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Protease-Resistant Peptide Design – Empowering Nature's Fragile Warriors Against HIV

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Abstract

Peptides have great potential as therapeutic agents, but their use is often limited by susceptibility to proteolysis and their resulting *in vivo* fragility. In this review, we focus on peptidomimetic approaches to produce protease-resistant peptides with the potential for greatly improved clinical utility. We focus on the use of mirror-image (D-peptide) and β -peptides as two leading approaches with distinct design principles and challenges. Application to the important and difficult problem of inhibiting HIV entry illustrates the current state-of-the-art in peptidomimetic technologies. We also summarize future directions for this field and highlight remaining obstacles to widespread use of protease-resistant peptides.

In drug discovery and development, peptide therapeutics have many advantages. Their polymeric nature makes synthesis straightforward, especially when compared to the synthetic schemes typically utilized for small molecules. Peptides are generally easier and less expensive to produce than recombinant proteins. Peptide therapeutics can also be more specific (and less toxic) than small molecules and excel at the challenging problem of disrupting large protein-protein interaction interfaces (i.e., “undruggable” targets). Due to advancements in genomics and proteomics, a plethora of natural peptide ligand sequences for important drug targets are available and provide a sensible starting point for the rational development of therapeutic compounds. In addition, a host of mature and emerging library-based screening techniques provides a means to rapidly discover novel peptide sequences with specific binding properties.

In spite of these enticing advantages, a major problem limiting development of peptide therapeutics is their proteolytic sensitivity and associated delivery challenges. Synthetic therapeutic peptides are typically relatively unstructured and are therefore rapidly degraded *in vivo*, often with half-lives on the order of minutes¹. Proteolysis commonly occurs in the GI lumen, intestinal brush border, enterocytes, hepatocytes, antigen-presenting cells, and plasma. Because of this *in vivo* fragility, oral delivery is generally not possible, necessitating frequent dosing by injection. Even when delivered parenterally, degradation in the blood combined with rapid renal filtration often results in drugs that are expensive, inconvenient, and unpleasant to administer.

Protease-resistant peptides would address many of these limitations. One of the most promising approaches is to modify the chemical structure of the peptide backbone (peptidomimetics)². Modifications that have been shown to substantially decrease proteolysis include N-methylation, ester linkages (α -hydroxy acids), insertion of additional methylene groups into the backbone (β -amino acids, γ -amino acids, etc.), and the use of D-

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amino acids. More significant changes to the peptide backbone include peptoids, azapeptides, oligoureas, arylamides, and oligohydrazides²⁻⁴.

In this review, we describe how modified peptide backbones can be used to design protease-resistant inhibitors with a special focus on the high-priority problem of designing protease-resistant HIV entry inhibitors. Although these modified backbones effectively address protease sensitivity, each is associated with a set of design challenges using rational design or library screening techniques. This review will not cover traditional strategies to reduce protease sensitivity, e.g., peptide capping, sequence alteration at susceptible sites, cyclization, or stapling, which have been extensively reviewed elsewhere⁵.

Inhibiting HIV Entry

An estimated 34 million people worldwide are infected with HIV, the causative agent of AIDS, resulting in nearly 2 million deaths per year and over 25 million cumulative deaths (UNAIDS). Dramatic progress has been made in reducing mortality since the inception of antiretroviral therapy against HIV enzymes reverse transcriptase, protease, and recently integrase. However, the relentless development of drug resistance necessitates ongoing development of therapeutics that target other stages in the viral lifecycle. In particular, there have been extensive efforts to develop potent, broadly active, and economical entry inhibitors for the prevention and treatment of HIV/AIDS⁶.

