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Emerging Drug Targets for Antiretroviral Therapy

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Abstract

Current targets for antiretroviral therapy (ART) include the viral enzymes reverse transcriptase and protease. The use of a combination of inhibitors targeting these enzymes can reduce viral load for a prolonged period and delay disease progression. However, complications of ART, including the emergence of viruses resistant to current drugs, are driving the development of new antiretroviral agents targeting not only the reverse transcriptase and protease enzymes but novel targets as well. Indeed, enfuvirtide, an inhibitor targeting the viral envelope protein (Env) was recently approved for use in combination therapy in individuals not responding to current antiretroviral regimens.

Emerging drug targets for ART include: (i) inhibitors that directly or indirectly target Env; (ii) the HIV enzyme integrase; and (iii) inhibitors of maturation that target the substrate of the protease enzyme. Env mediates entry of HIV into target cells via a multistep process that presents three distinct targets for inhibition by viral and cellular-specific agents. First, attachment of virions to the cell surface via nonspecific interactions and CD4 binding can be blocked by inhibitors that include cyanovirin-N, cyclotriazadisulfonamide analogues, PRO 2000, TNX 355 and PRO 542. In addition, BMS 806 can block CD4-induced conformational changes. Secondly, Env interactions with the co-receptor molecules can be targeted by CCR5 antagonists including SCH-D, maraviroc (UK 427857) and aplaviroc (GW 873140), and the CXCR4 antagonist AMD 070. Thirdly, fusion of viral and cellular membranes can be inhibited by peptides such as enfuvirtide and tifuvirtide (T 1249). The development of entry inhibitors has been rapid, with an increasing number entering clinical trials. Moreover, some entry inhibitors are also being evaluated as candidate microbicides to prevent mucosal transmission of HIV.

The integrase enzyme facilitates the integration of viral DNA into the host cell genome. The uniqueness and specificity of this reaction makes integrase an attractive drug target. However, integrase inhibitors have been slow to reach clinical development, although recent contenders, including L 870810, show promise. Inhibitors that target viral maturation via a unique mode of action, such as PA 457, also have potential. In addition, recent advances in our understanding of cellular pathways involved in the life cycle of HIV have also identified novel targets that may have potential for future antiretroviral intervention, including interactions between the cellular proteins APOBEC3G and TSG101, and the viral proteins Vif and p6, respectively.

In summary, a number of antiretroviral agents in development make HIV entry, integration and maturation emerging drug targets. A multifaceted approach to ART, using combinations of inhibitors that target different steps of the viral life cycle, has the best potential for long-term control of HIV infection. Furthermore, the development of microbicides targeting HIV holds promise for reducing HIV transmission events.

There are an estimated 38 million individuals infected by HIV worldwide, of whom an estimated 1.6 million reside in high-income countries and the majority in developing nations (UNAIDS, the Joint United Nations Programme on HIV/AIDS, estimates to the end of 2003).[1] In 2003, an estimated 5 million individuals were newly infected, more than in any previous year.[1] With no prospect for an effective vaccine or cure in sight, containment of the spread of HIV relies on measures to prevent transmission and treatment relies on antiretroviral therapy (ART).

Antiretroviral agents were first approved for use in HIV-infected individuals in 1987, within 3 years of the discovery of HIV as the causative agent of AIDS. ART aims to reduce viral load, providing symptomatic relief, delaying destruction of the immune system and consequently delaying disease progression. The first antiretroviral agent to be approved, zidovudine, is a nucleoside reverse transcriptase inhibitor (NRTI) that indirectly targets the viral enzyme reverse transcriptase. Subsequently, six other NRTIs have been approved, with more under development.[2] Two other groups within the reverse transcriptase inhibitor class of antiretrovirals have also been approved, currently comprising three non-nucleoside reverse transcriptase inhibitors (NNRTIs) and one nucleotide reverse transcriptase inhibitor (reviewed by Ruane and DeJesus[3] and Balzarini[4]). The viral enzyme protease is also a target for ART, with eight approved protease inhibitors (PIs) and more in development.[5,6]

Currently, highly active antiretroviral therapy (HAART) employs combinations of protease and reverse transcriptase inhibitors, and can reduce viral load to below detectable levels for prolonged periods.[7-10] This has markedly improved survival times for HIV-infected individuals and has reduced AIDS mortality rates, despite an increase in the number of HIV infections.[1,11] However, HAART can be complicated by considerable adverse effects, difficult drug regimens, substantial cost and the emergence of drug-resistant viruses. Thus, HAART may not ultimately be 'highly active' and the term 'HAART' is losing favour in preference to 'ART'.

Drug resistance is becoming increasingly problematic, with drug-resistant viruses being involved in transmission and some individuals harbouring viruses that are resistant to a number of different drugs.[12-15] The development of virus variants resistant to one inhibitor can often confer cross-resistance to other inhibitors within the same class. Thus, although numerous anti-HIV drugs are approved, an increasing number of people are left with little or no options of new therapeutics that can be added to their drug regimen and to which their virus will be fully sensitive. This highlights the need for the development of new antiretroviral agents targeting not only reverse transcriptase and protease but other viral targets as well. Indeed, a fusion inhibitor targeting the entry of HIV into cells (enfuvirtide; see section 1.1.3) was recently approved for use in combination therapy together with an optimised ART background regimen for individuals with multidrug-resistant viruses (reviewed by Greenberg et al.[16]).

