Review

HIV protease as a target for retrovirus vector-mediated gene therapy

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Abstract

The dimeric aspartyl protease of HIV has been the subject of intense research for almost a decade. Knowledge of the substrate specificity and catalytic mechanism of this enzyme initially guided the development of several potent peptidomimetic small molecule inhibitors. More recently, the solution of the HIV protease structure led to the structure-based design of improved peptidomimetic and non-peptidomimetic antiviral compounds. Despite the qualified success of these inhibitors, the high mutation rate associated with RNA viruses continues to hamper the long-term clinical efficacy of HIV protease inhibitors. The dimeric nature of the viral protease has been conducive to the investigation of dominant-negative inhibitors of the enzyme. Some of these inhibitors are defective protease monomers that interact with functional monomers to form inactive protease heterodimers. An advantage of macromolecular inhibitors as compared to small-molecule inhibitors is the increased surface area of interaction between the inhibitor and the target gene product. Point mutations that preserve enzyme activity but confer resistance to small-molecule inhibitors are less likely to have an adverse effect on macromolecular interactions. The use of efficient retrovirus vectors has facilitated the delivery of these macromolecular inhibitors to primary human lymphocytes. The vector-transduced cells were less susceptible to HIV infection in vitro, and showed similar levels of protection compared to other macromolecular inhibitors of HIV replication, such as RevM10. These preliminary results encourage the further development of dominant-negative HIV protease inhibitors as a gene therapy-based antiviral strategy. © 2000 Elsevier Science B.V. All rights reserved.

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1. Overview

The widespread use of small-molecule human immunodeficiency virus (HIV) protease (PR) inhibitors has resulted in the first significant reduction in the number of acquired immune deficiency syndrome (AIDS)-related deaths since the beginning of the epidemic (see [1] and references within). The initial success of PR inhibitors validates the enzyme as a target for antiviral therapy, although the emergence of PR inhibitor-resistant virus strains threatens to circumvent the efficacy of these drugs. As pharmaceutical manufacturers design new peptidomimetic and non-peptidomimetic PR inhibitors aimed at reducing the incidence of resistance, many laboratories continue to explore alternative strategies for controlling HIV replication. Some of these strategies involve macromolecular inhibitors that target specific HIV gene

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products, including Tat, Rev, and gag. Of particular interest are macromolecular inhibitors of the viral PR which portend to combine a proven drug target with a novel mechanism of inhibition. In most cases, macromolecules cannot be delivered to cells as soluble agents. Instead, these antiviral agents must be expressed in cells from adopted genetic information, thus requiring gene therapy technology.

Gene therapy is the process of introducing an exogenous gene into the somatic or germline cells of an organism to confer a desired phenotype upon the cell. This process may be performed to correct a genetic deficiency, express a conditionally lethal gene product, or express a protein which affords protection against intracellular parasites or environmental insults. Gene therapy technology will, arguably, facilitate the next series of scientific breakthroughs contributing to the treatment of human diseases caused by both microorganisms and genetic defects. Gene therapy-based approaches for the treatment of severe combined immunodeficiency syndrome associated with inherited adenosine deaminase (ADA) deficiency [2,3] and cystic fibrosis [4-6], have already shown great promise. Preliminary results with gene therapy treatment for hemophilia (resulting from either Factor VIII or Factor IX deficiencies), thalassemias, and familial hypercholesterolemia are also encouraging (reviewed in [7,8]).

In view of the qualified success of small-molecule HIV PR inhibitors, the delivery of macromolecular HIV PR inhibitors using a gene therapy-based approach may represent a powerful new tool in the arsenal against AIDS. Inhibition of HIV polyprotein processing through the inactivation of the viral PR leads to the production of immature, non-infectious virions [9]. By creating defective PR monomers which interact with the wild-type PR monomers in an infected cell, inactive PR heterodimers are produced that cannot function in virus polyprotein processing [10]. These genetically encoded inhibitors can be used for so-called ‘intracellular immunization’ [11] of T-cells to provide protection against HIV infection. This survey is intended to be a review of the structure, function, and inhibition of HIV PR with emphasis on the specific inhibition of the enzyme by engineered macromolecules. In the instances where recent reviews have covered certain aspects of this subject, the reader will be directed to these articles.

2. HIV protease

2.1. Genome organization and protease function

The prototypic retrovirus contains three open reading frames (ORFs), gag, pol, and env [12]. The gag (an abbreviation for group-specific antigen) ORF encodes the structural proteins that ultimately form the virus capsid, the pol ORF encodes three essential retrovirus enzymes (protease (PR), reverse-transcriptase (RT), and integrase (IN)), and the env ORF encodes the envelope surface proteins (refer to [13] and references within). In addition to these basic retrovirus genes, the genome of HIV encodes a number of accessory proteins that modulate virus gene expression. Rev binds to the Rev-responsive element (RRE) in unspliced (or singly spliced) virus mRNAs and participates in their transport to the cytoplasm. This activity circumvents the cellular mechanisms that prevent the nuclear export of incompletely spliced RNAs ([14-17] and references within). Tat is a transcriptional activator that binds to the Tat activation responsive (Tar) RNA structure in the HIV promoter located in the 5' long terminal repeat (LTR) of the integrated virus genome. The roles of the Vpr, Vpu, Nef, and Vif genes in the virus life cycle are less certain and the requirement for these proteins depends upon the specific growth condition of the virus (i.e., passage in immortalized cells, infection of primary cells, inoculation of an animal, etc.) (see [18] and references within).

During viral gene expression, incompletely spliced mRNAs initiated from the 5' LTR promoter of the integrated HIV-1 genome encode the 55-kDa gag polypeptide. The pol gene products are translated only following a translational frame-shift, that results in the pol genes being shifted to the same ORF as that of gag. Ribosomal frame-shifting occurs during translation with a frequency of ~5%, ensuring that the gag structural gene products are produced in excess with respect to the virus-encoded enzymes, including the PR, which are required only in catalytic quantities [19,20]. Translation of the downstream genes requires mRNA splicing.

Following translation, the PR must mediate cleavage at its C-terminus between PR and RT [21]. This cleavage presumably occurs in trans. Precisely when the cleavage event at the N-terminus of the PR oc-
curs is less certain. Proteolytic processing of the gag polypeptide occurs within the context of a dense, immature virus core particle and is concomitant with budding. The PR may be delivered to the assembling core particle as a C-terminal extension of gag, avoiding the need for a separate mechanism for PR localization. According to this model, an assembling HIV core particle contains gag multimers along with relatively few gag-PR fusion proteins, which are sufficient to conduct the chorus of maturation cleavages in the budding core particle.

