation of the Gag/Gag-Pol ratio result in major defects in virus replication, suggesting that interfering with ribosomal frameshifting represents a viable drug target. Alteration of the Gag/Gag-Pol ratio, by engineering vectors with *gag* and *pol* genes in the same open reading frame, results in major defects in assembly and budding (Karacostas et al. 1993; Park and Morrow, 1991). The block in virus assembly is partially overcome by inhibition of the HIV-1 PR, suggesting that increased HIV-1 PR activity is responsible for the defect (Karacostas et al. 1993). A later study, in which the impact of decreasing the ratio of Gag/Gag-Pol on virion production was determined by co-transfection of plasmids expressing Gag and Gag-Pol alone demonstrate that the maintenance of this ratio is not only important for HIV-1 replication but also for virion RNA dimer formation and stability (Shehu-Xhilaga et al. 2001a). Furthermore, a decrease in Gag-Pol translation results in major defects in virus maturation and HIV-1 infectivity (Dulude et al. 2006). The small molecule, 1,4-bis-[*N*-(3-*N*,*N*-dimethylpropyl)amidino]benzene tetrahydrochloride (RG501, Table 1), is thought to enhance ribosomal frameshifting of HIV-1 by binding to the RNA stem loop structure of the ribosomal frameshifting signal resulting in increased ribosomal pausing (Hung et al. 1998). The imbalance in the resulting Gag/Gag-Pol ratio is associated with inhibition of acute and chronic HIV-1 infection in CCRF-CEM cells and peripheral blood mononuclear cells.

## Targeting Gag and Gag-Pol Trafficking

During the late phase of the viral life cycle, Gag polyproteins are targeted to the plasma membrane, where they are believed to colocalise to lipid raft microdomains for assembly into immature virions (Morikawa et al. 1996; Bryant and Ratner, 1990; Bouamr et al. 2003; Ding et al. 2003; Holm et al. 2003; Tang et al. 2004). Membrane targeting of Gag is mediated by the N-terminal myristoyl group in concert with conserved basic amino acids at the N-terminus of the MA domain of Gag (Bryant and Ratner, 1990; Facke et al. 1993; Ono and Freed, 1999; Ono et al. 2000). Myristic acid is a saturated 14-carbon fatty acid, post transationally attached to the N-terminal glycine of both Gag and Gag-Pol (Veronese et al. 1988). Myristoylation of Gag but not Gag-Pol is critical for targeting these polyproteins to the plasma membrane (Park and Morrow, 1992; Smith et al. 1993). Mutations that interfere with Gag myristoylation inhibit viral budding and misdirect virion assembly to the cytosolic fraction (Gottlinger et al. 1989; Bryant and Ratner, 1990). However, complete inhibition of Gag myristoylation is necessary to block HIV-1 budding (Morikawa et al. 1996).

Myristoylation is a two-step process involving activation of myristate to myristoyl-CoA by acyl-CoA synthetase and transfer of the myristoyl moiety from the myristoyl-CoA substrate to the N-terminal glycine of Gag by the enzyme *N*-myristoyltransferase (NMT) (Morikawa et al. 1996; Veronese et al. 1988). This pathway has been utilized to deliver alternate myristoylation substrates that perturb viral assembly.

The myristic acid analogue 12-azidododecanoic acid is a potent inhibitor of HIV-1 production in acute and chronically infected T-cell lines, exhibiting a maximum inhibitory effect between 10–50 μM at noncytotoxic concentrations, however the mechanism of action is not defined (Devadas et al. 1992). Another analogue, 4-oxatetra-decanoic acid, reduces HIV-1 replication in a T-cell line at 18 μM (Langner et al. 1992). Heteroatom-substituted analogs of myristic acid such as 12-methoxydodecanoate (13-oxamyristate or 13-OxaMyr), 5-octyloxypentanoate (6-oxamyristate or 6-OxaMyr), 11-ethylthioundecanoic acid and 12-thioethyldodecanoic acid act as alternate substrates for Gag myristoylation (Bryant et al. 1989; Bryant et al. 1991; Parang et al. 1997) and can prevent membrane binding of the modified Gag proteins (Bryant et al. 1989; Bryant et al. 1991). Of the heteroatom substituted analogs, 13-OxaMyr is the most potent inhibitor. 13-OxaMyr is added to Gag with an efficacy similar to that of myristate and alters viral polyprotein processing, which is suggested to be a consequence of inhibiting Gag and

**Table 1.** Inhibitors of the late stages of HIV-1 replication.

| Inhibitor | Description | Reference |
|---|---|---|
| *Ribosomal frameshifting* | | |
| 1,4-bis-[N-(3-N,N dimethylpropyl)amidino]benzene tetrahydrochloride (RG501) | Small molecule | (Hung et al. 1998). |
| *Myristoylation and Trafficking* | | |
| 12-azidododecanoic acid<br>4-oxatetra-decanoic acid | Myristic acid analogue | (Devadas et al. 1992)<br>(Langner et al. 1992) |
| 12-methoxydodecanoate<br>5-ocytl-oxypentanoate<br>11-ethylthioundecanoic acid<br>12-thioethyldodecanoic acid | Heteroatom-substituted myristic acid analogues | (Bryant et al. 1989)<br>(Bryant et al. 1991)<br>(Parang et al. 1997) |
| 5-*cis*-tetradecenoic acid (physeteric acid)<br>5-cis,8-cis-tetradecenoic acid (goshuyic acid) | Unsaturated 14-Carbon fatty acids | (Lindwasser and Resh, 2002). |
| *Assembly—Gag/Gag Interactions* | | |
| CAP-1 | Small molecule | (Tang et al. 2003) |
| PAATLEEMMTA | CA derived peptide | (Niedrig et al. 1994). |
| GPG-$NH_2$ | CA derived tripeptide amide | (Hoglund et al. 2002) |
| CAI | Peptide | (Sticht et al. 2005)<br>(Ternois et al. 2005) |
| *Maturation—Gag processing* | | |
| 3-0-(3′-3′-dimethylsuccinyl)-betulinic acid (PA-457/bevirimat) | Small molecule | (Li et al. 2003)<br>(Zhou et al. 2004)<br>(Sakalian et al. 2006) |
| electrophilic disulfide-substituted benzamides (DIBAs) | NC Zn finger inhibitor | (Rice et al. 1995)<br>(Turpin et al. 1996) |
| 1,2-dithiane-4,5-diol,1,1-dioxide (NSC 624151) | NC Zn finger inhibitor | (Rice et al. 1997) |
| S-acyl 2-mercaptobenzamide thioester (SAMT) | NC Zn finger inhibitor | (Schito et al. 2006). |
| *Maturation—PR dimerisation* | | |
| Ac-TLNF-OH | PR C-terminal tetrapeptide | (Zhang et al. 1991) |
| Pal-YDL-OH<br>Pal-YD-(biphenylalaine)-OH<br>Pal-YDT-OH | Modified PR C-terminal lipopeptides | (Schramm et al. 1999) |
| Apam(2)-YD-thyroxine-OH | | (Dumond et al. 2003) |
| PQITL(GGG)CTLNF | Glycine linked PR interface tetra-peptides | (Babe et al. 1992) |
| HO-FNLTS-NH-$(CH_2)_n$-N-PQITLW-OH | Alkyl linked PR interface peptides | (Zutshi et al. 1997)<br>(Ulysse and Chmielewski, 1998)<br>(Zutshi and Chmielewski, 2000) |