The life cycle of HIV presents numerous potential targets for antiretroviral intervention, besides the reverse transcriptase and protease enzymes (see figure 1). Promising emerging targets include distinct stages of the HIV entry pathway as well as HIV integration and maturation. A number of entry inhibitors are in clinical and preclinical trials, as described in section 1.1, including inhibitors that aim to prevent HIV transmission between individuals. In addition, clinical trials are currently in progress with inhibitors targeting HIV integration and maturation

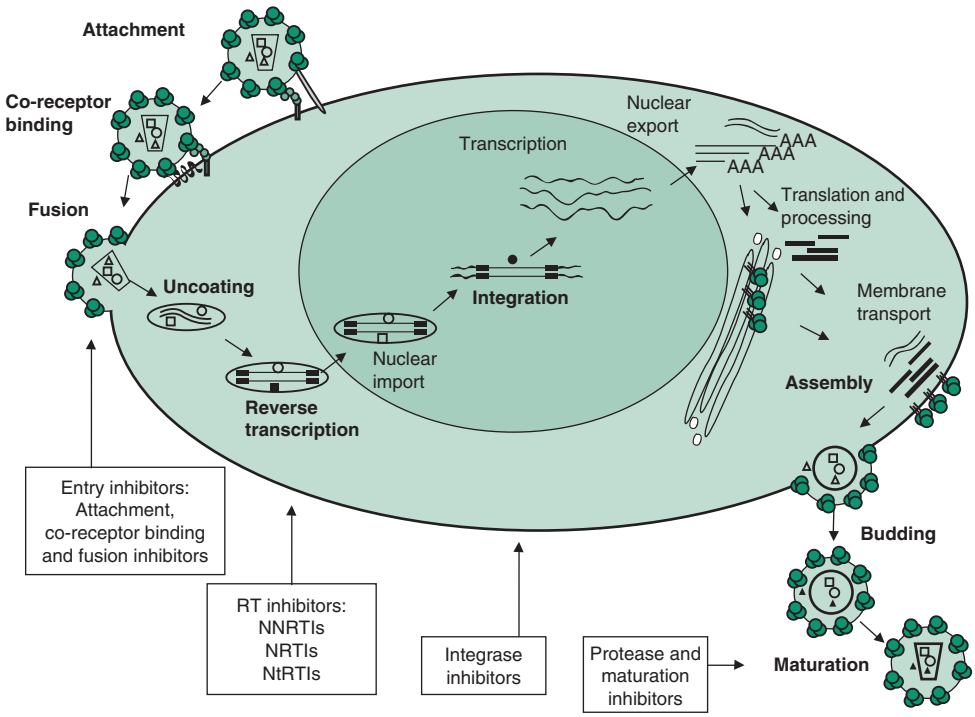

Fig. 1. The life cycle of HIV presents several targets for inhibition. Steps of the HIV life cycle targeted by approved antiretrovirals are fusion, reverse transcription and maturation. Emerging targets are attachment, receptor binding and maturation integration. Classes of antiretroviral agents targeting these steps are listed in the boxed text. Steps of the HIV life cycle that are potential drug targets, and are discussed in this review, are uncoating, assembly and budding. The viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR) are represented symbolically as squares, circles and triangles, respectively. Filled symbols indicate the step of the viral life cycle that these enzymes promote. **NNRTI** = non-nucleoside RT inhibitor; **NRTI** = nucleoside RT inhibitor; **NtRTI** = nucleotide RT inhibitor.

(see sections 1.2 and 1.3). Furthermore, recent progress in our understanding of cellular factors and pathways involved in HIV infection and replication also present numerous potential targets for ART (see section 2).

1. Emerging Targets

HIV entry into susceptible cells, the integration of HIV DNA into the host cell genome and virion maturation currently represent promising emerging targets for ART.[17-23] In addition, inhibitors that target entry are being developed as candidate microbicides to prevent the transmission of HIV between individuals.[17-23]

1.1 Entry

The envelope protein (Env) on the surface of virions mediates entry of HIV into target cells[24,25] (figure 2). Env is comprised of an outer protein, glycoprotein (gp)120, and a transmembrane protein, gp41, which are assembled as trimers of heterodimers on the surface of virions. Virions can attach to target cells in a relatively nonspecific manner following Env interactions with cell surface molecules, such as heparan sulfate proteoglycans (electrostatic) or dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN; carbohydrate binding) on some cell types.[26,27] Env then mediates entry following specific, sequential interactions with its cell surface receptor CD4 and a co-receptor molecule, primarily the chemokine receptors CCR5 or CXCR4[28,29] (figure 2). First, gp120 binds to CD4,

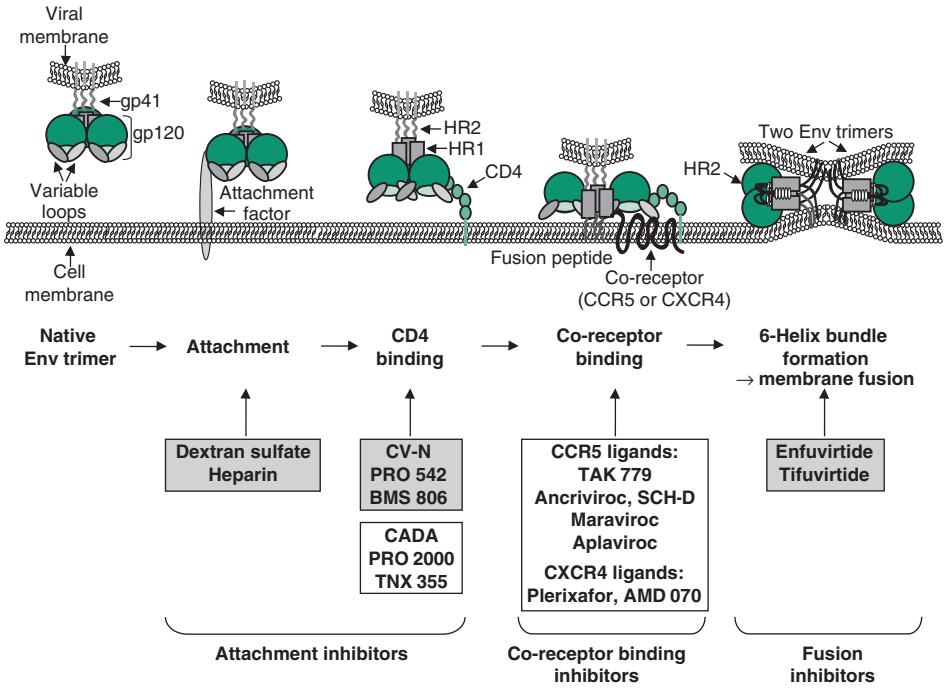

Fig. 2. HIV entry into target cells is mediated by the envelope protein (Env), comprised of glycoprotein (gp) 120 and gp41 subunits that assemble as trimers on the surface of virions. HIV can attach to target cells via nonspecific interactions with cellular attachment factors and specific interactions with the cell surface receptor CD4. CD4 binding induces conformational changes in Env that include exposure of a co-receptor binding site in gp120. Env can then interact with a co-receptor molecule, usually CCR5 or CXCR4, which induces further conformational changes in Env, probably resulting in fusion peptide insertion into the target cell membrane and the subsequent formation of a 6-helix bundle following interaction of the heptad repeat (HR) 1 and HR2 domains in three gp41 subunits (two gp120 subunits shown for clarity). 6-Helix bundle formation by a number of Env trimers is thought to cooperatively mediate fusion of viral and cellular membranes. This entry process presents three distinct steps for inhibition: cell surface attachment, co-receptor binding and fusion. Inhibitors of these steps that are discussed in this review, targeting Env (shaded boxes) and cell surface receptors (unshaded boxes), are indicated (model adapted from Moore and Doms,[17] with permission; © 2003 National Academy of Sciences, U.S.A.). **CADA** = cyclotriazadisulfonamide analogues; **CV-N** = cyanovirin N.