While the requirement for a frame-shift mutation for translation of pol gene products results in the production of low levels of PR during an infection, cells still appear to require more PR than is required for capsid maturation in vitro. This observation is based on the phenotypic effect of amino acid substitutions in HIV-1 PR that reduce catalytic activity without affecting substrate recognition. The catalytic constant ($k_{cat}$) of HIV-1 PR is decreased 4-fold when threonine-26 (Thr-26) is replaced by serine (T26S) and approximately 50-fold when Ala-28 is replaced by serine (A28S). These mutations have minimal effect on $K_m$ values. PR genes containing these mutations were cloned into a proviral vector for analysis of their effects on virion maturation and infectivity in vitro (the env gene was supplied in trans for these assays). The results showed that wt and T26S provirus vectors produced mature, infectious core particles with approximately equal efficiency, despite the 4-fold difference in PR activity. HIV provirus vectors encoding the A28S PR variant did not produce infectious core particles, although some proteolytic processing was detected. These observations suggested the existence of a minimum threshold level of PR activity (somewhere between 2 and 25% of normal levels), below which processing is insufficient to yield infectious particles. This finding further suggested that complete inhibition of the PR may not be required to produce a therapeutic effect in an infected individual [22].

The hypothesis that significant reductions in the catalytic efficiency of the PR do not drastically alter viral infectivity is confirmed by clinical data. Many of the point mutations present in rapidly emerging small-molecule PR inhibitor-resistant viruses have been identified and characterized. The PR genes of these viruses invariably contain mutations that result in decreased sensitivity to the inhibitors being administered. These mutations typically have an adverse effect on the catalytic efficiency of the enzymes [23,24]. For example, substitutions at amino acid residue 82, common in patients treated with ritonavir and indinavir, result in PRs with approximately 25% of wild-type (wt) activity (in terms of the specificity constant ($k_{cat}/K_m$)). Variant PRs with as little as 7% of wt activity are capable of viral replication [25], consistent with in vitro observations based on engineered mutations [22]. Despite this reduced efficiency, even multidrug-resistant viruses possess the fitness necessary for the productive infection of autologous cells (in the same patient) and heterologous cells (in a non-infected individual) [26]. Subsequent mutations may then be selected to increase the catalytic efficiency of the enzyme.

In order to compare the selective advantage of one PR to another, Erickson and colleagues have proposed the use of the expression ‘vitality’ to describe the selective advantage of a mutated PR compared to the wt PR. ‘Vitality’ is defined as $(K_i/k_{cat}/K_m)_{mutant}/(K_i/k_{cat}/K_m)_{wt}$, in which the typically higher $K_i$ value of the drug-resistant PR to a given inhibitor is multiplied by the typically lower catalytic efficiency of the PR variant (relative to wt kinetic parameters). The greater the vitality value, the higher the predicted selective advantage of the PR variant in vivo in the sustained presence of the small-molecule PR inhibitor [25]. Notably, Klabe and colleagues have found a low correlation between $K_i(k_{cat}/K_m)$ (relative enzyme resistance) and the IC$_{50}$(mutant)/IC$_{50}$(wt) (relative antiviral resistance). Furthermore, substituting ‘vitality’, which adjusts for the relative catalytic efficiency of the enzymes, instead of $K_i(k_{cat}/K_m)$, did not improve this correlation. These findings suggested that in vitro measurements of wt and variant HIV PR inhibition are not predictive of the in vivo phenotype of the corresponding viruses [27]. Clearly, there are other factors at work.

Studies involving the inhibition of HIV PR with dominant-negative inhibitors in cultured mammalian cells provided further evidence that a threshold level of HIV PR is required for virus infectivity (see also Section 2.3.4 below). These experiments demonstrated that the relationship between PR activity and virus infectivity was not linear [10]. Taken together with the in vitro studies of engineered PR
variants [22] and variants selected in vivo, the data suggest that: (1) wt PR activity is in excess in an infected cell; and that (2) reduction of PR activity below a non-zero threshold may essentially abolish the production of infectious virus particles. The postulated existence of this threshold level of activity for HIV infectivity is one feature that makes the enzyme an attractive target for inhibitors [10].

2.2. Structure

The single PR encoded in the genome of HIV-1 was initially classified in the aspartyl family based on homology to cellular aspartyl PRs, the inhibition of the enzyme with pepstatin, and inactivation by mutagenesis of the active site aspartate residues [28,29]. However, unlike the mammalian enzymes, X-ray diffraction data showed HIV PR to be an obligate homodimer (refer to Fig. 1). Each 99-amino acid monomer contributes one catalytic aspartate residue in the context of the sequence, Asp-25–Thr-26–Gly-27 [30,31]. The catalytic mechanism of HIV PR is dependent upon the presence of one protonated and one unprotonated aspartyl residue, similar to that of other studied aspartyl PRs. An ordered water molecule between Asp-25 and Asp-25' in the liganded dimer acts as the catalytic nucleophile [32–34].

The main dimer interface is formed by the N- and C-termini from each monomer interlocking to form a four-stranded, antiparallel β-sheet. The dimers also interact in the ‘flap’ region composed of dual two-stranded antiparallel β-sheets formed by residues 45–55 [30,31]. These flaps interact with each other and with substrate upon binding. The monomers become more compact upon substrate binding, with several regions of the molecule moving inward toward the bound ligand. The PR dimer loses symmetry following the binding of substrate, based on superimposition of the α-carbons of each monomer in the liganded and unliganded structures [35]. The presence of bound substrate also contributes to the stability of the HIV PR dimer through multiple interactions with both monomers. The result of these interactions is an extremely tight affinity between monomers in an enzyme–substrate complex (low
nanomolar range) that may depend greatly on ligand concentration in addition to salt concentration and pH [36]. The PRs of both HIV-1 and HIV-2 contain cysteine residues at position 95 (Cys-95) in the dimer interface region. The dimer interface regions of related retroviruses (e.g. simian immunodeficiency virus (SIV), Rous sarcoma virus (RSV) and feline immunodeficiency virus (FIV), and others) also contain cysteine or methionine residues. HIV-1 PR also has solvent-accessible cysteine residues at position 67 (Cys-67) which are highly conserved among virus isolates. While neither Cys-67 or Cys-95 have known catalytic roles, it has been proposed that these residues provide a mechanism of regulation for HIV PR activity in response to oxidative stress within the host cell [37,38]. Oxidative stress has been shown to stimulate HIV replication in vitro [39]. Experiments involving the selective oxidation of Cys-67 or Cys-95 with the sulphydryl reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or with glutathione have suggested a role for oxidative modification in regulating the activity of HIV PR [37,38]. However, there is currently no model which adequately explains these data. It is noteworthy that at least one class of irreversible small-molecule PR inhibitors, β-unsaturated ketones, reacts primarily with the cysteine residues of HIV-1, suggesting another potential means to inhibit PR activity [40,41].