The current HIV entry pathway model is shown in Fig. 1. Viral entry into host cells is mediated by the trimeric HIV envelope (Env) glycoprotein. Env contains the non-covalently associated surface gp120 and transmembrane gp41 subunits. gp120's primary function is to interact with cell receptors that mark HIV's preferred target cells (e.g., T-cells and macrophages), while gp41 induces membrane fusion. Host cell interactions are mediated by gp120 through association with the primary cell receptor (CD4) and chemokine co-receptor (either CXCR4 or CCR5, depending on viral tropism). Upon gp120 engagement with cell receptors, a complex series of structural rearrangements in gp120 propagate to gp41, activating it for membrane fusion (reviewed by⁷). At this stage, gp41 forms an extended prehairpin intermediate containing an N-terminal trimeric coiled coil (N-trimer) and C-terminal region (C-peptides) of unknown structure. Fusion is driven by collapse of this intermediate as three helical C-peptides pack antiparallel to the N-trimer (trimer-of-hairpins formation), drawing the viral and host cell membranes into close proximity. A similar fusion mechanism is utilized by many other enveloped viruses, including influenza, Ebola, and paramyxoviruses⁷.

C-peptide Inhibitors

This mechanism suggests that peptides derived from the N- and C-peptide regions of gp41 could prevent viral membrane fusion in a dominant-negative manner by preventing trimer-of-hairpins formation. Indeed, both N- and C-peptides inhibit HIV entry⁸⁻¹⁴. The N-trimer/C-peptide interaction is predominantly mediated by conserved interactions between the hydrophobic face of helical C-peptides and a hydrophobic groove formed between helices in the N-trimer. C-peptide inhibitors are more promising drug candidates because of their higher potency and better solubility compared to N-peptide inhibitors.

C-peptide inhibitors were first identified through screens of gp41-derived peptides^{9,11}. Fuzeon (Enfuvirtide, T-20) is a 36 amino acid L-peptide taken from the gp41 C-peptide region. Fuzeon inhibits HIV entry with nM potency and reduces viral loads by 2 logs¹⁵, leading to its approval as the first HIV entry inhibitor in 2003. Unfortunately, Fuzeon's clinical use has been limited by its short half-life. Fuzeon requires injection at very high doses (90 mg, twice daily) to overcome its proteolysis and rapid renal filtration. These

practical problems result in a drug that is expensive (~\$30,000 per year), can cause painful injection site reactions, and is only approved for patients experiencing treatment failure due to multi-drug resistance (“salvage therapy”). Fuzeon's high dosing requirements and *in vivo* fragility also limit options for less frequent dosing via depot formulation.

The gp41 “pocket” region

At the N-trimer's C-terminus lie three symmetry-related deep hydrophobic pockets. Each pocket has a volume of ~400 Å³ that is filled primarily by three C-peptide residues (Trp628, Trp631, and Ile635)^{16,17} (Fig. 2). The pocket is a promising inhibitory target because of its critical importance in membrane fusion and very high level of conservation across diverse HIV strains^{16,18}. Mutations in the pocket are often not well tolerated due to the requirement for compensatory mutations in the C-peptide region to restore binding. In addition, the pocket region is encoded by the structured RNA region of the Rev-response element (RRE), which contains a signal critical for nuclear export of viral RNA¹⁸. Interestingly, extensive efforts by numerous groups to discover small molecule pocket-binding inhibitors have had limited success, generally producing inhibitors with modest potency and/or significant toxicity (e.g.,^{19–23}). Based on this body of work, the gp41 pocket appears to be “undruggable” by small molecule inhibitors, a common problem for extended protein-protein interaction interfaces.

Fuzeon was discovered prior to the gp41 6-helix bundle crystal structure and does not bind to the gp41 pocket. However, next generation C-peptide inhibitors (e.g., C34, T-1249) do include pocket-binding residues and enjoy superior potencies and resistance profiles^{24–26}. The follow-on compound to Fuzeon, T-1249, performed very well in clinical trials, but was not developed further due to unspecified formulation problems, which we speculate includes challenges in the economic synthesis of this 39-residue peptide and a requirement for four 1 mL injections, once per day, as used in a phase I/II trial²⁵.