*(Continued)*

**Table 1.** (*Continued*)

| Inhibitor | Description | Reference |
|---|---|---|
| Molecular tongs | Scaffold constrained PR interface peptides | (Bouras et al. 1999)<br>(Breccia et al. 2003)<br>(Merabet et al. 2004)<br>(Hwang and Chmielewski, 2005)<br>(Bannwarth et al. 2006) |
| β-sheet peptide/peptidomimetic | PR interface derived peptidomimetics | (Song et al. 2001) |
| PQITL-RKKRRQRRRPPQV-SFNF-C/ATLN (P27/P27A) | PR C-terminal fusion peptide | (Davis et al. 2006) |
| BocFψ[$CH_2$NH]FEF-NH-$CH_2$-CO-TLNF-OH | Linked PR C-terminal tetrapeptide—active site inhibitor | (Uhlikova et al. 1996)<br>(Skalova et al. 2003) |
| Pentaester 13e | Didemnaketal A analogue | (Fan et al. 1998) |
| Ursolic Acid | Triterpene | (Quere et al. 1996) |
| NHGRNLLTQI (S8) | PR LES peptide | (Broglia et al. 2005)<br>(Broglia et al. 2006) |
| IVQVDAEG (p51) | Random peptide | (Park and Raines, 2000). |
| Vpr-(spacer)-TLNF-OH | Vpr, PR C-terminal fusion peptide | (Cartas et al. 2001). |
| *Maturation—RT dimerisation* | | |
| [2′,5′-bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1′,2″-oxathiole-2″,2″-dioxide) thymine (TSAO-T) | Small molecule | (Sluis-Cremer et al. 2000)<br>(Rodriguez-Barrios et al. 2001) |
| N-(4-tert-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone) (BBNH) | Small molecule | (Arion et al. 2002)<br>(Sluis-Cremer and Tachedjian, 2002)<br>(Himmel et al.) |
| KETWETWWTE (Pep-7) | RT connection subdomain peptide | (Morris et al. 1999)<br>(Depollier et al. 2005) |
| TLMALELKGKLLLAGLAPSAFLPLSFP EGL (TLMA2993) | Designed peptide targeting RT connection subdomain | (Campbell et al. 2002)<br>(Hosokawa et al. 2004) |
| *Maturation—IN Dimerisation* | | |
| INI 1 | Host cell factor | (Yung et al. 2001)<br>(Sorin et al. 2006)<br>(Ariumi et al. 2006)<br>(Kalpana et al. 1994) |
| *Budding and Egress* | | |
| 5-(N,N-hexamethylene)amiloride (HMA)<br>5-(N,N-dimethyl)amiloride (DMA) | Amiloride analogue | (Ewart et al. 2002)<br>(Ewart et al. 2004) |
| Vpu Binding protein (UBP) | Host cell factor | (Callahan et al. 1998)<br>(Handley et al. 2001)<br>(Harila et al. 2006)<br>(Neil et al. 2006) |
| TSG101 | Host cell factor | (Garrus et al. 2001)<br>(Demirov et al. 2002)<br>(Goila-Gaur et al. 2003) |
| zLLL/MG-132 Lactocystin | Proteosome inhibitor | (Schubert et al. 2000)<br>(Ott et al. 2003) |

**Abbreviations:** Ac: acetylation; Pam: palmitoyl; Apam: 2-aminopalmitic acid

Gag-Pol trafficking (Bryant et al. 1991). In an acutely infected T-cell line 13-OxaMyr reduces HIV-1 replication in the 40–80 μM range (Bryant et al. 1989). 13-OxaMyr also inhibits viral production in chronically infected H9/IIIB cells, which is consistent for an inhibitor that targets the late stage of HIV-1 replication (Bryant et al. 1991). 13-OxaMyr exhibits a synergistic anti-HIV-1 effect with AZT suggesting its potential for use in combination therapy (Bryant et al. 1991). The therapeutic efficacy of 13-OxaMyr can be further enhanced by conjugation with glycerophospholipid L-∝-phosphatidylethanolamine (Pidgeon et al. 1993). The selectivity of these heteroatom analogs for the target protein is dependent on the position of the substituted heteroatom, thus they can be exploited as a therapeutic antiretroviral strategy. Nevertheless, heteroatom-substituted myristic acid analogs are still expected to adversely affect a substantial range of cellular processes that depend on protein N-myristoylation (Lindwasser and Resh, 2002).

An alternative strategy for targeting Gag myristoylation is the exogenous treatment of cells with unsaturated 14-carbon fatty acids including 5-*cis*-tetradecenoic acid (14:1*n*-9, physeteric acid) and 5-*cis*,8-*cis*-tetradecadienoic acid (14:2*n*-6, goshuyic acid) (Lindwasser and Resh, 2002). As lipid rafts have preference for saturated fatty acids, treatment with unsaturated analogs interferes with membrane targeting of Gag and consequentially viral assembly and production (Lindwasser and Resh, 2002). These inhibitors also interfere with certain Src-kinase mediated cellular pathways, although they appear to have no effect on cell proliferation (Campbell and Vogt, 1995). It is suggested that direct dietary intake of physeteric acid and goshuyic acid could be a useful therapeutic strategy for the treatment of HIV-1 infections. However, the effect of long term intake of these unsaturated fatty acids and their effect on N-myristoylated signaling proteins such as Src, G-proteins, Arf and heterogeneously N-acylated retinal proteins needs to be assessed (Lindwasser and Resh, 2002).

Recent studies also indicate a role for phosphatidylinositide 4,5-bisphosphate [PI(4,5)P2] in regulating Gag localization (Ono et al. 2004). In HIV-1, binding of PI(4,5)P2 to the MA domain in Gag activates the "myristyol switch" and also acts as the point of membrane attachment (Saad et al. 2006). The binding site of PI(4,5)P2 on MA is highly conserved amongst HIV-1 strains and therefore represents an attractive antiviral target (Shkriabai et al. 2006; Saad et al. 2006).

## Targeting HIV-1 CA

CA plays an important role in the HIV-1 life-cycle by promoting Gag-Gag interactions during virion maturation. The N- and C-terminal domains of this protein serve distinct functions. As shown by mutational analysis, the N terminal domain of CA (N-CA), otherwise known as the NTD, is responsible for maintaining the proper conformation of CA during the assembly process (Worthylake et al. 1999; Li et al. 2000). The C-terminal domain of CA (C-CA) or the CTD, is critical for Gag-Gag interactions during assembly and maturation (Gamble et al. 1996; Gamble et al. 1997) and described mutations in this region have major consequences on virion maturation and infectivity (von Schwedler et al. 2003; Ganser-Pornillos et al. 2004). The NMR structure of CA has demonstrated that the protein consists mainly of seven α-helices, two β-hairpins and a loop structure (Momany et al. 1996; Gitti et al. 1996). Five of the α-helices form a coiled-coiled structure while one of the β-hairpins is located on the surface of the N-terminal domain of the protein (Momany et al. 1996). The second β-hairpin is predicted to be formed after cleavage by the HIV-1 PR (Tang et al. 2002). Cleavage of CA from its neighbouring proteins is necessary for core condensation and conical capsid shell formation (Vogt, 1996; Wiegers et al. 1998). Compounds that bind to these regions would be expected to disrupt proper CA shell formation and virion infectivity making CA an important and attractive target for the development of antiretroviral agents.