2.3. Inhibition

Several small-molecule inhibitors of HIV-1 PR have been developed in recent years. All of the compounds in clinical use are competitive inhibitors which mimic the presence of the substrate in the active site of the PR dimer. Alternative approaches to PR inhibition include molecules that covalently modify the active-site aspartates as well or target the dimer-interface region of the PR. Finally, defective PR monomers that form inactive dimers with the wt monomers have been investigated for the ability to reduce virus replication.

2.3.1. Competitive small-molecule inhibitors

At present, five small molecule inhibitors, saquinavir (Hoffman-LaRoche), ritonavir (Abbott), indinavir (Merck), nelfinavir (Agouron), and amprenavir (Vertex/Glaxo-Wellcome) have US Food and Drug Administration (FDA) approval and are in clinical use. These PR inhibitors are typically the cornerstone of combination drug therapies which may also include reverse transcriptase inhibitors. Other compounds, including ABT-378 (Abbott), PNU-140690 (Pharmacia and Upjohn), DMP-450 (DuPont-Merck-Triangle Pharmaceuticals), and DMP-851 (DuPont-Merck) are at various stages of development. The synonyms for these compounds, sources, inhibition constants (Kᵢ), and molecular structures are shown in Table 1. The first available small-molecule PR inhibitor was saquinavir, marketed by Hoffman-LaRoche as Invirase. Saquinavir was arguably the first truly effective drug for the treatment of HIV infection in patients. The inhibitor is peptide-mimetic with respect to the Phe–Gly motif present at most HIV polyprotein cleavage sites [42]. The low bioavailability (~4% for a 600-mg dose) of saquinavir makes delivery of effective concentrations of the drug problematic. As with other early HIV PR inhibitors, saquinavir does not pass the blood–brain barrier. Left unchecked, HIV replication in the central nervous system may result in dementia [43,44].

Ritonavir was the second small-molecule PR inhibitor to be approved for use in the United States. Abbott Laboratories manufactured the peptidomimetic inhibitor as Norvir [45]. Indinavir (Crixivan), manufactured by Merck was the third FDA-approved HIV PR inhibitor and the first peptidomimetic transition state analog for HIV therapy [46]. Structure-based drug design was largely responsible for the next PR inhibitor, nelfinavir (Agouron Pharmaceuticals) [47]. Sold as Viracept, this compound is non-peptidomimetic in that it does not possess a substituted peptide-like bond intended to mimic the scissile peptide bond of a substrate polypeptide. Nelfinavir demonstrates synergistic effects on virus replication when combined with earlier peptidomimetic inhibitors [48] and is the first compound to cross the blood–brain barrier with reasonable efficiency. Vertex Laboratories also used structure-based design in developing amprenavir (sold as Agenerase), a non-peptidomimetic sulfonamide-based inhibitor which can be effective against viruses that are resistant to other PR inhibitors. The drug was recently approved by the FDA after only two years of devel-
Table 1
Current FDA-approved small-molecule HIV-1 PR inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Generic name (Trade name)</th>
<th>Source</th>
<th>$K_i$ (nM)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro-31-8959</td>
<td>saquinavir (Invirase)</td>
<td>Hoffman-La Roche</td>
<td>0.33(^2)-0.99(^1)</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>MK-639</td>
<td>indinavir (Crixivan)</td>
<td>Merck</td>
<td>0.40(^2)</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>ABT-538</td>
<td>ritonavir (Norvir)</td>
<td>Abbott</td>
<td>0.19(^2)</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>AG-1343</td>
<td>nelfinavir (Viracept)</td>
<td>Agouron</td>
<td>2.0(^1)</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>141W94, VX-478</td>
<td>amprenavir (Agenerase)</td>
<td>Glaxo-Welcomo/Vertex</td>
<td>0.11(^3)</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
</tbody>
</table>

References: \(^1\)[74], \(^2\)[146], \(^3\)[27,77].

opment, which relied heavily on three-dimensional computer-assisted design.

Another non-peptide, sulfonamide-based inhibitor with a $K_i$ of 10 nM (PNU-140690 (Pharmacia and Upjohn)) is in an earlier stage of development [49]. A C-2-symmetric inhibitor approximately 10-fold more effective than ritonavir is also in the early stages of clinical testing (ABT-378 (Abbott)) [50]. Additional inhibitors in early stages of development include: DMP-450 (DuPont-Merck-Triangle Pharmaceuticals), a symmetric cyclic urea non-peptide inhibitor with a $K_i$ of 10-20 nM [51] and DMP-851 (DuPont-Merck), an asymmetric cyclic urea inhibitor with a $K_i$ of approximately 20 pM. The pharmacokinetics and metabolism of these HIV PR inhibitors have recently been reviewed [52].

2.3.2. Irreversible small-molecule inhibitors

Following the initial clinical success of competitive small-molecule HIV PR inhibitors, a branch of research was aimed at the development of irreversible PR inhibitors. These drugs are an attractive alternative to competitive inhibitors as they lead to the permanent inactivation of the enzyme in an infected cell. This characteristic results in decreased dependence on high concentrations of inhibitors in the tissues and somewhat eschews the problem of low bioavailability. It was also possible that the irreversible inhibitors would be less sensitive to the development of resistance mutations.

An early epoxide inhibitor, EPNP (1,2-epoxy-3-(4-nitrophenoxy)propane) (Fig. 2A), had been shown to esterify one or both catalytic aspartate residues of
the prototypic aspartyl PR, pepsin [53]. The ability of EPNP to form a covalent adduct with bacterially-expressed HIV PR in a time-dependent manner was among the first evidence that a retroviral PR belonged to the aspartyl PR family [54]. Epoxides and β-unsaturated carbonyl groups were also tested as reactive functionalities using the haloperidol framework [40,41,55–57]. Haloperidol is an anti-psychotic drug identified in a structure-based DOCK [58] search as a potential inhibitor of HIV PR. The unmodified butyrophenone inhibits HIV-1 and HIV-2 PRs with IC$_{50}$ values of 125 and 140 μM, respectively. Epoxide and β-unsaturated carbonyl derivatives of haloperidol showed $K_{\text{inact}}$ (the inhibitor concentration resulting in half-maximal inactivation) values of 10–500 μM. The functional groups modified the active site aspartates as well as Cys-95 in the dimer interface [40]. An irreversible inhibitor based on the FMO motif, cyclic cis-1,2-disubstituted epoxide (Fig. 2B), inhibited HIV PR with a $K_{\text{inact}}$ of 65 μM [59]. Additional structures were also investigated as scaffolds for covalent inhibitors, including biliverdin, bilirubin, xanthobilirubin [60], curcumin, and boron derivatives of these compounds [61]. In general, all irreversible inhibitors tested suffered from at least one of several shortcomings, including: (1) low affinity of the scaffold for HIV PR; (2) a high level of toxicity in cell culture; and/or (3) functionalities which were too reactive and/or too easily metabolized in vivo.