Fuzeon and T-1249 show that a peptide fusion inhibitor can be very effective against HIV, but the impact of such drugs will be limited until the problems of short half-life and high dosing (and the resulting high cost) can be overcome. In this review, we focus on two distinct strategies that have yielded promising protease-resistant peptide fusion inhibitors with the potential to overcome Fuzeon's *in vivo* fragility.

Rational drug design with modified peptide backbones

While there is much interest in the *de novo* development of peptides with defined structural and functional characteristics, this work is hampered by limitations in currently available modeling strategies. Thus, as illustrated below, most successful rational designs of protease-resistant peptides start from sequence and structural information from existing peptide ligands.

In the realm of rational design of modified peptide therapeutics, β -peptides and mixed α/β -peptides are among the most promising. β -peptides are composed of β -amino acids, which contain an extra backbone methylene group (between the amino and α -carbon, specified as a β^2 -amino acid, or between the carboxylate and α -carbon, specified as a β^3 -amino acid) (Fig. 3). Short β -peptide sequences can adopt robust secondary structures analogous to α -helices formed by α -amino acids. If a natural helical peptide ligand is known, a β -peptide mimic can be generated by the precise placement in three dimensions of key side chains onto a β -peptide scaffold. Two β -peptide scaffolds that have been extensively utilized are the 12-helix and 14-helix, named after the number of atoms between hydrogen bonding groups (these and other β -residue-containing scaffolds are reviewed elsewhere^{3,27–30}). The specific structural motif adopted by a particular β -peptide is dictated by the nature of the substituent

β -amino acids³¹. β -peptides composed of monosubstituted, acyclic β -amino acids or cyclic six-member ring β -amino acids preferentially adopt the 14-helix structure, while the 12-helix structure is favored by peptides composed of five-member ring cyclic β -amino acids. The helical parameters of the 12- and 14-helices are discussed and compared to α -helices in^{27,31}.

In a 14-helix composed of β^3 -amino acids, side chains at residues i , $i+3$, and $i+6$ are presented along the same face of the helix, and are reasonably superimposable with side chains at residues i , $i+4$, and $i+7$ of an α -helix³². This property can be exploited to display epitopes that mimic an α -helical face and has been applied to the development of low-mid μ M HIV entry inhibitors that bind to the gp41 pocket region^{33,34}. In an analogous approach, β -peptide inhibitors of HCMV entry were developed using the 12-helix scaffold³⁵. To map an α -helix epitope onto the 12-helix, side chains at positions i , $i+4$, and $i+7$ on the α -helix are placed at positions i , $i+3$, and $i+5$ on the 12-helix. Although acyclic residues diminish 12-helix propensity, they provide the easiest avenue for side chain attachment, so a minimum number of acyclic β^2 or β^3 residues were introduced into the structure at specific points to mimic side chain presentation of the native α -helix. This approach enabled the rapid discovery of inhibitors with modest potency, but its main challenge is the lack of a route forward, by rational design or high-throughput screening, to optimize these initial hits.

A sequence-based approach utilizing mixed α/β -peptides has been applied to develop an HIV entry inhibitor that structurally and functionally mimics C-peptides (~10 turn α -helix)³⁶. In this approach, a subset of C-peptide residues were strategically replaced with homologous β^3 -amino acids following an $\alpha\alpha\beta\alpha\alpha\beta$ pattern, which, despite the additional methylene units, does not significantly alter secondary structure of the helix³⁷. Upon folding, this pattern generates an α -helix-like conformation with a β -residue stripe that runs down the side of the helix distal to the interaction surface, minimizing disruption of the binding interface. Upon replacing 11 of the 38 residues with β^3 -amino acids, the resulting α/β -peptide had >10,000-fold diminished affinity for its binding target relative to the α -peptide counterpart.