A proof of concept study, demonstrating the potential of inhibiting CA-CA interactions as an antiretroviral target has been published (Tang et al. 2003). Computational high throughput screening of a small molecule library and NMR analysis for binding specificity resulted in the identification of CAP-1 and CAP-2 which bind to an apical site on the NTD of both immature and mature CA (Tang et al. 2003). While CAP-2, is toxic to U1 cells, CAP-1 reduces viral infectivity by 95% at 100 μM. The released virions lack cone shaped cores and resemble viral particles that have been observed in HIV-1 expressing mutations that disrupt CA-CA interactions (Dorfman et al. 1994; Reicin et al. 1996; von Schwedler et al. 2003;

Lanman et al. 2003). Despite aberrant viral morphology CAP-1 does not affect viral particle release or proteolytic processing (Tang et al. 2003). CAP-1 and CAP-2 bind to a common site within the NTD thus preventing CA-CA interactions and proper Gag assembly.

Peptides derived from HIV-1 CA have also been described to affect viral morphogenesis by interfering with capsid formation (Niedrig et al. 1994). The synthetic peptide, PAATLEEMMTA, inhibits HIV-1 replication in cell culture assays at 20–200 μg/ml and results in the production of immature and aberrant viral particles (Niedrig et al. 1994). Tripeptide amides derived from the carboxyl terminus of CA inhibit HIV-1 replication, with the three most potent peptides interacting with CA as demonstrated by capillary electrophoresis analysis (Hoglund et al. 2002). Glycyl-prolyl-glycine-amide (GPG-$NH_2$) interferes with the formation of HIV-1 particles with a normal conical core structure (Hoglund et al. 2002). G-$NH_2$ is an active metabolite of GPG-$NH_2$ indicating that the latter acts as a pro-drug (Andersson et al. 2005). However, the development of HIV-1 resistance to either G-$NH_2$ or GPG-$NH_2$ has been elusive suggesting that the peptides mediate their effects through a host cell or other factor (Andersson et al. 2004).

CAI, a small peptide selected by phage display screening, acts as an inhibitor of the assembly of immature Gag *in vitro* (Sticht et al. 2005; Ternois et al. 2005). CAI binds to the C-terminus of CA

($K_d \sim 800$ μM), thus preventing the necessary conformational changes in CA that lead to the formation of mature cores (Sticht et al. 2005). The structure of CAI complexed with CA has revealed that the CAI binding region is a highly conserved hydrophobic pocket within the C terminus of CA where the peptide forms an extra α-helix, which binds to the four α-helices of CA (Ternois et al. 2005). The resulting protein-peptide complex is therefore a five α-helix bundle with reduced CA-CA dimerization contacts that destabilizes the dimer interface. Binding of CAI to the C-CA not only affects the assembly of the immature capsid particles but also reduces the amount of correctly assembled mature capsids *in vitro*, thus acting as a promising two-step inhibitor (Sticht et al. 2005).

The C-terminal domain of Gag in the context of Gag-Pol is essential for its interaction with Gag and its incorporation into the virion (Srinivasakumar et al. 1995; Chiu et al. 2002; Chien et al. 2006). This sequence includes a highly conserved "major homology region" (MHR) in the CA domain of Gag and the adjacent CA-SP1 (Srinivasakumar et al. 1995; Chien et al. 2006). These sequences are also critical for HIV-1 Gag assembly as they drive Gag oligomerization. However, the magnitude of the virion incorporation defect of Gag-Pol MHR deletion mutants varies between different studies making the value of targeting this region of Gag-Pol unclear with respect to inhibition of the late stages of HIV-1 replication (Mammano et al. 1994; Srinivasakumar et al. 1995; Chiu et al. 2002; Chien et al. 2006).

Sequences involved in Gag and Gag-Pol interactions are assumed to be similar to those involved in Gag-Gag interactions. However, virions generated in the presence of CAP-1 are unlikely to affect Gag/Gag-Pol interactions as defects in proteolytic processing in the virus or virion associated RT activity were not observed (Tang et al. 2003). The proline rich region of p6 has also been implicated in the packaging of cleaved Pol proteins into virions, which is suggested to be mediated by host cell proteins (Dettenhofer and Yu, 1999; Cen et al. 2004). Identifying the host cell factor implicated in the virion incorporation of cleaved Pol will be necessary for establishing this process as a viable drug target.

## Targeting HIV-1 NC

The HIV-1 NC (NCp7) contains two highly conserved zinc finger motifs C-$X_2$-C-$X_4$-H-$X_4$-C (X, any amino acid). The zinc fingers of NC are critical in the early and late stages of HIV-1 replication with mutations in the zinc chelating amino acids resulting in formation of non-infectious virus (Aldovini and Young, 1990). The zinc fingers of NCp7 are required for initiation, elongation and efficient template switching during reverse transcription (Rodriguez-Rodriguez et al. 1995; Tanchou et al. 1995). NCp7 is also involved in HIV-1 genomic RNA dimerization, IN cleavage activity and coats the viral RNA genome protecting it from nucleases (Lapadat-Tapolsky et al. 1993).

Given the critical role of NCp7 zinc fingers in HIV-1 replication it is not surprising that agents that covalently modify the zinc chelating residues of NCp7 have been described as inhibitors of HIV-1 replication (Rice et al. 1995). The electrophilic

disulfide-substituted benzamides (DIBAs) inactivate cell free virus and inhibit the early and late stages of HIV-1 replication by interfering with reverse transcription and viral particle maturation (Rice et al. 1995; Turpin et al. 1996). In the U1 cell line treatment with DIBAs results in the inhibition of virus particle release, processing of Gag, and the production of virions with reduced infectivity (Turpin et al. 1996). The defect in viral particle release and maturation was attributed to the formation of intermolecular cross-linkages between the zinc fingers of adjacent Gag molecules, thereby preventing efficient cleavage by the HIV-1 PR (Turpin et al. 1996).

The non-dissociable tethered dithiane compound 1,2-dithiane-4,5-diol,1,1-dioxide, (NSC 624151) also mediates similar defects in Gag processing (Rice et al. 1997). Although cellular proteins also contain zinc fingers, these inhibitors appear to preferentially target retroviral zinc fingers. This may be explained by the inaccessibility of these inhibitors to the appropriate cellular compartments where zinc finger containing cellular proteins are located. The *in vivo* anti-HIV-1 activity of zinc finger inhibitors has been demonstrated in a transgenic murine model where infectious HIV-1 is induced from an integrated provirus (Schito et al. 2003). A recent study in a nonhuman primate model demonstrated a reduction in the levels of SIV/DeltaB670 in peripheral blood mononuclear cells during therapy with the zinc finger inhibitor, S-acyl 2-mercaptobenzamide thioester (SAMT), although there was no effect on viral load (Schito et al. 2006). Further studies are in progress to optimise the bioavailability and pharmacokinetics of this promising inhibitor.

## Targeting HIV-1 PR

Much of our understanding of how the PR domain in Gag-Pol is activated and the processing cascade of Gag and Gag-Pol are due to the contributions of Kaplan and colleagues (Kaplan et al. 1994; Pettit et al. 2005). Strict regulation of PR function is critical for efficient production of mature viral particles. Premature activation, partial inhibition, or over-expression of HIV-1 PR leads to major defects in viral assembly and the production of non-infectious viral particles (Krausslich, 1991; Kaplan et al. 1993; Karacostas et al. 1993). Hence novel inhibitors designed to prevent or perturb PR dimerization could potentially inhibit the mature PR homodimer and the immature Gag-Pol embedded PR.