In another study, an irreversible, pseudo-C-2-symmetric PR inhibitor with an epoxide functional group (Fig. 2C) demonstrated an IC$_{50}$ of 20 nM in tissue culture [62]. A sulfone derivative of this epoxide compound had an improved IC$_{50}$ of 6.6 nM against HIV-1 as assayed in transformed human CD4$^+$ lymphocytes [63] (Fig. 2D). The irreversible nature of PR inhibition was demonstrated by dialysis studies. It is uncertain whether these compounds will be pursued as therapeutic agents.

2.3.3. Dimer disrupters

An alternative to targeting the active site of HIV PR is to preclude the formation of the active site by preventing dimerization of the PR monomers. Several reports have described the inhibition of HIV PR activity by synthetic peptides corresponding to the N- and/or C-termini of the PR monomer [36, 64–66]. In the first report to include proof of the mechanism of inhibition, it was shown that the tetrapeptide Ac-Thr-96-Leu-97-Asn-98-Phe-99-COOH (corresponding to the four C-terminal residues of the enzyme, 96–99) demonstrated a $K_i$ of 45 μM. Sedimentation equilibrium experiments suggested association of the tetrapeptide inhibitor with PR monomers [36]. Another report showed that members of a panel of synthetic peptides corresponding to the N-terminus or C-terminus of HIV PR monomers were able to inhibit HIV-1 PR activity at high μM concentrations (IC$_{50}$). More interestingly, synthetic peptides representing both N- and C-termini separated by a flexible three-glycine linker (Fig. 2E) demonstrated IC$_{50}$ values of ~40 μM. While these inhibitors showed promise, inhibition was observed only in vitro when HIV-1 PR was refolded in the presence of the synthetic peptides at artificially low concentrations of NaCl [65].

The use of a C$_{18}$ alkyl-tethers between the peptide moieties of similar interfacial peptide inhibitors resulted in an inhibitor with an IC$_{50}$ of 4 μM [67]. Efforts were then made to improve the potency of the inhibitors by decreasing the flexibility of the alkyl tether; double or triple bonds were introduced to remove torsional degrees of freedom. Interestingly, increased rigidity tended to decrease the potency of the inhibitors, which could then be partially restored by lengthening the tether with additional polyethylene groups. This result suggested that rigidity, at least with respect to simple alkyl chains, may not be beneficial to the design of dimer interface inhibitors [68]. Increasing the hydrophilicity of the tether

Fig. 2. Inhibitors of HIV PR. (A) DPNP. (B) Irreversible FMO-based, cyclic cis-1,2-disubstituted epoxide inhibitor [59]. (C) Irreversible, pseudo-C-2-symmetric PR inhibitors with epoxide functional group [62]. (D) Sulfone derivative of C [63]. (E) Synthetic peptide representing two dimer interface domains [65]. (F) N-terminally cross-linked interfacial peptide which mimics the dimer interface of HIV PR [67]. (G) Schematic of the HIV PR dimer interface region (i) and the proposed mechanism of dimer disruption for the synthetic peptide (E) (ii), and the N-terminally crosslinked molecule (F) (iii). E can interact with a single terminus of one monomer (or of two monomers). F can interact with both termini of a single monomer. (H) Dominant-negative, macromolecular inhibitor harboring the active site mutation, D25N [10]. Arrows indicate the orientation of the amino acid chains (N→C).
A) \[
\text{O}_2\text{N}-\text{Ph}-\text{O}-\text{Ph}
\]

B) \[
\text{O}-\text{N}-\text{Ph}
\]

C) \[
\text{H}_2\text{N}-\text{N}-\text{Ph}-\text{N}-\text{Ph}-\text{N}-\text{Ph}
\]

D) \[
\text{O}-\text{N}-\text{Ph}-\text{SO}_2\text{N}
\]

E) \[
\text{NH}_3\text{-Pro}_1\text{-Gln}_2\text{-Ile}_3\text{-Thr}_4\text{-Leu}_5\text{-Gly}_6\text{-Cys}_7\text{-Thr}_8\text{-Leu}_9\text{-Asn}_{92}\text{-Phe}_{99}\text{-COOH}
\]

\[
\text{O}^\text{N}-\text{Pro}_1\text{-Gln}_2\text{-Ile}_3\text{-Thr}_4\text{-Leu}_5\text{-Trp}_6\text{-OH}
\]

\[
\text{O}^\text{NH-Ser}_{95}\text{-Thr}_{96}\text{-Leu}_{97}\text{-Asn}_{98}\text{-Phe}_{99}\text{-OH}
\]

G) (i), (ii), (iii)

H) WT WT ACTIVE HOMODIMER

WT D25N INACTIVE HETERO DIMER
also resulted in a less effective inhibitor [69]. A novel chemical synthesis approach has recently allowed the synthesis of N-terminally cross-linked interfacial peptides. The most effective inhibitor, shown in Fig. 2F, demonstrated an IC50 of 2 μM [67]. These molecules mimic the natural directionality of the terminal amino acid residues in the dimer interface of the PR (Fig. 2G). Methods for the delivery of these molecules to infected cells have not been investigated. The expression of short peptides is amenable to a gene therapy-based approach; however, N-terminally cross-linked peptides cannot be delivered genetically due to the synthetic nature of the tether.

2.3.4. Dominant-negative inhibitors

The intracellular expression of defective PR monomers to function as dominant-negative, macromolecular inhibitors represents an additional method of HIV PR inhibition (Fig. 2H). The effectiveness of such inhibitors was initially demonstrated in cultured mammalian cells [10]. The first dominant-negative inhibitors harbored single amino acid substitutions of the active-site aspartate residue 2(Asp-25→Asn or D25N). Transient transfection of mammalian cells with the wt HIV-1 provirus plasmid, pHIV-gpt [70], along with similar plasmids encoding the D25N mutation, resulted in decreased production of mature virus particles. These results were based on immunoassays using a polyclonal antibody to p24 which recognized mature p24 as well as unprocessed p55 and partially processed forms (e.g. p39) of the gag polypeptide. Transfection of an increasing amount of D25N-containing pHIVgpt with a constant amount of wild-type pHIVgpt resulted in decreased processing of p55 and core particle maturation. Additionally, cotransfection of a plasmid encoding the HIV env gene (pHIVgpt contains a drug selectable marker (gpt) in place of env) allowed the quantitation of infectious virus particles following transfection of pHIVgpt in the absence or presence of D25N PR [10]. These experiments suggested that a directly proportional relationship did not exist between the relative amount of gag processing, and also indicated that only a threshold level of PR activity was required for virus infectivity (see also, Section 2.1) [10,22]. Expression of defective PR monomers without upstream gag sequences resulted in little protection in transfection experiments, suggesting that delivery of the defective monomers to assembling HIV core particles relied upon the gag polypeptide (Rosé and Craik, unpublished results).