triggering structural rearrangements in Env that allow the formation or exposure of a co-receptor binding site. Then gp120 interacts with CCR5 or CXCR4 co-receptors. Co-receptor binding results in further structural rearrangements that probably include insertion of the hydrophobic fusion peptide, at the amino-terminus of gp41, into the target cell membrane and formation of a hairpin-like fold in each gp41 subunit following interaction of the two heptad repeat (HR) regions, HR1 and HR2. The HR1 regions in each gp41 subunit interact to form a central coiled-coil rod and the HR2 regions fold over and pack into grooves formed between the HR1 coils, creating a six-helix bundle in the context of an Env trimer.[30,31] Bundle formation acts to bring the viral and cell membranes into juxtaposition and creates pores in the target cell membrane.[32] Ensuing conformational changes in a number of Env trimers are likely to cooperatively mediate fusion of viral and cellular membranes,[33] allowing the viral core entry into the cytoplasm of the target cell.

The steps of this entry process present three distinct targets for inhibition: (i) attachment of virions to the cell surface via nonspecific interactions and CD4 binding; (ii) co-receptor binding; and (iii) fusion (figure 2). An obvious advantage of targeting entry is that infection of new target cells can be prevented. However, a number of practical implications for entry inhibition need to be considered, including co-receptor tropism (discussed 1.1.2), and

the fact that both viral and cellular factors can markedly impact entry inhibition.[34-36] As such, cellular factors, which include differences in co-receptor density, can markedly impact the susceptibility of a given virus to some entry inhibitors.[35,37] Additionally, the susceptibility of different inhibitor-naive viruses to a specific entry inhibitor can vary by as much as 3 logs based on determinations of the concentration of inhibitor that produces 50% inhibition (IC_{50}) values, which is reflective of Env being the most variable HIV protein.[34,38,39] In contrast, susceptibility to reverse transcriptase inhibitors and PIs usually differs by only a few-fold.[40] These issues indicate that viral phenotyping for co-receptor tropism as well as baseline susceptibility to prospective entry inhibitors could aid in the appropriate inhibitor selection.

The considerable variability and plasticity of Env, coupled with the remarkable ability of HIV to adapt to selective pressures, means that as for other inhibitors the selection of entry inhibitor resistant variants will be inevitable. However, the ability of enfuvirtide therapy to reduce viral load for a prolonged period in some individuals (see section 1.1.3) validates entry as a viable target for inhibition.

1.1.1 Attachment Inhibitors

HIV attachment inhibitors comprise agents that interact with the virus or the target cell to block virion absorption/attachment or Env-receptor interactions. Attachment inhibitors can be subdivided into those with nonspecific or specific modes of action.

Nonspecific Attachment Inhibitors

Inhibitors of HIV adsorption/attachment to the cell surface include soluble polyanions, such as dextran sulfate and heparin, that target basic regions of gp120 to block cell surface binding and co-receptor interactions.[41,42] Cyanovirin-N (Cellegy Pharmaceuticals), an 11 kDa protein derived from the cyanobacterium (blue-green algae) *Nostoc ellipsosporum*, is another example of an absorption/attachment inhibitor with a different mechanism of action. Cyanovirin-N blocks infection of diverse HIV strains at low nanomolar concentrations by interacting with certain high mannose carbohydrate residues on gp120 to interfere with CD4 and co-receptor interactions.[43-46] These types of inhibitors are candidate topical microbicides[21,22,47] (see section 1.1.4).

Specific Attachment Inhibitors

Specific inhibitors of CD4 binding can also be categorised under attachment inhibitors. CD4 binding is an attractive target for inhibition as all strains of HIV interact with CD4 as a primary receptor. HIV stains have been adapted *in vitro*, however, that can bypass the requirement of CD4 binding and can mediate entry via direct interactions with a co-receptor molecule[48-52] (reviewed by Bhattacharya et al.[53]). Furthermore, primary HIV-2 isolates have been identified that can utilise CCR5 or CXCR4 directly for CD4-independent infection.[54,55] Therefore, acquisition of a CD4-independent phenotype is a possible escape mechanism from inhibitors targeting CD4-binding. CD4 independence has the potential to broaden the target cell population susceptible to infection. However, CD4-independent infection is usually less efficient than infection via CD4 and CD4-independent viruses are more susceptible to neutralisation.[56,57]

Inhibitors directly targeting CD4 binding include compounds that down-modulate CD4, ligands to CD4 (both of which have possible ramifications for immune function) and agents that bind to Env to prevent Env-CD4 binding or functional Env-CD4 interactions. Compounds that specifically down-modulate CD4 from the cell surface include cyclotriazadisulfonamide (CADA) analogues, thought to act at a (post)translational step, which block HIV infection at low micromolar concentrations[58,59] (reviewed by Vermeire et al.[60,61]). Naphthalene sulfonate polymers target Env-CD4 interactions via a different mechanism to prevent HIV entry. PRO 2000 (Indevus Pharmaceuticals, Inc.) binds CD4 with nanomolar affinity, exhibits potent anti-HIV activity and is currently in trials as a promising candidate microbicide (see section 1.1.4).[62]

TNX 355 (Hu5A8; Tanox, Inc.) is a humanised IgG4 CD4-specific monoclonal antibody (MAb) which binds to domain 2 of CD4 at a site distinct from Env and major histocompatibility complex

proteins, and potently inhibits HIV infection *in vitro*.[63] TNX 355 does not prevent Env-CD4 binding but may act by inhibiting CD4-induced conformational changes.[64,65] Preliminary trials in HIV-infected patients revealed that a single infusion of TNX 355 at 10 or 25 mg/kg could reduce viral load by >1 log at 2–3 weeks post-infusion.[66] A transient dose-dependent increase in CD4+ T cells was also observed, possibly due to mobilisation from lymphoid tissues.[66] A phase II trial is in progress. The clinical application of TNX 355 may be constrained by the requirement of infusion. As such, issues including patient acceptability and convenience may limit the utilisation of TNX 355 to individuals with few other treatment options.