## Targeting the PR Dimer Interface with Interface Peptides

HIV-1 PR is a homodimeric aspartyl protease formed by the symmetrical association of two 99 amino acid subunits. The crystal structure reveals a compact, predominantly β-strand structure with a short α-helix region near the C terminus (Wlodawer et al. 1989). Dimerization of the PR monomers generates both the substrate-binding pocket and the catalytic centre and is essential for PR activity (Cheng et al. 1990). The PR dimer has a dissociation constant of 50 nM and Gibbs free energy of dimer stabilisation of 10 kcal/mol (25 $^{o}$C, pH 3.4). Nearly 75% of the binding energy is contributed by the four-stranded β-sheet formed by the N- and C-termini (Todd et al. 1998).The four-stranded β-sheet comprising the N- and C-termini from each PR monomer represents an attractive drug target for the following reasons: 1. It is the major stabilising region of the active dimer, 2. The region is relatively free of known PR resistance mutations, 3. The sequence is highly conserved in most HIV-1 and HIV-2 isolates and 4. It provides a unique target minimising potential toxicity issues for eukaryotic aspartyl proteases (Gustchina and Weber, 1991).

A standard methodology for analysing potential PR inhibitors that prevent PR dimerization (dissociative inhibition) or target and bind to the PR active site (competitive inhibition) has been described (Zhang et al. 1991). An example of a dissociative inhibitor is the C-terminal tetrapeptide, Ac-T-L-N-F, which exhibits activity in the micromolar range ($K_i$ 45 μM) (Zhang et al. 1991). Other studies have also shown the capacity of N- and C-terminal peptides, or 'interface' peptides, to bind to PR monomers and thus prevent PR dimerization and activity (Babe et al. 1991; Franciskovich et al. 1993; Schramm et al. 1991; Schramm et al. 1996). The identification of these lead peptides provides proof of concept that targeting the PR β-sheet region constitutes a viable strategy for the development of novel inhibitors of HIV-1 PR.

The potency of C-terminal tetrapeptides are increased by truncation to a core tripeptide, amino acid modification, and the addition of a linear hydrophobic moiety such as palmitoyl to the amino

terminus of the peptide. The lipid moiety is thought to increase the dissociative activity of the peptides by directing it to the hydrophobic PR interface (Schramm et al. 1999). However, despite their capacity to inhibit PR activity at low nanomolar concentrations, these lipopeptides are poorly soluble and susceptible to protein degradation. Further modifications have been made to the lipopeptides by making them less peptide-like (Caflisch et al. 2000) and by modifying the lipid moiety to increase their solubility while retaining potency (Dumond et al. 2003).

Cross-linking interfacial peptides represent another strategy, with the aim to increase the affinity of the peptides by presenting them in a conformation similar to a PR monomer. The first interface tetrapeptides tethered with a glycine linker display greater potency (PF1, $IC_{50}$= 40 μM) compared to free tetrapeptides ($IC_{50} \geq 150$ μM) (Babe et al. 1992). This approach has evolved to linking peptides with flexible alkyl tethers (Zutshi and Chmielewski, 2000) and semirigid alkyl based tethers (Ulysse and Chmielewski, 1998), which increase the distance between the peptides to approximate that of the PR termini in the dimer (~10 Å). The conformational freedom of these linked peptides was addressed by the use of pyridinediol and naphthalene based molecularly constrained scaffolds (Bouras et al. 1999; Song et al. 2001; Merabet et al. 2004; Bannwarth et al. 2006). Known as 'molecular tongs', these compounds are designed to position the interface peptides to clamp the termini of a PR monomer (Fig. 2). These studies have culminated in a set of optimised tongs with symmetrical peptidomimetic sequences based on an optimised PR C-terminal sequence. The tongs inhibit the activity of HIV-1

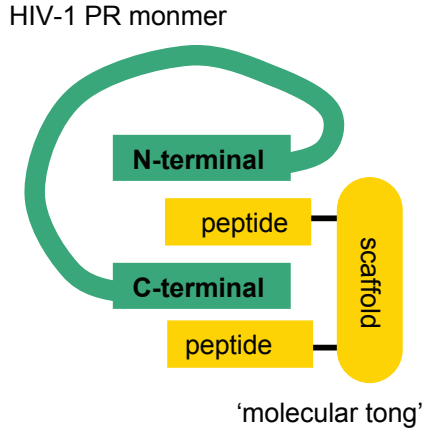


**Figure 2.** A molecular tong bound to the C-terminus of the HIV-1 PR monomer.

PR that are either sensitive or resistant to PIs with Ki values from 0.4–4.8 μM in cell free assays (Bannwarth et al. 2006).

Other variations on the theme of interface peptides include combining the advantages of lipopeptides and molecular tongs. Interface peptides have been linked to lipophilic groups by a rigid bicyclic guanidinium scaffold (Breccia et al. 2003). The most potent compound demonstrates PR inhibitory activity similar to tethered peptides and molecular tongs. Cross-linked interfacial peptides have been designed to irreversibly inhibit HIV-1 PR by formation of a disulfide bond between the peptide and the conserved PR residues C-95 and C-67, and demonstrate a $K_i$ in the low micromolar range (Zutshi and Chmielewski, 2000). The C-terminal tetrapeptide has also been tethered to a peptidic PR active site inhibitor, combining both dissociative and competitive methods of inhibition in one molecule (Uhlikova et al. 1996).

Random peptides that are dissociative inhibitors of HIV-1 PR have been described. The bacteriophage lambda repressor protein was utilised to develop a powerful two-hybrid PR dimerization assay. From a library of $5 \times 10^8$ random peptides, 300 were identified as potential PR dimerization inhibitors. The most potent peptide identified, p52, was a pure dissociative inhibitor with low $K_i$ of 780 nM (Park and Raines, 2000).

Ultimately, one of the major hurdles in developing peptidic inhibitors is to obtain a biologically stable compound that can be delivered inside the cell. One mechanism to achieve this is to fuse the peptide to amino acid sequences that promote either encapsidation into viral particles or entry into the host cell. Virus protein R (Vpr) is a HIV-1 accessory protein packaged into virions by its *trans* association with the Gag p6 motif. Both viral and cellular proteins have been successfully delivered into viral particles as Vpr fusion proteins (Wu et al. 1997). Inhibition of HIV-1 replication has been reported by the fusion of Vpr to viral PR recognition sequences (Serio et al. 2000). Expression of the PR C-terminal tetrapeptide as a Vpr fusion [Vpr-(spacer)-T-L-N-F-OH] attenuates HIV-1 replication in chronically infected cells and in single-round replication assays (Cartas et al. 2001).

Most recently, inhibition of HIV-1 replication has been demonstrated by delivering PR interface peptides as a fusion peptide utilising the HIV-1 Tat derived cell permeable protein transduction domain

(Davis et al. 2006). Peptides P27/A are PR dimerization inhibitors that inhibit the activity of wild-type and drug resistant PR in cell free assays with $IC_{50}$ values in the 0.28–0.58 μM range. These peptides are successfully delivered into chronically HIV-1 infected cells and reduce viral particle production. This was observed by a reduction in p24, rather than inhibition of Gag processing which suggests that the peptide may interact with the Gag-Pol embedded PR and disrupt the ordered processing of Gag-Pol leading a decrease in viral particle production (Davis et al. 2006).