The use of molecular modeling led to the design of more 'potent' defective HIV PR monomers containing three amino acid changes D25K, G49W and I50W (abbreviated KWW, refer to Fig. 1). Energy minimization calculations (using SYBYL (Tripos, St. Louis)) suggested that the large, bulky side chain substitutions in the binding pocket may be able to minimize defective homodimer formation and promote heterodimer formation, thereby selectively interacting with wt monomers to inhibit virus activity. These molecules were more effective inhibitors than D25N in cultured mammalian cells. Notably, the inhibitory effect of the G49W and I50W substitutions was observed even when the catalytic aspartate was present at position 25, suggesting that the cryptophans hinder the accessibility of the PR's natural substrates to the active site [71]. Another study demonstrated the effectiveness of KWW in cultured human T-lymphocytes. Results from this latter study also suggested that mutagenesis of the gag-pol frameshift sequence (creating a continuous gag-pol ORF) improved the efficacy of the KWW inhibitors, presumably due to overproduction of the defective PR as part of a gag-pol fusion protein [72].

In vitro experiments have demonstrated the formation of HIV PR heterodimers, providing strong evidence for the mechanism of dominant-negative inhibition. In one such study, wt PR monomers, along with defective, histidine-tagged monomers (i.e. D25N or KWW), were separately expressed in *Escherichia coli*, purified, denatured, and renatured together at equimolar concentrations. Following isolation by nickel-chelate chromatography, PR dimers were analyzed by SDS-polyacrylamide gel electrophoresis. Both tagged defective monomers and non-tagged wt monomers were present in the imidazole-eluted column fractions. The presence of wt monomers in the bound material is compelling evidence for heterodimer formation [147]. Another study used the yeast two-hybrid assay to examine heterodimer formation in *Saccharomyces cerevisiae*, providing evidence that heterodimer formation can occur in eukaryotic cells [148].
2.3.5. Resistance to HIV protease inhibitors

Even before the clinical application of small-molecule PR inhibitors, data obtained in vitro suggested that mutations could arise in HIV that significantly reduced the susceptibility of the PR to inhibition [48,73–75]. These mutations also arise in patients, in some cases, rendering the small-molecule inhibitors ineffective [45,76–81]. Resistance presumably results from the high rate of RNA replication [82,83] combined with the high error rate of the viral RNA-dependent DNA polymerase (reverse transcriptase or RT) [84]. Several studies have described reduced levels of viral fitness resulting from small-molecule PR inhibitor-associated resistance mutations. These findings were initially interpreted to mean that the molecular genetic constraints imposed upon the viral PR (i.e. to allow proper biological function in the cell) may slow or prevent the adaptation of HIV beyond the emergence of attenuated, PR inhibitors-resistant variants [23,24]. In view of the observation that as little as 7% of wt catalytic efficiency is required for HIV replication [22,25], this conclusion was tenuous.

Indeed, the sexual transmission of a multi-drug-resistant (mdr) HIV variant has been reported [26]. This observation dampens hopes that drug-resistant variants lack the fitness necessary for efficient transmission and leads to speculation that emerging mdr variants may represent a significant threat to the efficacy of current antiviral therapies.

An additional complication associated with present antiviral therapies is the in vivo selection of more efficient variants of HIV PR. It has been speculated that the inhibitor off-rate, as opposed to the $K_i$, is responsible for resistance to small-molecule inhibitors [22,85]. This model is particularly useful in rationalizing resistance mutations that map 10 Å from the substrate binding pocket which effect the off-rate of the inhibitors more than the efficiency of substrate hydrolysis. Furthermore, clinical exposure to PR inhibitors may actually select for variants of HIV PR with increased $k_{cat}/K_m$ values, making the design of subsequent generations of PR inhibitors more difficult. Perhaps even more disturbing is the emergence of viruses harboring mutated PR cleavage sites, in addition to a mutated PR gene. HIV-1 strain IIIB passaged in vitro in the presence of the peptidomimetic inhibitors BILA 1906 BS and BILA 2185 BS [86] were shown to accumulate mutations in both the nucleocapsid (NC, p7/p) and p1/p6 cleavage sites of the gag polyprotein. Further analysis showed that these mutations resulted in a better substrate for HIV PR in in vitro cleavage assays [87]. Similar mutations were found in the genomes of HIV variants isolated from patients receiving indinavir [81]. Mutation of the two loci (p7/p1 and p1/p6) in the gag substrate may represent a drug-selected shift toward a different mechanism of substrate recognition that will require the redesign of small-molecule PR inhibitors. Alternatively, the fact that other gag cleavage sites have not mutated suggests the existence of molecular genetic constraints which may limit the ability of the virus to evolve different mechanisms of substrate recognition [87]. These issues will clearly require additional study to understand their implications for antiviral therapy. Several excellent reviews covering resistance to small-molecule PR inhibitors have been published, including (but not limited to) [78,80,88].

3. Gene therapy delivery of dominant-negative HIV protease monomers

3.1. Introduction to gene therapy

Gene therapy involves the delivery of exogenous therapeutic genes to a cell using one of a number of transfection or transduction techniques. Methodologies for the delivery of genes to target cells fall into two categories: (1) mechanical delivery; and (2) vector-mediated delivery. Mechanical transfer technologies include direct DNA microinjection, ballistic gold particle delivery, liposome-mediated transfection, and receptor-mediated gene transfer ([89] and references within). A detailed discussion of mechanical gene delivery methods is beyond the scope of this review. Gene delivery vectors based on modified cloned virus genomes have been described using derivatives of adenovirus [90–95], adeno-associated virus [96,97], herpesvirus [98–101], vaccinia virus [102], picornaviruses [103], alphaviruses [104], papovaviruses [105], and retroviruses ([11,70,106–110] and others). Salient features of these vectors are listed in Table 2.

Adenovirus vectors can be grown to high titers ($10^{12}$–$10^{13}$ particles/ml) and can be used to infect
both replicating and quiescent cells [111,112]. The vectors usually do not integrate into the host cell genome and the episomal transgene is eventually lost. Adeno-associated viruses (AAD, e.g. parovirus) have the advantage of stably integrating into the host genome but preparation of high titer vector stocks requires the quantitative removal of helper virus (i.e. replication-competent adenovirus) [8]. Adenovirus vectors (and to a lesser degree, adenov-associated virus vectors) tend to elicit strong cellular and humoral immune responses in animals, resulting in the eventual destruction of the transduced cells [113]. Herpesvirus infections tend to cycle unpredictably between latent and lytic phases, making them of questionable value for gene therapy. Vaccinia viruses cause cytopathic effects and also elicit strong immune responses. Neither of the large, double-stranded DNA viruses, herpesvirus or poxvirus, integrates into the host chromosome.