As a second step in the design, specific β^3 -residues were replaced by cyclic β -residue homologues. The cyclic residues were incorporated to reduce the entropic penalty associated with helix formation due to the inherent torsional flexibility of β^3 -residues. β^3 analogues of alanine in the α/β -peptide were replaced with a nonpolar, five member ring constrained β -residue (ACPC), while β^3 analogues of arginine were replaced with a polar, heterocyclic analogue of ACPC (APC). These replacements improved affinity by ~400-fold over the peptide with acyclic residues. Though the binding affinity never recovers to that of the original α -peptide ligand, the resulting α/β -peptide was nearly as potent as the α -peptide, but with the added advantage of being 280-fold more resistant to proteolytic degradation by proteinase K. The apparent discrepancy of having diminished binding affinity, yet α -peptide-like potency is likely due to the potency plateau observed for many HIV entry inhibitors (see the discussion of the “resistance capacitor” below).

The original report indicated that the N-terminal Trp-Trp-Ile motif of the α/β -peptide does not engage the C-terminal hydrophobic pocket of gp41, but subsequent crystallographic analysis indicated that the pocket-binding motif on the α/β -peptide is indeed able to engage the pocket. The authors suggest that the lack of engagement in the original structure was an artifact caused by crystal packing, and that the newer structure more faithfully portrays the binding of the α/β -peptide (see discussion in supplementary materials of³⁸).

Genetically-encoded library-based screens

An alternative to rational design is screening of random peptide libraries. These high-throughput methods identify novel peptides with a desired function (typically binding to an immobilized target). Commonly used screening techniques include phage, ribosome, and mRNA display, but these methods all rely on cellular translation machinery and are therefore not yet fully compatible with peptidomimetics in their standard forms. Though there have been many advances and refinements in the field of synthetic peptidomimetic library generation (e.g., split and pool synthesis, physically addressable synthesis by photolithography), these synthetic libraries are typically limited to $<10^6$ members³⁹ compared to the billion to trillion member libraries that can be generated with genetically-encoded libraries.

D-peptides

D-peptides are entirely composed of D-amino acids, which are mirror-image stereoisomers of the L-amino acids found in naturally occurring L-peptides. D-peptides are a promising therapeutic platform because they are highly resistant to natural proteases⁴⁰. In elegant work by the Kent group⁴¹, D-HIV protease was shown to cleave only D-peptide substrates, showing that proteases exhibit highly stereospecific substrate discrimination.

The symmetry relationship between L- and D-peptides can be exploited in mirror-image display techniques⁴² in which a mirror-image version of the target molecule is generated by solid-phase synthesis using D-amino acids. Randomized L-peptide libraries are then screened against this D-target. The winning L-peptides are identified by DNA sequencing and then the corresponding D-peptides are synthesized. By symmetry, the D-peptide will have the same activity towards the natural L-target as the L-peptide had against the mirror-image D-target (Fig. 4).

A major limitation of mirror-image display is the requirement for chemical synthesis of the D-target. Synthesis of D-peptides is currently done using traditional solid phase peptide synthesis (SPPS)⁴³. Routine use of SPPS chemistries for the production of peptides is limited to ~50 residues, though this limit varies widely depending on the required purity and sequence/structure properties of the peptide in question (e.g., extended beta-strand peptides can aggregate during SPSS). Despite these challenges, syntheses of very long peptides have been reported (e.g., the synthesis of the 140-residue IL-3 protein⁴⁴).

Larger D-peptide targets can be obtained using peptide ligation techniques to link multiple synthesized peptide fragments. A variety of ligation chemistries have been developed (see⁴⁵ for a very thorough review), but the most common technique is cysteine-mediated native chemical ligation (NCL). NCL requires the presence of an N-terminal cysteine on one peptide fragment and a C-terminal thioester on the other (see⁴⁶ for a summary of popular recombinant and synthetic methods for the generation of peptides bearing a C-terminal thioester) and results in the ligation of the two segments via a native peptide bond. SPPS of thioester-containing peptides has traditionally been carried out via Boc chemistry, but recent advances have enabled the robust synthesis of thioesters using the easier and more popular Fmoc chemistry⁴⁷ and commercially available Dawson Dbz resin (Novabiochem). Other means of accessing peptide thioesters via Fmoc chemistry have been recently reviewed⁴⁸.