## PR Folding Inhibitors

Local elementary structures (LES) are comprised of strongly interacting, highly conserved amino acids that are usually hydrophobic. These amino acids are suggested to direct the folding of a protein into its native conformation. Short peptides corresponding to or mimicking the LES are hypothesised to act as folding inhibitors, preventing the protein achieving its native conformation (Broglia et al. 2005). A peptide has been identified from a LES in the HIV-1 PR (peptide S8, amino acids 83–93) that inhibits PR activity with a $K_i$ of 2.58 μM and results in disorganisation of the PR secondary structure by reducing the β-sheet content from 30% to 14% (Broglia et al. 2005; Broglia et al. 2006). Current efforts are directed towards developing a shorter less hydrophobic peptide or mimetic based on the S8 lead peptide.

## Catalytically Inactive PR Subunits as Dominant Negative Inhibitors of PR activity

Catalytically inactive PR monomers act in a dominant negative fashion to inhibit wild-type HIV-1 PR by forming inactive heterodimers in recombinant protein assays (Babe and Craik, 1991). When virus expressing a PR active site mutation is co-transfected with wild-type HIV-1, both viral replication and virus infectivity are reduced (Babe et al. 1995). Computer modelling has been used to successfully design an optimised dominant-negative PR expressing D25K, G49W and I50W (KWW) (McPhee et al. 1996), which also reduces viral replication and infectivity (Junker et al. 1996). Biochemical studies on recombinant dominant negative PRs confirm that the mechanism of action is by formation of inactive heterodimers (Rozzelle et al. 2000). Interestingly, the mutant PRs cannot homodimerize and they fold only when expressed with wild-type PR. PR heterodimers are also more stable that the wild-type homodimer (Rozzelle et al. 2000). Hence inactive PR heterodimers form the dominant species. Such a dominant negative strategy for the inhibition of HIV-1 PR would require *in vivo* delivery by a gene-therapy system, the therapeutic use of which is unlikely in the near future.

## Non-Peptide Inhibitors of HIV-1 PR

A screen of a crude extract from the marine organism magenta ascidian didemnum identified two didemnaketals, A (a bicyclic ketal) and B (a linear heptaprenoid), that inhibit HIV-1 PR activity with $IC_{50}$ values of 2 μM and 10 μM, respectively (Potts et al. 1991). These compounds are unsuitable drug candidates, but have given rise to a novel class of pentaesters, the most potent of which is a dissociative inhibitor of PR with a $K_i$ of 2.1 μM (Fan et al. 1998).

A novel class of PR dimerization inhibitors were identified by searching the Cambridge structural database for pharmacophores that mimic the action of previously identified inhibitory interface peptides (Quere et al. 1996). Several triterpene structures were identified, of which ursolic acid acts as a dissociative inhibitor of PR with an $IC_{50}$ of 2 μM. It has been suggested that triterpene could provide another basic scaffold for building more effective peptidomimetics. Interestingly, another member of the triterpene family, PA-457, acts as a novel inhibitor of HIV-1 maturation which is discussed later in this review. A β-sheet mimetic was tested for its ability to inhibit PR homodimerization by perturbation of β-sheet formation (Song et al. 2001). The β-sheet mimetic had a relatively high $IC_{50}$ of 30 μM and the method of inhibition appears to be complex, however the structure provides a non-peptidic lead compound for PR inhibitors.

## Targeting Gag Processing

The rate and the specificity of Gag cleavage by the HIV-1 PR is dependent on the amino acid composition of the different cleavage sites recognized by the viral PR (Swanstrom, 1997). Based on the order of proteolysis by HIV-1 PR, these sites are classified as primary (p2/NC), secondary (MA/CA and p1/p6) or tertiary (CA/p2

or CA/SP2) cleavage sites. The lack of processing of any of these sites by PR results in the formation of aberrant particles (Swanstrom, 1997). In particular, inhibition of cleavage at the CA/p2 site has severe consequences for core formation, stability and virion infectivity (Pettit et al. 1994; Wiegers et al. 1998; Pettit et al. 1998; Shehu-Xhilaga et al. 2001b). The α-helical structure that stretches between the C-terminus of CA and the N-terminus of SP1 is critical for virion assembly and p2 function (Accola et al. 1998). Clearly, these PR cleavage sites are potential targets for antiretroviral drug design.

In this regard, a compound that interferes with viral maturation by blocking CA/p2 cleavage has been identified. 3-O-(3′,3′-Dimethysuccinyl) betulinic acid (PA-457 or bevirimat) potently inhibits HIV-1 maturation and infectivity (Li et al. 2003; Zhou et al. 2004). PA-457 specifically blocks the cleavage of CA/p2 in cell based (Li et al. 2003) and in cell free assays (Zhou et al. 2005; Sakalian et al. 2006), thus inhibiting core condensation and virion maturation. Inhibition of Gag processing at the CA/p2 junction results in the generation of the uncleaved p25 product in transfected cells at 0.1 μg/ml of PA-457 (Li et al. 2003). Consistent with the proposed mechanism, PA-457 resistant HIV-1 selected in long term cultures in the presence of betulinic acid contain mutations in the regions that flank the P-P' scissile bond (Adamson et al. 2006; Zhou et al. 2006). These mutated sites in Gag are recognized by the viral PR during proteolysis. In addition, other single amino acid substitutions have been identified that confer resistance to PA-457 and are exclusively located either at the C terminus of CA or within the first three amino acids of the p2 spacer peptide (Adamson et al. 2006). Interestingly, they all conferred resistance independently and were located within the boundaries of the CA/p2 proteins, a region well known to promote Gag multimerization (Adamson et al. 2006). These data suggest that there is more than one mechanism by which these mutants have acquired resistance to PA-457. PA-457 has successfully undergone Phase 1 and 2 clinical trials and is currently in a Phase 2b trial to test the efficacy of different doses of PA-457 in combination with approved HIV-1 inhibitors as part of an optimised regimen in patients failing therapy due to the emergence of drug resistant virus.

## Targeting the RT Domain in Gag-Pol

Like other HIV-1 enzymes, RT subunits must oligomerize to form an active enzyme. The biologically relevant form that is present in the virion is an asymmetric heterodimer comprised of the p66 (66 kDa) and the p51 (51 kDa) subunits (Jacobo-Molina et al. 1993; Kohlstaedt et al. 1992). The RT heterodimer is extremely stable and has an extensive protein surface area (4800 $Å^2$) that is buried upon subunit dimerization. Thermodynamic measurements of the association between the p66 and p51 RT subunits have estimated Gibbs free energy of dimer stabilization of approximately 10–12 kcal/mol$^{-1}$, corresponding to a dissociation constant of approximately $3 \times 10^{-7}$ M (Venezia et al. 2006). For an extensive review on HIV-1 RT dimerization see Srivastava et al. 2006.

Regions both upstream and downstream of the PR region in Gag-Pol have been investigated for effects on PR activation (Bukovsky and Gottlinger, 1996; Partin et al. 1991; Louis et al. 1999). Large deletions within or C-terminal truncations of RT in the context of Gag-Pol result in an increase in virion associated Gag processing intermediates, suggesting a defect in PR activity (Cherry et al. 1998; Liao and Wang, 2004; Quillent et al. 1996). These studies suggest that modulating RT dimerization in the context of Gag-Pol may have a negative impact on PR activation and HIV-1 maturation.