PRO 542 (Progenics Pharmaceuticals, Inc.) is a recombinant tetrameric CD4-IgG2 fusion protein in which the Fv portions of human IgG2 heavy and light chains have been replaced with domains 1 and 2 of human CD4.[67] PRO 542 targets the CD4 binding site on Env and exhibits broad and potent activity *in vitro*.[67,68] Phase I/II trials have demonstrated good tolerability and viral load reductions in HIV-infected adults and children infused with a single or four weekly doses at 10 or 25 mg/kg.[69-71] A phase II trial is currently underway. The requirement of intravenous administration may limit the clinical application of PRO 542; however, it is being considered for salvage therapy in patients with advanced disease.[69]

A small molecule inhibitor that binds to gp120 and blocks functional Env-CD4 interactions was recently described by researchers at Bristol-Myers Squibb.[72,73] BMS 806 (BMS 378806) was originally reported to inhibit gp120-CD4 binding;[72,73] however, further studies by Sodroski and colleagues[74,75] indicate that, rather than preventing CD4 binding, BMS 806 interferes with CD4-induced conformational changes in Env that create/expose HR1 epitopes in gp41 required for HR2 binding and subsequently fusion. BMS 806 has potent antiviral activity (nanomolar range) against many clade B HIV-1 isolates but reduced, poor or no activity against viruses from other clades.[73] Furthermore, as with other HIV inhibitors, resistance mutations can be rapidly selected *in vitro*. However, oral bioavailability and good pharmacological properties in animal models make BMS 806 the first representative of a promising novel class of HIV inhibitors.[73,76]

1.1.2 Co-receptor-Binding Inhibitors

Following virus attachment/adsorption to the cell surface and CD4 binding, Env then interacts with a co-receptor molecule, typically CCR5 and/or CXCR4, to mediate infection.[28,29] CCR5 using viruses (R5 tropic) are involved in HIV transmission, highlighted by the fact that individuals who do not express CCR5 on the surface of their cells, due to a 32 base pair deletion in both copies of their *ccr5* gene (*ccr5Δ32* homozygotes), are highly resistant to HIV infection.[77-80] Later on in infection, approximately 20–50% of individuals acquire viruses that can use CXCR4 in addition to CCR5 (dual tropic R5/X4 or mixed R5 and X4), and a small percentage of individuals acquire viruses that use CXCR4 but not CCR5 (X4 tropic).[81,82] Acquisition of R5/X4, R5 and X4, or X4 virus correlates with, but is not a prerequisite for, disease progression.[81,82]

Inhibitors of co-receptor interactions represent a second broad category of entry inhibitors that include proteins, small molecules and antibodies. These co-receptor-binding inhibitors interact either with CCR5, CXCR4 or Env to prevent functional Env-co-receptor interactions. The potential of blocking co-receptor binding to inhibit HIV infection was realised soon after the discovery that CXCR4 could mediate entry of T-cell tropic HIV strains and that these viruses could be blocked by a CXCR4 specific MAb.[51,83] Indeed, C-C chemokines had previously been shown to exhibit antiviral activity and led to the rapid identification of CCR5 as the other major HIV co-receptor.[84-92] The chemokines macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4 and RANTES (Regulated on Activation, Normal T Expressed and Secreted)/CCL5 bind to CCR5, and stromal cell-derived factor-1 alpha (SDF-1) binds CXCR4 to prevent infection of R5 and X4 tropic viruses, respectively.[86,93,94] Chemically modified chemokine derivatives have been developed with more potent

HIV inhibitory activity, such as the CCR5 ligand aminooxypentane (AOP)-RANTES which efficiently downregulates CCR5 from the cell surface.[95,96]

Inhibitors of CCR5 Binding

CCR5 is an attractive drug target as *ccr5Δ32* homozygote individuals exhibit few consequences of being effectively CCR5-negative. The utility of antibody and chemokine derivatives as HIV therapeutics is likely to be limited because of a lack of oral bioavailability, production costs, etc., and orally bioavailable small-molecule co-receptor ligands[23,97,98] currently have the most therapeutic potential as co-receptor-binding inhibitors.

TAK 779 (Takeda Chemical Industries, Ltd) was the first identified small molecule CCR5 antagonist that inhibited R5 virus infection at nanomolar concentrations; however, it lacked oral bioavailability and is not a clinical candidate.[99] TAK 779 binds in a cavity between the transmembrane helices of CCR5.[100]

Ancriviroc (Schering C, SCH-C, SCH 351125; Schering-Plough Corp.) is an oxime-piperidine CCR5 antagonist that is also thought to bind between the transmembrane helices and inhibit ligand binding by altering the conformation of the extracellular domains.[101,102] Ancriviroc is potently active against diverse R5 isolates (IC_{50} values in the low nanomolar range), can be delivered orally and has good pharmacological properties in animal models.[102] In addition, ancriviroc has been shown to act synergistically with other HIV inhibitors, including a PI, NRTIs and enfuvirtide.[103] In phase I trials, viral load reductions of >0.5 log were observed in 10 of 12 volunteers.[104] Ancriviroc was found to be generally well tolerated, although prolongation of corrected QT intervals (QTc interval; the time between depolarisation and repolarisation of the heart muscle adjusted for heart rate) at high doses raised concerns.[104] Ancriviroc is no longer under clinical development and has been superseded by SCH-D (Schering D, SCH 417690; Schering-Plough Corp.), a piperazine-based CCR5 antagonist that exhibits increased antiviral potency without the cardiac effects noted with ancriviroc.[105,106] SCH-D has been shown to be well tolerated and active in phase I clinical trials.[105] Phase II trials are underway and phase III trials are planned.