As is the case with most RNA viruses, picorna viruses and alphaviruses do not integrate into the host genome, again resulting in the eventual loss of the transgene. Retroviruses are the only group of RNA viruses that stably integrate into the host genome as an essential part of the replication cycle. Not surprisingly, retroviral vectors have become the most commonly used gene therapy delivery vehicles. Most retroviruses (e.g. Moloney murine leukemia virus (MoMLV) and human T-cell leukemia viruses (HTLV-1 and HTLV-2)) infect only mitotically active cells [114]. A subgroup of retroviruses, the lentiviruses, are of particular interest for gene therapy because of their ability to infect quiescent cells [115–117]. Lentiviruses include pathogens which cause acquired immunodeficiency in humans, primates, cattle, and cats (HIV-1/2, SIV, BIV, FIV, respectively), paralysis in sheep (VMV), encephalitis/arthritis in goats (CAEV), and anemia in horses (ELAV) [114]. Unlike the classical retroviruses (e.g. Moloney murine leukemia virus (MoMLV)), lentiviruses encode characteristic subsets of accessory proteins, including (in the case of HIV-1) proteins such as Nef, Rev, Tat, Vif, Vpr, Vpu, or Vpx. [17].

3.2. The evolution of lentivirus gene therapy vectors

The evolution of lentivirus vectors has been marked by at least two major technological advances, which allows the classification of these vectors into three generations (Fig. 3). First-generation vectors are represented by the pHIVgpt vector, described by Page et al. [70]. The vectors consist of a complete copy of the integrated HIV genome with the notable absence of a functional env gene. In the case of pHIVgpt, env was replaced by an in vitro selectable marker (guanine phosphoribosyltransferase (gpt)) which can be used to select for integration of the vector following stable transfection into cells. Disruption of the env gene also prevents the production of infectious virus particles, thereby providing a margin of safety for in vitro experiments (Fig. 3A). Furthermore, a heterologous env gene can be supplied in trans to allow packaging of the vector RNA in the envelope of a different virus (such as the glycoprotein of vesicular stomatitis virus (VSV-G)) [118]. Following pseudotyping with the amphotrophic VSV-G coat protein, the resulting vector can be used to infect, or transduce, in gene therapy terminology, a variety of cell types. These cells need not display the specific receptors required for attachment of the HIV envelope. Vector pseudotyping with
VSV-G also allows the concentration of vector stocks by centrifugation [119].

Second-generation vectors are simplified versions of the earlier vectors, encoding only viral genes that are essential for replication and packaging (Fig. 3B). In the case of the HIV-1-based vectors, the accessory genes Vpr, Vif, Vpu, and Nef are deleted. Rev, Tat, and all *cis*-acting regulatory signals (e.g. Tar and the RRE) remain intact [120]. Tat is still required to activate transcription from the HIV LTR and Rev is required to ensure the transport of unspliced, vector-derived mRNAs to the cytosol for translation and packaging.

Third-generation lentivirus vectors encompass a particulate coding strategy that has become known as split-genome construction. The minimal vectors require only: (1) a RNA packaging signal, designated $\Psi$, located near the 5′-LTR in the gag-coding region of the genome [121–123]; (2) a RRE; (3) 5′- and 3′-LTRs derived from a simple retrovirus; and (4) a transgene under the control of an independent promoter (Fig. 3C). The LTRs from simple retroviruses, such as MoMLV, do not require Tat activation, obviating the need for the Tat gene. The Rev gene has been also been eliminated in some third-generation
Table 3
Macromolecular inhibitors of HIV replication

<table>
<thead>
<tr>
<th>Macromolecular antiviral agent</th>
<th>References</th>
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<tbody>
<tr>
<td>Transdominant Tat</td>
<td>[149,150]</td>
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<tr>
<td>Transdominant Rev</td>
<td>[151,152]</td>
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<tr>
<td>Transdominant fusion polypeptide, Trevel</td>
<td>[152]</td>
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<tr>
<td>Transdominant gag structural proteins</td>
<td>[153]</td>
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<tr>
<td>Transdominant PR</td>
<td>Reviewed herein</td>
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<tr>
<td>Ribozymes target to HIV-specific RNAs</td>
<td>[154,155]</td>
</tr>
<tr>
<td>Tat activation response (TAR) element decoys</td>
<td>[155]</td>
</tr>
<tr>
<td>Rev responsive element (RRE) RNA decoys</td>
<td>[157]</td>
</tr>
<tr>
<td>Intracellular antibodies directed against Rev</td>
<td>[158]</td>
</tr>
<tr>
<td>Intracellular antibodies directed against gp120</td>
<td>[159]</td>
</tr>
<tr>
<td>Intracellular antibodies directed against Tat</td>
<td>[141,160]</td>
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</table>

A drawback of expressing dominant-negative viral proteins as macromolecular inhibitors is the amount of antiviral agent required to produce an effect. Most dominant-negative inhibitors of HIV replication are loss-of-function variants that are typically required in stoichiometric (not catalytic) quantities. It follows that the required amount of a dominant-negative inhibitor in a cell must be roughly equivalent to the amount of the virus target gene product produced in a cell. Thus, expression of the antiviral agent will require a promoter at least as efficient as the HIV-1 LTR, and the mRNA must be translated efficiently in the cell. The promoters of HIV-1 and MoMLV have been shown to direct sufficiently high levels of expression of antiviral genes to achieve a therapeutic effect [128,129]. It is worth noting here that macromolecular inhibitors of HIV replication do not need to be delivered to all cells of a target population. For example, in procedures where T-cells are removed from patients, treated, and returned, the total number of transduced cells is likely to represent somewhere between 0.1% and 10% of the PBMC in circulation. The crucial issue is whether a sufficient repertoire of T-cells has been protected to reconstitute a functioning immune system. Nevertheless, the higher the levels of transduction the more likely that this goal will be achieved. In addition, in in vitro experiments testing the efficacy of a particular gene product as an anti-HIV agent, virus challenge experiments become almost meaningless unless close to 100% of test cells express the gene product.

Most of the data pertaining to gene therapy for AIDS has been based on studies with a defective variant of the HIV-1 Rev gene product. Rev, an essential auxiliary protein, is translated from a highly
Table 4
Variables in testing antiviral agents

<table>
<thead>
<tr>
<th>Variables</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type transfected or transduced</td>
<td>PBMC, CEM, SupT1, Jurkat</td>
</tr>
<tr>
<td>Manner of introduction of the agent into cells</td>
<td>transfection, transduction, mechanical delivery</td>
</tr>
<tr>
<td>Strain of HIV-1 used in the challenge</td>
<td>IIIB, HXB2, NL4.3, SF2, SF13</td>
</tr>
<tr>
<td>Multiplicity of infection</td>
<td>0.00002–0.05</td>
</tr>
<tr>
<td>Method of measuring virus replication</td>
<td>p24 production, plaque assay, RT assay, RT-PCR</td>
</tr>
</tbody>
</table>

spliced viral mRNA containing nucleotide sequences from the env region of the genome. Reports from several groups have demonstrated that Rev interacts with a cis-regulatory RNA motif, the Rev-responsive element (RRE), in unspliced (or singly-spliced) mRNAs and participates in the export of these RNAs to the cytoplasm. ([14–17,130] and references within). An engineered variant of Rev, designated RevM10, functions as a dominant-negative inhibitor of HIV replication in immortalized human T-cells [131], primary blood mononuclear cells (PBMC) [132–134], and CD34+ stem cells [135–137].