The importance of the RT region in Gag-Pol for both RT maturation and viral particle production has been demonstrated by the study of RT point mutations that prevent RT heterodimerization and p66 homodimerization. Mutations at W401, a component of the highly conserved tryptophan repeat motif in the connection subdomain, blocks RT dimerization *in vitro* (Tachedjian et al. 2003; Tachedjian et al. 2005b). When expressed in HIV-1 it manifests as defects in reverse transcription, aberrant processing of RT, and low levels of infectivity (Wapling et al. 2005). The L234A mutation, located in the RT primer grip region, prevents RT dimerization and decreases Gag-Pol stability (Tachedjian et al. 2000; Yu et al. 1998). This mutation reduces PR incorporation into virions, increases the accumulation of Gag processing intermediates, and results in the production of non-infectious virus particles (Tachedjian et al. 2000; Yu et al. 1998). These examples demonstrate the potential for

targeting the Gag-Pol embedded RT for blocking HIV-1 maturation.

## Inhibitors that Modulate HIV-1 RT Subunit Interaction

Apart from classical NNRTIs, there exists a class of unconventional NNRTIs that bind to HIV-1 RT and inhibit enzyme activity by decreasing the overall stability of the heterodimer without dissociating the complex (Sluis-Cremer et al. 2000; Sluis-Cremer and Tachedjian, 2002; Sluis-Cremer et al. 2006; Camarasa et al. 2006). The TSAO-T derivatives ([2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) thymine) destabilise both RT heterodimers and p66 homodimers by inducing changes at the RT dimer interface (Sluis-Cremer et al. 2000; Rodriguez-Barrios et al. 2001). The putative binding site is at the RT dimer interface and overlaps in part with the NNRTI binding pocket (Rodriguez-Barrios et al. 2001). Resistance mutations to the TSAO drugs are readily generated in cell culture indicating that these drugs are specific inhibitors of the HIV-1 RT (Balzarini et al. 1993). While TSAO represent the first class of small molecules that destabilize the RT heterodimer, preclinical studies demonstrate that the pharmacological profile of TSAO inhibitors is unfavourable for further clinical development (Camarasa et al. 2006).

The N-acylhydrazone derivative N-(4-tert-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) binds to both the DNA polymerase and RNase H domains of RT and inhibits both enzymatic activities of the RT (Arion et al. 2002). Similar to TSAO, BBNH prevents RT activity through destabilizing, but not dissociating the subunits. BBNH derivatives that bind to the DNA polymerase domain alone are sufficient to induce dimer destabilization (Sluis-Cremer and Tachedjian, 2002). The recently resolved structure of HIV-1 RT bound to a BBNH derivative has confirmed that the binding site is in close proximity to, but distinct from both the polymerase active site and NNIBP. It is thought that BBNH destabilizes the RT heterodimer by inducing changes in the primer-grip motif, which is an important region for RT dimer stability (Himmel et al. 2006; Srivastava et al. 2006).

By targeting the RT dimerization interface, it is possible that unconventional NNRTIs may have an affect on the late stage of virus replication. In particular, the TSAO drugs that destabilize the p66 homodimer, may also perturb the process of RT maturation to the heterodimer, and arguably even target the RT domain in Gag-Pol and interfere with PR activation. However, these possible effects have not been described. Further elucidation of the impact of nonclassical NNRTIs on RT maturation, and the mechanism of destabilization would be advantageous for designing more potent inhibitors of both RT function and RT maturation.

## Classical NNRTIs as RT Inhibitors Acting at the Late Stage of Viral Replication

Interestingly, several classical NNRTIs have been shown to confer a concentration dependant increase in RT heterodimer formation, corresponding with a loss of RT polymerase function (Tachedjian et al. 2001; Venezia et al. 2006). Efavirenz (EFV) is a strong enhancer of RT dimerization, and also enhances the formation of p66 and p51 homodimers (Tachedjian et al. 2005a). The exact mechanism for increasing RT subunit interactions is unknown but it is suspected that the binding of EFV to the RT mediates conformational changes in the p66 subunit that promotes interaction with p51 (Tachedjian et al. 2001). EFV has also demonstrated the capacity to enhance the homodimerization of p66 *in vitro*, and a 90kDa model Pol protein in an inducible bacterial expression system (Sluis-Cremer et al. 2004; Tachedjian et al. 2005a). It has recently been demonstrated that NNRTIs enhance Gag-Pol dimerization, resulting in premature PR activation and a decrease in viral particle release (Figueiredo et al. 2006). In HIV-1 transfected cells, EFV, TMC120 and TMC125 increased Gag and Gag-Pol processing, and caused up to 45% decrease in viral particle production. Similar effects were not observed for NNRTIs that do not significantly enhance p66 homodimerization and NRTIs (Figueiredo et al. 2006). Hence, NNRTIs that are potent enhancers of RT dimerization also affect the late stage of viral replication, which represents a novel inhibitory mechanism for these drugs. However, the concentrations required to mediate this effect are two to three orders of magnitude higher then concentrations that block RT function. This is likely due to reduced binding affinity of the NNRTIs to the proposed target which is the RT domain of Gag-Pol. Strategies to identify drugs that are more potent

inhibitors of this late stage in the viral life-cycle could be identified by screening for molecules that enhance Gag-Pol dimerization. Such a screen could be enhanced by incorporating mutations in the RT that are known to confer decreased susceptibility to current NNRTIs in order to select for drugs that have the potential to block NNRTI resistant strains of HIV-1.

## Peptide Based Inhibitors of RT dimerization

Two strategies have been utilised to generate peptides designed to target the RT dimer interface in order to block HIV-1 RT function, including peptides corresponding to regions that are known to have an important role in RT dimerization (Debyser and De Clercq, 1996; Depollier et al. 2005; Divita et al. 1994; Morris et al. 1999b; Morris et al. 1999a). The most successful of these peptides, Pep-7, corresponds to RT residues 395–404, derived from the highly conserved tryptophan repeat motif (W398–W414) (Depollier et al. 2005). Pep-7 interacts with p51, and destablizes both the RT heterodimer and the p66 homodimer. Similar to unconventional NNRTIs, Pep-7 is unable to induce RT dissociation (Depollier et al. 2005). Pep-7 based peptides are potent suppressors of HIV-1 replication at noncytotoxic concentrations (Morris et al. 1999b). The method of inhibition in HIV-1 infected cells has not been elucidated. However, given that Pep-7 cannot induce RT subunit dissociation, it has been suggested that it acts at the late stage of virus replication by preventing the formation of an active RT heterodimer (Morris et al. 1999b).

Rational strategies utilising the available RT structures to direct the design and manufacture of mimetic peptides targeting subunit interaction is a recent development (Campbell et al. 2002; Hosokawa et al. 2004). These studies have led to the synthesis of a peptide, TLMA2993, which also targets the RT connection subdomain. TLMA2993 inhibits RT activity at micromolar concentrations (Campbell et al. 2002). Cells stably transfected with this peptide are protected from HIV-1 infection in a concentration dependant manner due to inhibition of reverse transcription, as observed by a decrease in HIV-1 DNA (Hosokawa et al. 2004).

## Targeting the IN Domain in Gag-Pol

IN catalyses the insertion of viral DNA into the host chromosome and thus inhibits an early crucial step in the virus life cycle. IN is also implicated in reverse transcription, nuclear import of the pre-integration complex, viral assembly and budding (Engelman et al. 1995; Hehl et al. 2004). Despite the numerous roles of IN in HIV-1 replication, new approaches for inhibiting viral replication have focused on targeting the catalytic activity of IN that is required for proviral DNA integration. Two IN inhibitors, MK-0518 and GS-9137 (JTK-303) have entered clinical trials (Cotelle, 2006; Makhija, 2006) and have shown efficacy in Phase III clinical trials (Stephenson, 2007).