GlaxoSmithKline are developing a spirodiketopiperazine-based CCR5 antagonist, aplaviroc (GW 873140, AK 602, ONO 4128), which preserves CC-chemokine interactions.[107] Aplaviroc is active against R5 strains at low nanomolar concentrations and is currently in phase II clinical development.[107] Aplaviroc is orally available, can reduce viral load by >1 log and preliminary results indicate that it is well tolerated.[108] Phase III trials are planned.

Another CCR5 inhibitor with good potential is being developed by Pfizer. Maraviroc (UK 427857, MVC) is potently active against diverse HIV-1 isolates from different clades[109] and does not compete with chemokine binding.[109] *In vitro* escape mutants have been difficult to generate and maintained continued CCR5 use.[110] Maraviroc is currently in clinical trials and has been shown to be well tolerated and exhibit potent antiviral activity following short-term monotherapy in HIV-infected patients.[111] Pfizer is currently the furthest along with the clinical development of a CCR5 binding inhibitor, with maraviroc in phase II/III trials.

Concerns about the use of CCR5 inhibitors include the potential for immune modulation and for viruses to escape by selecting for X4 tropism, which is associated with disease progression.[81,82] *In vitro* studies have shown that R5 viruses can indeed adapt to use CXCR4 to escape inhibitors targeting CCR5 binding.[112] However, another mechanism of escape involves continued use of CCR5 via altered Env-CCR5 interactions.[113,114] The use of a CCR5 inhibitor in macaques experimentally infected with R5 and X4 viruses rapidly reduced circulating R5 virus and resulted in a rapid but transient increase in circulating X4 virus in two of three animals that was not sustained.[115] Analysis of virus from an individual receiving short-term maraviroc therapy with no change in viral load revealed the presence of R5 and X4 tropic virus at the onset of treatment.[116] Circulating R5 virus was suppressed during treatment but returned to the dominant circulating species after the cessation of treatment.[116] The long-term effects of

CCR5 inhibitor use on co-receptor tropism, viral load and HIV pathogenesis are currently unknown. Therefore, evaluation of the clinical efficacy of CCR5 inhibitors will require careful monitoring of co-receptor tropism (discussed in the section on Considerations for the Use of Co-receptor-Binding Inhibitors), as well as monitoring of other viral and immunological parameters.

Inhibitors of CXCR4 Binding

CXCR4 is also a potential target for antiretroviral intervention.[23,117] CXCR4 and SDF-1 represent a monogamous receptor-chemokine couple;[93,94] therefore, there is concern that inhibitors interfering with this relationship may have adverse consequences. This is underscored by the fact that SDF-1 or CXCR4 knock-out mice have developmental defects and are non-viable.[118,119]

A number of CXCR4 ligands that block HIV infection have been identified (reviewed by Schols[117] and De Clercq[120]). Plerixafor (AMD 3100; AnorMED, Inc.) is a bicyclam compound first shown to inhibit T-cell tropic HIV strains before the identification of co-receptors.[121] Plerixafor was subsequently shown to be a small-molecule CXCR4 antagonist that potently blocks infection of X4 viruses.[122-124] Inhibition of X4 (but not R5) viruses was observed in phase I clinical trials following infusion of plerixafor, as was dose-dependent leukocytosis that was attributed to CXCR4 antagonism.[125,126] In addition, the use of plerixafor in individuals harbouring circulating R5 and X4 or R5/X4 viruses selected for R5 circulating viruses.[126,127] Development of plerixafor for HIV therapy has been halted because of adverse effects and the lack of oral absorption. A follow-up compound, AMD 070 (AMD 11070; AnorMED, Inc.), is an orally available CXCR4 antagonist with potent anti-HIV activity that is currently being evaluated in clinical trials.[128,129] As for plerixafor, a dose-dependent increase in white blood cell count has been observed for which the long-term consequences are unknown.[129] AMD 070 administration was generally well tolerated in phase I trials and phase Ib/IIa trials are planned for HIV-infected individuals.[129] AMD 070 has shown additive or synergistic activity in combination with enfuvirtide, NRTIs and a PI *in vitro*.[128]

Considerations for the Use of Co-receptor-Binding Inhibitors

Implications specific for the use of co-receptor binding inhibitors include that viral phenotyping, or possibly genotyping, for co-receptor tropism will almost certainly be required. For example, an individual harbouring R5 virus would need to be treated with a CCR5-, not CXCR4-, specific inhibitor to reduce viral load. Furthermore, continued monitoring of viral co-receptor tropism is likely to be indicated to screen for inhibitor escape via selection of change in co-receptor use, which has the potential to impact pathogenesis. This scenario could potentially be avoided by the use of a combination of CCR5 and CXCR4 inhibitors, although there is the possibility that viruses could adapt to utilise one of a number of alternative co-receptors. Some HIV-1 strains and many HIV-2 isolates can use alternative co-receptors, in addition to CCR5 and/or CXCR4, for infection *in vitro*.[29,54] The role of these alternative receptors for infection and pathogenesis *in vivo* is currently unclear but likely to be minor; however, the use of CCR5 and CXCR4 inhibitors could potentially select a more important role for these receptors *in vivo*.

Evaluation of the clinical efficacy of co-receptor inhibitors raises novel concerns for ART. The utility of co-receptor-binding inhibitors, as well as the best approach to selection, treatment and monitoring of co-receptor use will obviously have to be determined through clinical trials.