The first clinical study involving the ex vivo transduction of RevM10 into PBMC isolated from HIV+ patients was performed by gold-particle-mediated delivery of plasmid DNA. The transfer vector harbored RevM10 under the control of the Rous sarcoma virus (RSV) promoter and HIV tat responsive element (TAR) along with a selectable marker (the NeoR gene) under control of a SV40 promoter. Transduced cells from the three patients involved in the study showed a 4–5-fold decrease in HIV replication, based on the p24 levels from transduced cells grown in vitro. The longer half-life of RevM10-transduced cells in the patients, compared to cells transduced with a control vector, indicated that cells expressing RevM10 exhibited a selective advantage for PBMC in HIV+ patients, presumably due to the protective effect of the transduced antiviral gene. Using this mechanical gene delivery method, expression of RevM10 in patients’ cells was relatively short-lived and the protective gene could not be detected after approximately 3 weeks [132]. A subsequent study, involving retrovirus vector-mediated delivery of RevM10, showed that the protective gene could be detected in patients’ PBMC for longer than 6 months following gene transfer [129]. Data showing the levels of protection afforded by RevM10 in vivo in the absence and presence of other inhibitors of HIV replication (e.g. small molecule inhibitors), awaits the completion of comprehensive clinical trials. The efficacy of other macromolecular inhibitors (Table 3) has also been demonstrated in cell culture; however, studies from different research groups have been difficult to compare due to the large number of variables involved in gene transfer protocols (Table 4).

3.4. Delivery of dominant-negative protease inhibitors using a gene therapy-based approach

A split-genome, Moloney murine leukemia virus

![Diagram](image)

Fig. 4. Vectors involved in the delivery of dominant-negative PR inhibitors to PBMC. pKT3-PLAP contains the HIV-1 gag and PR coding regions in-frame with expression driven by the Moloney murine leukemia virus (MoMLV) LTR. The MoMLV LTR does not require Tat activation. Rev is expressed when the gag-PR coding sequence is released as an intron. Placental alkaline phosphatase (PLAP) is a cell surface protein which allows magnetic bead selection of anti-PLAP-labeled cells. pRevM10 contains the defective Rev gene [131], phutat2kZ encodes the humanized single-chain anti-Tat intrabody [141], pTK-PLAP encodes only the cell-surface selectable marker, PLAP. The truncated nerve growth factor receptor (NGFR) is another protein used as a cell-surface marker and is encoded in place of PLAP in some constructs (Todd, Anderson, Ramstedt, Eberhardt, Jolly and Craik, submitted).
(MoMLV)-based, gene therapy vector was chosen for the delivery of dominant-negative PR inhibitors to primary human CD4+ cells (Todd, Anderson, Jolly, Ramstedt, Eberhardt, Craik; submitted). This vector offered several advantages over the first-generation pHIV-gpt ([70], Fig. 3A) and pHIV-ΔEdhfr [72] vectors we have used previously to express D-N PR inhibitors. These advantages included: (1) the substitution of HIV LTRs with MoMLV LTRs, eliminating the need for HIV Tat transactivation and therefore all HIV accessory genes, except Rev; (2) the use of the cell surface selectable markers placental alkaline phosphatase (PLAP) or truncated nerve growth factor receptor (NGFR), which allowed antibody labeling and magnetic bead selection of cells containing the vector and avoided cell selection using cytotoxic compounds (e.g. G418, mycophenolic acid); and (3) the elimination of the HIV-1 reverse transcriptase and integrase genes in the plasmid as these genes are not required in this system. These pKT3-PLAP vectors, as well as control vectors, are depicted in schematic form in Fig. 4.

Vector-producing cell lines (VPCL) were made by cotransfecting vectors designed to express macromolecular inhibitors (or control vectors) along with a plasmid encoding vesicular stomatitis virus glycoprotein (VSV-G) [118]) into 293 2–3 cells (Fig. 5). These cells contain an integrated MoMLV gag-pol gene with a non-functional packaging signal under control of a CMV promoter [118]. The resulting VSV-G-pseudotyped vectors produced by the 293 2–3 cells were used to transduce the canine packaging cell line, DA [138], which constitutively expresses MoMLV packaging proteins, including an amphotropic envelope. The pool of VPCL were enriched for PLAP-expressing cells using magnetic beads. These cells make useful titers of vector on the order of \( \sim 10^6 \) transducing units/ml as measured by a rapid antibody-based colorimetric assay for the cell surface marker.

Vector harvested from magnetic bead-selected
VPCL was used to transduce target cells, CD8⁺-depleted primary blood mononuclear cells (PBMC) pooled from the buffy coats of four donors. Transduction of PBMC was performed by spinning the cells and vector together at 1200×g speed (known as centrifugation, [139]). This method is approximately 5-fold more efficient than standard stationary transfection methods. Transduced PBMC were enriched for PLAP or NGFR expressing cells using magnetic bead selection, yielding nearly 100% transduced, minimally cultured cells ready to be expanded for virus challenge.

The transduced and bead-selected PBMC expressing: (1) the dominant-negative PR inhibitor; (2) RevM10 ([131,132,134,140]; and others); (3) an intracellular, single-chain anti-Tat antibody (intrabody, [141]); or (4) only the cell surface marker were infected with HIV-1 strains IIIB or SF2 and a multiplicity of infection of 0.05 or 0.006, respectively. HIV replication was measured by assaying the amount of processed p24 in the cell media by enzyme-linked immunosorbent assay (ELISA). RevM10 and the intrabody were included in the study to allow for comparison of different macromolecular agents under similar assay conditions.

The results of p24 ELISA assays are summarized in Fig. 6. The results indicated that the D-N PR inhibitors are approximately as effective as the intrabody or RevM10 in protecting transduced PBMC against HIV infection. Expression of the D-N PR inhibitor resulted in a 36-fold reduction in the amount of p24 released from HIV-1 IIIB-challenged cells. Similar results were observed in RevM10-expressing cells, while the degree of protection resulting from the expression of the intrabody was approximately the same. These results suggest that the D-N PR inhibitor is approximately as effective as RevM10 in inhibiting the replication of HIV-1 strain IIIB. A more modest degree of protection was afforded the replication of HIV-1 SF2 in D-N PR-expressing PBMC (2–2.5-fold), which was also similar to that observed with intrabody (and presumably RevM10).