Since IN is expressed as part of Gag-Pol, agents that bind to the IN domain in this polyprotein are likely to impact on the late stages of replication. Consistent with this notion, mutations in IN have been reported to effect virion formation (Shin et al. 1994). Truncations of IN at the C-terminus of Gag-Pol result in aberrant virion core structures, with a reduction in the overall levels of cell-associated viral Gag, suggesting a defect in Gag-Pol processing (Engelman et al. 1995; Bukovsky and Gottlinger, 1996).

IN requires oligomerization for activity. Therefore, inhibitors of IN function that mediate their effects through negating IN subunit interactions are also likely to interfere with viral assembly. This would be manifested by interfering with Gag-Pol/Gag-Pol interactions leading to subsequent effects on HIV-1 PR activation (Muriaux et al. 2004). In this regard peptide inhibitors of IN dimerization have been reported, however their effects on the late stages of the virus life-cycle remains to be determined (Maroun et al. 2001; Zhao et al. 2003).

Certain host cell factors are incorporated into the virion by interaction with the IN domain of Gag-Pol. A cellular factor that has been implicated in affecting the late stage of the virus life cycle is integrase interactor 1 (INI1). INI1 was identified in a yeast two-hybrid screen for host cell proteins interacting with HIV-1 IN (Yung et al. 2001;Yung et al. 2004; Kalpana et al. 1994). INI1 mutants that abrogate interaction with IN or cells deficient in INI1 exhibit a substantial reduction in viral production (Yung et al. 2001). INI1 affects several steps during HIV-1 replication (Ariumi et al. 2006; Sorin et al. 2006; Yung et al. 2001) and is also packaged into HIV-1 particles (Kalpana et al. 1994). A 110-amino-acid fragment of INI1 (S6) with a minimal IN-interaction domain inhibits viral production (Yung et al. 2001; Yung et al. 2004). The inhibitory effect of S6 on HIV-1 production is mediated by

binding of the ectopically expressed S6 to the Gag-Pol embedded IN. Furthermore, stable expression of a transdominant S6 mutant inhibits infection in T-cells. S6 represents a potential lead for the development of inhibitors of the late stage of HIV-1 replication (Yung et al. 2001).

## Proteosome Inhibitors

Intracellular degradation of misfolded, damaged or unwanted proteins is mediated by the proteosome, which is a multisubunit proteolytic complex of 26S (Schubert et al. 2000). Proteins are tagged for proteolytic destruction by the covalent attachment of a chain of ubiquitin polypeptides on lysine residues of the protein (Schubert et al. 2000). Proteosome inhibitors inhibit the late stages of the HIV-1 life-cycle by interfering with viral particle release and maturation (Schubert et al. 2000). Decreased budding has also been demonstrated for retroviruses expressing the PPPY- or PTAP containing late domains but not those that use the YPDL type late domain (Schubert et al. 2000; Ott et al. 2003). The effect is not dependent on the viral particle assembly site (i.e. cytoplasm or plasma membrane) or on monoubiquitination of Gag (Ott et al. 2003). In addition to a decrease in viral particle release (4-fold), virions released from cells treated with proteosome inhibitors have approximately a 10-fold decrease in infectivity (Schubert et al. 2000). The impact of proteosome inhibitors is dependent on an active HIV-1 PR and the presence of the p6 late domain but is independent of Vpu function. Inhibition of HIV-1 maturation and budding is observed with reversible (zLLL also known as MG-132) and irreversible (lactocystin) proteosome inhibitors (Schubert et al. 2000).

Proteosome inhibitors also interfere with the activity of the HIV-1 viral infectivity factor (Vif) on the antiviral function of apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) in virus producing cells (Stopak et al. 2003; Sheehy et al. 2003; Mehle et al. 2004; Yu et al. 2003). Wild-type viruses expressing Vif are able to prevent incorporation of APOBEC3G into the virion by promoting its degradation in the cytoplasm of the producer cell. Inhibition of APOBEC3G incorporation in the virus prevents hypermutation in newly synthesized viral DNA following infection of target cells due to C to U modifications during minus stand DNA synthesis mediated by APOBEC3G. Proteosome inhibitors interfere with Vif dependent degradation of APOBEC3G suggesting that these inhibitors can impede the mechanisms used by the virus to evade the innate defences of the host cell (Stopak et al. 2003; Sheehy et al. 2003; Mehle et al. 2004; Yu et al. 2003).

Targeting an essential cellular process like the proteosome is anticipated to be cytotoxic and not well tolerated *in vivo*. Nevertheless, the highly specific proteosome inhibitor epoxomicin, which also inhibits HIV-1 maturation, is well tolerated in mice (Meng et al. 1999). The proteosome inhibitor, PS-341, is approved as a last resort treatment of multiple myelomas and is associated with adverse effects (Kane et al. 2006). The use of proteosome inhibitors in HIV-1 infected individuals needs to be considered in the context of the potential risk benefit and the net effect on inhibition of HIV-1 replication as proteosome inhibitors also enhance the early step of the virus life-cycle by preventing degradation of the reverse transcription complex mediated by TRIM5α (Schwartz et al. 1998; Wu et al. 2006; Wei et al. 2005).

## Targeting HIV-1 Egress Mediated by Vpu

The HIV-1 accessory protein, Vpu is a 16 kDa type 1 integral membrane protein that is indispensable for viral pathogenesis (Li et al. 2005). Vpu plays two distinctive roles in the viral life-cycle that include down regulating host cell CD4 receptors (Willey et al. 1992) and enhancing viral particle release from the cell surface, the latter associated with its ion channel forming properties (Schubert et al. 1996a). Vpu is unique to HIV-1/SIVcpz viruses (Binette and Cohen, 2004). Interestingly the two closely related retroviruses HIV-2 and SIV, which lack Vpu, are less pathogenic (Bour and Strebel, 2003).

The role of Vpu in the viral budding process is coupled to its ion channel forming properties, which is predicted to be a pentameric structure composed of five transmembrane domains (Grice et al. 1997). The Vpu ion channel is thought to function by altering the electric potential at the plasma membrane or alternatively by overcoming host restriction factors for viral release (Neil et al. 2006). The HIV-1 Vpu is a member of viral ion channel proteins called viroporins and is structurally similar to the M2 ion channel protein of influenza (Gonzalez and Carrasco, 2003; Hout et al. 2006). An interesting feature of Vpu is its role in

viral particle release from nondividing cells such as macrophages (Deora and Ratner, 2001). In this regard the rate of host cell proliferation is a determining factor for Vpu mediated viral particle release (Deora and Ratner, 2001).