1.1.3 Fusion Inhibitors

Fusion inhibitors represent the first class of entry inhibitors for which there is an approved drug. Enfuvirtide (Fuzeon® [1], T 20; Trimeris Inc., Roche Laboratories) was approved early in 2003 for use in combination with other antiretrovirals in treatment-experienced individuals with evidence of HIV-1 replication despite ongoing ART (reviewed by Greenberg et al.[16]). Enfuvirtide is administered by

1 The use of trade names is for product identification purposes only and does not imply endorsement.

subcutaneous injection of 90mg twice daily and, apart from the occurrence of injection site reactions, is generally well tolerated. Administration of enfuvirtide using a needle-free injection device is under investigation.[130] Enfuvirtide is a 36-amino acid peptide based on the sequence of the HR2 region of gp41. As such, enfuvirtide binds to HR1, preventing HR2 binding and subsequent conformational changes required for fusion.[131] The HR1-enfuvirtide binding site becomes accessible to enfuvirtide following Env interaction with CD4 and/or co-receptor, and is lost following HR1-HR2 interaction; thus, enfuvirtide targets a structural fusion intermediate conformation of Env.[75,132,133] Enfuvirtide has also been reported to interact with gp120, at the base the V3 loop of X4 and R5/X4 viruses, to inhibit CXCR4 binding.[134]

Addition of enfuvirtide to an optimised background regimen of reverse transcriptase and PIs can reduce viral load by >1 log for a prolong period.[135,136] Viral rebound is associated with the acquisition of mutations at amino acid 36–45 in the HR1 enfuvirtide-binding site region of gp41 (reviewed by Miller and Hazuda[137] and Greenberg and Cammack[138]). HR1 mutations reduce enfuvirtide binding efficiency, increasing the amount of peptide required for inhibition.[139] Amino acid changes in HR2 have also been noted and may represent functional compensatory mutations.[140-142]

Besides HR1 amino acids, sequences in the V3 loop as well as the co-receptor binding site of gp120 that alter Env-co-receptor binding affinity can also influence enfuvirtide susceptibility *in vitro*.[35,36,38,39] Mechanistically, mutations that reduce co-receptor binding affinity confer delayed Env fusion kinetics, increasing the temporal window for enfuvirtide binding and thus enhancing enfuvirtide susceptibility.[35,36] It will be interesting to see whether changes in gp120 that can affect enfuvirtide susceptibility will also be selected *in vivo*.[35,36,38,39,134]

Cessation of enfuvirtide therapy can result in the rapid selection of viruses with wild-type HR1 sequences, supporting *in vitro* observations that HR1 mutant viruses exhibit reduced fitness.[37,140,143-145] Interestingly, a recent report has described an enfuvirtide-dependent HIV variant isolated from a patient on enfuvirtide therapy.[141] This variant virus has mutations in HR1 and HR2, and in the absence of enfuvirtide is proposed to undergo receptor-induced conformational changes too readily, resulting in a non-functional Env.[141]

Enfuvirtide-resistant viruses can remain sensitive to inhibitors targeting CD4 and co-receptor binding *in vitro* as well to a second fusion inhibitor tifuvirtide (T 1249; Trimeris, Inc.) *in vitro* and *in vivo*.[37,146,147] Furthermore, synergy has been observed *in vitro* between enfuvirtide and other entry inhibitors targeting CD4 or co-receptor binding.[103,148,149]

Tifuvirtide is a 39-amino acid peptide based on a combination of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) HR2 sequences that correspond to a region overlapping that of enfuvirtide.[150] As for enfuvirtide, tifuvirtide requires subcutaneous administration and, apart from the occurrence of injection site reactions, is generally well tolerated.[136,150] As with enfuvirtide, tifuvirtide therapy can select for resistance mutations in HR1.[150] Tifuvirtide can inhibit HIV-2 as well as HIV-1 isolates and is more potent than enfuvirtide, which may correlate with a shifted binding site in HR1 that covers a highly conserved deep hydrophobic pocket.[36,37,146] However, despite greater potency, the ability to inhibit HIV-2 infection and activity against enfuvirtide resistant strains, the development of tifuvirtide has been halted because of formulation difficulties.

Other peptide-based and small-molecule inhibitors of fusion can target gp41 (reviewed by Root and Steger[151]), but these are not currently in clinical development. Small-molecule inhibitors, with their reduced production costs and oral availability, hold the most promise for widespread use.

1.1.4 Use of Entry Inhibitors to Prevent HIV Transmission

Heterosexual transmission accounts for the majority of HIV infections worldwide. In the absence of condom use, microbicides represent a promising and inexpensive alternative to reduce transmission rates.[152,153] Besides reverse transcriptase inhibitors

and virucidal compounds such as detergents, a number of agents that target HIV entry are being investigated as potential microbicidal agents to prevent the mucosal transmission of HIV.[21,22,47] These include small molecules, antibodies and proteins that interfere with virus attachment and various steps of the entry process. Inhibitors that interfere with virus attachment to dendritic cells and thus dendritic cell-mediated transport of virus to draining lymph nodes also need to be considered.[22,154,155]

Cyanovirin-N and sulfated polymers, such as PRO 2000 (see section 1.1.1), can protect against intra-vaginal and rectal SHIV (chimera between SIV and HIV) challenge in nonhuman primates.[156-158] Phase I and II clinical trials have found vaginal PRO 2000 gel to be generally well tolerated[159,160] and phase II/III trials are planned.

Specific inhibitors of receptor interactions have also been shown to be protective against mucosal transmission in animal models, including the small molecule CCR5 ligand CMPD 167 (Merck; no longer in development) and the amino-terminally modified RANTES analogue, PSC-RANTES.[161,162] The broadly neutralising MAb IgGb12 that interferes with CD4 binding can also protect against mucosal transmission.[163,164] In a model of transmission using cervical tissue *ex vivo*, a combination of IgGb12 and PRO 542 could protect against localised mucosal infection as well as uptake and transmission of virus by migratory cells.[155]

1.2 Integration

Joining of the viral genome and the host's chromosomal DNA (cDNA) is the hallmark of a retroviral infection. This step of the retroviral life cycle is catalysed by the integrase enzyme and is termed integration. Integrase, expressed as part of the *gag-pol* transcript is structurally and biochemically closely related to the DNA transposase family of enzymes (reviewed by Haren et al.[165]). Like reverse transcriptase, integrase is a promising therapeutic target because of the uniqueness of its enzymatic activity. However, unlike reverse transcriptase, this drug target remains clinically unutilised even though extensive drug development has occurred.[18,166] Mechanistically, integrase has two activities: 3′-processing and strand transfer. 3′-processing removes the terminal 2 nucleotides proximal to each 3′-end of the proviral cDNA genome. The recessed 3′ ends of this substrate then act as nucleophiles in the strand transfer reaction, attacking the host cDNA and resulting in the insertion of the proviral cDNA into the host chromosome. The insertion of the viral cDNA creates single-strand nicks, which are then presumably repaired by the host DNA repair system.[167,168] It is becoming increasingly apparent that integrase does not act alone during this process. Indeed, many host proteins in addition to other viral proteins, including matrix, nucleocapsid, reverse transcriptase and vpr, form the pre-integration complex (PIC) immediately after uncoating of the capsid.[169-171] The PIC in HIV-1 contains factors that allow nuclear localisation, and may provide some level of specificity for the selection of integration sites within the host genome and enhancement of activity.[172,173] The host proteins high mobility group A1 (HMGA1) and barrier-to-autointegration factor (BAF) present in HIV-1 PICS and have been reported to stimulate *in vitro* activity of integrase between 10- and 500-fold, respectively.[172,174,175] Thus, two distinct targets exist for interrupting integration: (i) inhibiting the activity of the integrase enzyme directly through conventional small molecule inhibitors; or (ii) designing agents that block the formation of the PIC, which may block nuclear entry and/or greatly decrease the activity of integrase. This second potential target is analogous to the NNRTIs in that antiviral agents directed toward non-catalytic surfaces of integrase may still be efficacious and, more importantly, exhibit unique drug-resistance profiles.