The levels of protection demonstrated by the dominant-negative PR inhibitor are consistent with those of others in RevM10 protection experiments using both PBMC [132–134] or CD34⁺-derived cells [135–137]. Studies using immortalized T-cells often show greater reductions in P24 levels but PBMC-based assays are generally accepted as more relevant ([131] and Jolly and Anderson, unpublished results). While the correlation between cell culture data and clinical data is uncertain, studies have suggested that cell culture assays can predict in vivo effects [129,142]. It is also noteworthy that mathematical models of HIV infection in patients suggest that modest levels of inhibition can have marked effects on viral load over an extended period of time [82].

Just as combinations of small-molecule inhibitors of HIV replication are typically given to AIDS patients, combinations of gene therapy approaches (e.g. KWW along with RevM10 or hutat2k2) may be necessary to sufficiently reduce the levels of HIV replication in patients. It is also possible that gene therapy-based antiviral agents will be used in combination with existing small-molecule PR inhibitors. At least one study has demonstrated greater than additive levels of protection against a laboratory strain of HIV-1 when RevM10 and a small-molecule inhibitor are combined in vitro [143]. The ultimate test of macromolecular inhibitors will be in
CD34+ hematopoietic stem cells that will continuously repopulate a patient’s immune system with genetically altered cells. Similar studies have been undertaken for RevM10 [135–137]. These studies are now being considered for the dominant-negative PR inhibitor.

4. Conclusions and perspectives

In 1928, Sir Alexander Fleming’s accidental discovery of penicillin ushered in the era of antibiotics and eventually allowed clinicians to combat numerous systemic and localized bacterial infections. In 1935, the German chemist Gerhard Johannes Paul Domagk determined that one of several hundred commercial dyes was effective in curing mice of a bacterial infection, representing the first successful screening of a library of compounds to find one with defined biological properties. By the 1950s, clinicians had batteries of antimicrobial compounds to use to treat bacterial infections and humanity had won a decisive victory against parasitic microbes.

In 1956, Jonas Salk developed an inactivated (killed) vaccine against poliovirus, the causative agent of the once-dreaded disease, poliomyelitis. The Sabin live attenuated vaccine strain soon followed. With the advent of vaccines against other viral diseases (smallpox, rabies, measles, mumps, and rubella) it seemed that human and animal diseases caused by viruses would soon be as easily treated as those caused by bacteria (the emergence of antibiotic-resistant strains of bacteria notwithstanding). However, with the exception of some slowly progressing virus infections (e.g. rabies), vaccination is not a treatment for an established infection. Several compounds are effective in controlling the replication of some human viruses (e.g. acylovir, gancyclovir, foscarinet, vidarabine, idoxuridine, and trifluridine against herpes simplex virus and amantidine and rimantadine against influenza virus); however, resistance to these drugs develops rapidly in vivo.

Whether due to the inherent difficulty in treating established viral infections or the fact that many common viral infections are non-life-threatening, 20th century medicine has failed to develop effective treatments for the vast majority of virus infections. The emergence of AIDS in the late 1970s and early 1980s was a startling blow to the medical community. When vaccines failed to be protective against the causative agent of AIDS, HIV [144,145], healthcare workers were virtually helpless to intervene in preventing disease progression. Today, the number of infected individuals approaches 40 million. Most cases of HIV infection occur in subequatorial Africa and Asia where the disease progresses virtually unchecked.

The resources spent on both basic and applied HIV research since the 1980s have been staggering, but the scientific knowledge gained from the study of this virus has benefited all areas of scientific research. Even in the cancer field, which ostensibly lost funding to AIDS research, has benefited from AIDS-related basic research involving many aspects of eukaryotic gene expression and intermolecular interactions. Structure-based design of small molecules has come of age and it is now possible to identify lead compounds by rational methods. At a practical level, the time required to develop new drugs, perform clinical trials, and gain FDA approval has been significantly reduced as the research community and the FDA have mobilized in response to the AIDS epidemic.

Gene therapy holds the greatest promise for treating diseases in which the patient lacks a gene (or contains a defective gene) encoding a soluble secreted protein (e.g. insulin, Factor VIII, Factor IX). In this case, the transduction of an exogenous gene into a small population of cells may be sufficient to produce sufficient protein to correct the genetic defect. Gene therapy as an antiviral strategy as outlined here is somewhat more challenging but may well be useful in the clinical situation even if only a small fraction of susceptible T-cells in the body are transduced, provided that these cells represent the HIV-susceptible population and are sufficient to create a working immune system. However, in the in vitro challenge experiments described here it is necessary to obtain target populations that are close to 100% transduced or HIV-I replication in the remaining nontransduced cells is likely to obscure any protection in the transduced cells.

Targeting highly mutable proteins with small-molecule inhibitors has proven difficult. The integration of retrovirus genomes into host cell genomes, combined with the high error rate of RNA-dependent DNA polymerases, and the most valuable commodity-time, will continue to thwart the efforts of phar-
maceutical companies. In order to perform a given function in the cell, a viral gene product must possess certain properties. In the case of HIV PR, one of these properties is the ability to form a homodimer with another PR monomer. The use of defective PR monomers as inhibitors of HIV-1 replication seems likely to be a difficult strategy for the HIV-1 genome to evade by evolution of point mutations. In order to defeat the dominant-negative inhibitor, the virus must devise a new mechanism for PR dimerization.

With the exception of the larger DNA viruses (e.g., herpes viruses and pox viruses), the coding capacity of most viruses is extremely limited. For the expression of the small number of genes typically required for virus replication, there are two main coding strategies: (1) maintaining multiple transcriptional units; or (2) expressing a polyprotein and PR. The latter coding strategy has been adopted by small RNA viruses, including the retroviruses. Many aspects of viral gene expression require only the basal host machinery and are therefore difficult to inhibit without destroying the host cell. However, the processing of virus-encoded polyproteins by a virus-encoded PR provides an opportunity to target a uniquely viral enzymatic activity which is absent in the host cell. Any requirement for specificity that can be exploited in the design of antiviral compounds represents an Achilles' heel for virus replication [146]. The dimeric nature of HIV PR, likely a strategy to further conserve coding capacity, represents an ideal target for therapeutic intervention. Not only is the activity of the PR a target for small-molecule inhibitors, the dimerization of the PR is a target for defective monomers (decoys). Small-molecule inhibitors have verified the PR as a target. The next step is developing inhibitors that will be less affected by another well-known feature of RNA viruses – a high mutation rate. Preliminary results suggest that defective monomers, delivered to cells by retrovirus-mediated gene transfer, may be an effective approach to HIV PR inhibition.

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