Analogues of amiloride (a sodium channel blocker) inhibit Vpu ion channel activity. The amiloride analogues, 5-(N,N-hexamethylene) amiloride (HMA) and 5-(N,N-dimethyl)amiloride (DMA) inhibit Vpu mediated virus budding and viral replication in macrophages (Ewart et al. 2002; Ewart et al. 2004). The inhibitory effects are observed in the absence of cytotoxicity. Both analogs exhibit strong inhibition of HIV-1 replication as measured by viral p24 levels in culture supernatants (Ewart et al. 2004). HMA at 4 μM suppresses viral p24 in culture supernatants to undetectable levels for more than 10 days in culture (Ewart et al. 2004). While amiloride analogues demonstrate activity in macrophages, they fail to inhibit HIV-1 replication in T-cells (Ewart et al. 2002). Nevertheless, Vpu ion channel inhibitors have the potential for use in combination therapy, targeting viral reservoirs and drug-resistant variants. An amiloride derivative, BIT225, is currently being pursued for drug development (Biotron Limited, Sydney, NSW, Australia). BIT225 represents a promising antiretroviral therapeutic although the evaluation of its *in vivo* efficacy will present a challenge since inhibition of HIV-1 replication appears to be restricted to nonproliferating cells.

Recent studies also suggest that rimantadine, an ion channel blocker of influenza A viruses, can be a useful lead compound for designing Vpu inhibitors. Rimantadine and amantadine belong to class of polycyclic amines that are active against the M2 ion channel of influenza A but not against HIV-1 Vpu (Hout et al. 2006). Studies indicate that mutating histidine at residue 19 to alanine results in a rimantadine sensitive Vpu ion channel demonstrating the potential of this class of inhibitor as HIV-1 ion channel blockers (Hout et al. 2006).

Vpu is also implicated to interact with certain host cell restriction factors that interfere with viral particle egress. The host cell protein, Vpu-binding protein (UBP), is suggested to be a negative factor for virus assembly (Callahan et al. 1998; Bour and Strebel, 2003). UBP is a 34-kDa protein that exhibits competitive binding with Vpu and Gag (Callahan et al. 1998; Handley et al. 2001). Overexpression of UBP has been reported to significantly suppress viral particle release suggesting that UBP is a negative factor that requires displacement by Gag or Vpu (Callahan et al. 1998). It is suggested that Vpu mediates its effect on viral egress by facilitating membrane targeting of Gag precursors (Handley et al. 2001; Harila et al. 2006; Neil et al. 2006). Supporting this notion, Vpu-defective particles appear in internal membrane-bound compartments suggesting a Gag targeting defect (Klimkait et al. 1990).

Another host cell restriction factor implicated in viral release is an acid-sensitive potassium channel-forming protein, TASK-1, which is down regulated during viral infection (Hsu et al. 2004). Due to the structural homology of TASK-1 and HIV-1 Vpu, TASK-1 has been suggested to form hetero-oligomers with Vpu which interferes with both TASK-1 mediated conductance and Vpu ion channel function (Hsu et al. 2004). Further delineation of the how these host cell factors interact with Vpu is required in order to design small molecule inhibitors that inhibit viral particle release.

## Targeting HIV-1 Egress Mediated by the p6 Late Domain

In the recent years, major advances have been made in our understanding of how HIV-1 and other retroviruses are released from infected cells. In the early 1990s it was reported that mutations in the p6 late domain inhibit virion particle release (Gottlinger et al. 1991). Moreover, a PTAP sequence, encompassing amino acids 7 to 10 of p6, is critical for virus particle production (Huang et al. 1995). The PTAP motif binds specifically to the host cell protein, tumor suppressor gene 101 (TSG101), resulting in the recruitment of components of the endosomal sorting complex required for transport-I (ESCRT-I) (VerPlank et al. 2001; Martin-Serrano et al. 2001; Garrus et al. 2001; Demirov et al. 2002). Deletion of the PTAP motif results in approximately 80% reduction in HIV-1 particle release. Similarly, overexpression and silencing of TSG101 abolish viral egress (Garrus et al. 2001; Demirov et al. 2002; Goila-Gaur et al. 2003).

NMR structure analysis of a 14 amino acid peptide derived from p6 encompassing the PTAP motif complexed with the UEV domain of TSG101 has revealed that the PTAP motif binds to a groove in TSG101. This binding creates two main pockets: the "A-P" pocket through contact of amino acids 7–10 and the "P" pocket through the binding of amino acids 9–10 of the peptide (Pornillos et al.

2002a; Pornillos et al. 2002b). Interruption of this viral host protein-protein interaction with compounds that bind to this pocket in TSG101 and compete with Gag would potentially inhibit viral particle release and the rate of cell-cell HIV-1 transmission (Bieniasz, 2006).

So far there has been one report that describes the synthesis and selection of small molecules with up to five fold better binding capacity than a peptide that contains the sequence of the wild type PTAP motif in the L domain of HIV-1 (Liu et al. 2006). In this study, the authors describe an approach previously employed to obtain peptoids in which a key proline residue is substituted with glycine at the N-terminus of the parent Pro-rich sequence in order to improve binding specificity to Src homology 3 (SH3) domains (Nguyen et al. 2000). Similarly, the proline rich PTAP domain in p6 was considered a good candidate for the N-substituted glycine residue approach. In this study, the Tsg101 binding affinity ($K_d$) of the 9-mer wild-type PTAP containing peptide was 50 μM (Liu et al. 2006). Binding constants for the highest affinity peptoid-hydrazones (designated 11q and 11p) were 17.5 and 9.8 μM, respectively. The highest affinity peptoid hydrazone was found to be the *n*-butyl-containing 11p peptide, with a five-fold increase in binding affinity compared to the wild-type 9-mer PTAP peptide. The capacity of these peptides to compete with HIV-1 Gag for TSG101 binding and their effect on HIV-1 egress *in vitro* and *in vivo* remains to be determined.

Although disruption of viral-host cell interactions is a very attractive approach to abolish virion release and infectivity, interfering with the host cell machinery could have major consequences for the host (Bieniasz, 2006). TSG101 is a multifunctional protein and plays a critical role in cell proliferation as shown by studies conducted in TSG101 deficient mice (Ruland et al. 2001). TSG101 is involved in cellular transcription and plays a central role in endosomal sorting of cargo protein that is destined for degradation by the proteasome. TSG101 is recruited to the endosomal sorting pathway by a specific interaction with the host cell protein, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which binds to TSG101 through a PSAP motif (Lu et al. 2003). Disruption of this interaction inhibits delivery of epidermal growth factor receptor (EGFR) to the late endosomes. In recruiting TSG101, it is believed that HIV-1 mimics Hrs in order to enter the endosomal sorting pathway and negotiate its release from the infected cell (Pornillos et al. 2003). Thus, targeting TSG101 would result in the accumulation of proteins at the plasma membrane and the disruption of protein sorting within the infected cell. Specific inhibition of HIV-1 budding by targeting the late domain binding site on TSG101 will require preferential inhibition of p6 binding compared to Hrs.

## Conclusion

Considerable progress has been made in understanding the late steps of the viral life-cycle leading to the production of infectious viral particles. Many of these processes rely on protein:protein interactions either between viral proteins or viral proteins and host cell factors. The interactions between the host and viral proteins have either a role in facilitating virus replication, as is observed for the viral p6 and Tsg101, or are necessary to overcome negative effects of the host on virus replication, as mediated by Vif. HIV-1 also relies on posttranslational modifications mediated by the host cell machinery in order for viral polyproteins to be trafficked to the appropriate compartment of the cell for viral assembly and budding. Arguably, one of the most effective drugs used to treat HIV-1 infected individuals are the HIV-1 PR inhibitors that block viral maturation. This underscores the effectiveness of targeting the late stage of virus replication. Nevertheless, while some of the strategies described in this review inhibit virus replication in cell culture assays, they require improvements in potency, specificity and delivery before being viable for use in the clinic.

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