The more accessible of these two targets is the direct inhibition of integrase activity by small-molecule inhibitors. Merck Research Laboratories has developed a series of anti-integrase agents that specifically block the strand transfer activity of integrase. These agents share a common 'drug core' consisting of a diketo carboxylate typified by L 731988 that directly interferes with the divalent metal-binding active site of integrase.[176,177] These

inhibitors display IC_{50} values in the low micromolar range, have high oral bioavailability and low toxicological profiles.[178] Like drugs directed toward the other retroviral enzymes, reverse transcriptase and protease, resistance mutations to integrase inhibitors develop during extended drug exposure. As expected, these mutations cluster near the catalytic residues of integrase (D66, D166 and E152).[179] However, Hazuda and coworkers[178] have recently reported a new, structurally and mechanistically related class of integrase inhibitors that substitute a hydroxy-naphthyridine-carboxamide (L 870810 and L 870812) core for the diketo carboxylate. This class of inhibitors displays similar efficacy profiles and oral bioavailability, and have shown promising activities (3–4 log decrease in viral load and a 1–2 log increase in CD4+ T cells) when administered in a SHIV Rhesus macaque model.[178] L 870810 is in early clinical trials. Importantly, the hydroxy-naphthyridine-carboxamide class exhibits a different resistance profile to the diketo carboxylate class of drugs, which would allow for modified anti-integrase drug regimens to continue after resistance develops to one class of inhibitors.[177] Next to reverse transcriptase, protease and entry inhibitors, integrase inhibitors represent the next class of antiretroviral agents most likely to be utilised for the treatment of HIV-1 infection.

1.3 Maturation

PIs, a core component of HAART, prevent the essential proteolytic processing of the Gag and Gag-Pol polyproteins. This processing leads to the structural maturation of the virus particle and the activation of the viral enzymes concomitant with viral budding. Resistance to PIs occurs as a consequence of continued viral replication in the presence of drug. Therefore, alternative drug leads that target viral maturation are needed to blunt current protease drug-resistant viruses. PA 457, a derivative of betulinic acid developed by Panacos Pharmaceuticals, uniquely blocks viral maturation.[180,181] Unlike conventional PIs that act at the enzyme active site, PA 457 appears to act on the substrate of protease, specifically preventing cleavage between the junction of capsid (CA) and spacer peptide 1 (SP1), thus blocking conversion of the HIV capsid precursor, CA-SP1 (p25) to the mature capsid protein (p24).[182] In an *in vitro* virus replication assay PA 457 displayed an IC_{50} of 10.3 nmol/L, comparable with an IC_{50} of 8.8 nmol/L for the PI indinavir.[181] More importantly, PA 457 retained its potency against isolates resistant to approved reverse transcriptase and PIs.[181] PA 457 escape mutants develop an A to V amino acid substitution at the CA-SP1 cleavage site. Interestingly, this mutation was previously reported to reduce viral fitness.[183] PA 457 is currently in phase II clinical testing and has been granted fast-track review status by the US FDA.

2. Potential Drug Targets

2.1 Innate Antivirals

2.1.1 Tripartite Motif (TRIM)-5α

It was noted more than 30 years ago that certain mouse cell lines were resistant to infection by the Friend murine leukaemia virus (reviewed by Stoye[184]). This resistance was traced to a single dominant gene locus, *Fv1*. A resistance phenotype was linked to residue 110 of the capsid protein and it was hypothesised that *Fv1* was preventing, through an unknown mechanism, the uncoating of the viral core post-entry, thereby blocking integration.[185,186] Interestingly, *Fv1* was identified as the capsid protein of an endogenous retrovirus residing within the genome of resistant mouse cells.[187] More recent reports have indicated the presence of other 'intrinsic' antiretroviral factors, *Ref1* and *Lv1*, within human and non-human primate cells, some with the ability to block HIV-1 infection (*Lv1*) [reviewed by Bieniasz[188]]. Similar to *Fv1*, *Ref1* was identified in human cells as a restrictor of murine leukaemia virus (MLV) infection and had the same capsid-dependent escape phenotype.[186] *Lv1* was identified in non-human primates as a potent restrictor of HIV-1 infection.[189,190]

Very recently, Sodroski and colleagues[191] utilised a genetic selection strategy to identify the

Rhesus macaque gene product encoded by *Lv1* as tripartite motif (TRIM)-5α, responsible for the restriction of HIV infection in old-world monkey cells. TRIM proteins contain RING domains, B-boxes and coiled-coil motifs, have unknown functions and reside within cytoplasmic bodies.[192] There are 37 identified TRIM family members within the human genome. Interestingly, several laboratories have shown that, unlike Rhesus macaque TRIM5α, human TRIM5α does not block HIV-1 infection. However, human TRIM5α can convey resistance to certain MLV strains in a fashion identical to the *Ref1* phenotype. Indeed, the *Ref1* antiretroviral resistance factor is the human homologue of TRIM5α.[193-195] It has been conjectured that species-specific TRIM5α may act in a similar fashion to *Fv1* by preventing uncoating of the capsid or, alternatively, sequestering incoming viral cores in discrete cytoplasmic compartments, thereby preventing nuclear entry and integration.[191] It remains to be determined if variants of TRIM5α may have therapeutic utility for HIV infection.

2.1.2 APOBEC3g

When the *vif* accessory gene of HIV-1 is deleted, the virus cannot replicate *in vivo*.[196,197] However, HIV-1-lacking *vif* (HIV-1Δ*vif*) is able to replicate in many tissue culture cell lines.[196,198,199] Furthermore, when HIV-1Δ*vif* permissive and non-permissive cells were fused, the non-permissive phenotype was dominant.[200] This indicated the presence of a Vif repressible restriction factor in non-permissive cells.

In 2002, Malim and coworkers[201] determined that Vif interacted with a host cell gene product called APOBEC3G, the apolipoprotein B messenger RNA-editing enzyme catalytic polypeptide-like 3G, a cytidine deaminase.[201] A salient feature of APOBEC3G is that it is incorporated into budding viruses in a nucleocapsid-dependent manner.[202,203] Thus, APOBEC3G cannot prevent initial infection, only subsequent spread. As the name suggests, APOBEC3G acts on nascent reverse transcripts, converting cytosine residues in the minus strand of DNA to uracil.[204-207] Thus, G to A lethal mutations are induced in the viral genome, blocking transmission. Vif induces the demise of APOBEC3G by triggering ubiquitination and subsequent degradation of the protein.[208,209] Yu and colleagues[209] recently reported that Vif simultaneously interacts with APOBEC3G and the cul5-SCF ubiquitin ligase complex to initiate the degradation cascade via the proteosome. Importantly, Vif function was traced to a highly conserved motif responsible for interaction with the cul5-SCF complex, and dominant negative inhibitors of cul5-SCF greatly decreased HIV-1 infectivity in the presence of vif and APOBEC3G.[209]

Small-molecule inhibitors directed toward the Vif-APOBEC3G interaction may prevent degradation of APOBEC3G and thus promote its incorporation into nascent virions.

2.2 HIV Assembly and vpu

Viral assembly and budding remains a significant, yet unexploited target for antiretroviral therapy. The p6 domain of HIV Gag contains a highly conserved PTAP motif critical for virus release. This motif interacts with the host protein TSG101, which then recruits host machinery to catalyse viral budding.[210,211] Proteins involved in endosomal sorting and the formation of multi-vesicular bodies (ESCRT-I, II, III, AIP1 and VPS4) perform critical roles in this process.[210,212-215] In addition to the PTAP motif, ubiquitination of P6 probably plays a significant role in the process.[216-218] Depletion of TSG101, expression of dominant negative forms of VPS4 or treatment with proteosome inhibitors reduce viral infectivity significantly. Unfortunately, these existing *in vitro* anti-budding treatments are not practical *in vivo* because of significant cellular toxicity and/or technical hurdles. However, as yet undeveloped small-molecule inhibitors of the TSG101-p6 interaction may prove valuable in controlling virus release.

Other viral genes besides *gag* play an important role in virus budding. The Vpu accessory protein of HIV-1, although not a component of virions, enhances virus release from many human cells, but has no effect on particle production within simian cell lines.[219-221] Recently, it was shown that heterokaryons of HeLa and Cos-7 cells were restrictive for

HIV-1Δ*vpu* budding.[222] As in the *vif* situation, this effect was overcome by the restoration of *vpu* accessory gene boosting viral release in heterokaryons 9-fold compared with the HIV-1Δ*vpu* virus.[222] Spearman and colleagues[222] speculate that an unknown cellular inhibitor may block Gag trafficking through the vesicular sorting pathways in the absence of Vpu.

3. Conclusions

Emerging targets for ART include various stages of the HIV entry process, the viral enzyme integrase and, with the development of inhibitors with novel mechanisms of action, maturation. Inhibitors of these novel targets are most likely to be fully active against viruses containing resistant mutations to current drugs and, thus, as for enfuvirtide, will present new treatment options for individuals with multidrug-resistant viruses. Therefore, inhibitors against new targets will complement and diversify current treatment options, and may act additively or synergistically with other inhibitors. Entry inhibitors are also being developed as microbicides to prevent HIV transmission.

The success of enfuvirtide therapy has validated entry as a target for inhibition. The development of entry inhibitors for ART has the advantage of preventing infection of new cells that could add to the viral reservoir. Additionally, the distinct stages of the entry pathway mean that escape from a given entry inhibitor will not necessarily confer class resistance. Indeed, enfuvirtide-resistant viruses can remain sensitive to inhibitors targeting attachment, co-receptor binding and fusion.[37] In addition, entry inhibitors with different targets have the potential to act in concert to potently reduce viral load *in vivo*, while minimising the incidence of escape. However, entry inhibition raises a number of unique points and concerns that require consideration.

Env is the most variable HIV protein and the susceptibility of different inhibitor-naive viruses to a specific entry inhibitor can vary considerably. Thus, the determination of baseline susceptibility to specific entry inhibitors may be appropriate. The plasticity of Env confers the potential to adapt readily to selective pressure. As seen with enfuvirtide and for other antiretrovirals, resistance mutations will occur. However, resistance to specific entry inhibitors has the potential to change viral tropism as well as pathogenicity. Furthermore, entry inhibitors targeting cellular attachment factors or receptors have the capability to interfere with normal cellular and immunological functions. Implications specific for the use of co-receptor-binding inhibitors include the fact that viral phenotyping for co-receptor tropism will almost certainly be required.

Inhibitors targeting integrase have the advantage that integration is a viral-specific event and inhibition of integration may be obtainable without adverse cellular consequences. Inhibitors targeting different steps of the integration process could also remain active against escape variants to a different target. Maturation inhibitors targeting the protease enzyme are an established component of HAART and the development of inhibitors with novel modes of action, such as targeting the substrate of protease, will complement current regimens.

In summary, the existence of viruses resistant to current antiretrovirals, and transmission of drug-resistant strains, means that the development of new drugs with novel targets is becoming increasingly urgent. Antiretroviral drug discovery and development is the focus of much research and a number of inhibitors to emerging targets are in preclinical and clinical trials. Investigational agents may provide virological, immunological and clinical benefits in patients with resistance to current drugs. Furthermore, future first-line HAART is likely to combine inhibitors that target multiple stages of the viral life cycle, which may provide better prospects for enhanced containment of viral replication. In addition, the development of topical microbicides holds promise for reducing HIV transmission rates.

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