By strategically utilizing masked N-terminal cysteines (e.g. thioproline), multiple peptide fragments can be joined together sequentially or in a single-pot reaction^{49–53}. This strategy has been used in the D-peptide synthesis of the 81-residue snow flea antifreeze protein⁵⁴. NCL leaves a Cys residue at each ligation site, but this “scar” can be removed by desulfurization of the cysteine residue to alanine^{55,56}. Furthermore, several creative

adaptions of NCL allow residues other than an N-terminal cysteine to be present at the ligation junction, such as N-terminal, thiol-containing auxiliary groups that can be removed via reduction⁵⁷, UV irradiation^{58,59}, or treatment with acid^{60,61} after they have facilitated peptide bond formation. In another approach, modified versions of phenylalanine⁶², valine⁶³, or lysine⁶⁴ bearing a thiol substituent were incorporated at the N-terminus of a peptide fragment and yielded the respective native amino acid at the ligation site following NCL/desulfurization.

Once a D-target has been synthesized, it can be used in conjunction with mirror-image display to screen peptide libraries for novel sequences of interest (see our work on HIV below and⁶⁵). The unifying feature that underlies all of the library-based display techniques discussed here is the physical linkage of a peptide to its corresponding genotype (RNA or DNA). This linkage allows the library to be subjected to multiple rounds of interrogation/library amplification leading to enrichment of sequences that bind to a target of interest. In these techniques, library diversity is generated in the nucleotide coding sequence, and cellular machinery efficiently translates this information into a peptide library. The display techniques most suitable for screening high-diversity libraries can be broken down into two broad categories: viral display and cell-free display systems (briefly described here, but for a more extensive review see^{66–70}).

Viral display

Phage display continues to be the workhorse of the viral display techniques because of its ease of use, versatility, and low cost. Since phage display requires a bacterial transformation step, library size is typically limited to $\sim 10^9$ – 10^{10} . The most commonly utilized phage display system is the non-lytic M13-family filamentous phage, in which the peptide library is expressed as an N-terminal fusion with the pIII minor coat protein. Up to five copies of pIII are present on the phage surface, making both polyvalent and monovalent display techniques possible. Polyvalent display provides a strong avidity effect, which is highly advantageous for screening naïve peptide libraries containing only rare low affinity binders. In contrast, monovalent display reduces avidity and allows for more stringent selection of peptides with high affinity. In an early round of phage display, library diversity is high, but each sequence is represented by only a few phage. As with any library display method, the application of selection pressure must be sufficient to drive selection for tighter binders, but not so severe as to eliminate rare tight binding sequences due to stochastic factors. In later rounds, as phage library diversity drops and each remaining sequence is represented by numerous phage, selection pressure can be steadily increased. Insufficient selection pressure can select for “cheater” phage that do not bear authentic tight binding peptides (e.g., phage with growth advantages).

Besides filamentous phage display, techniques employing various eukaryotic viruses, including retroviruses, baculovirus, Adeno-associated virus, and Adenovirus have been or are currently being developed for displaying peptide libraries⁶⁷. Other display techniques (e.g., bacterial, yeast, or mammalian cell display) have several advantages over phage display (e.g., more sophisticated folding machinery, post-translational modifications, ability to use FACS sorting), but are more complex and typically limited to less diverse libraries (reviewed by^{66,67,71,72}).

Cell-free display

One of the major advantages of cell-free techniques (reviewed by⁷³) is that they are carried out *in vitro*. Because a transformation step is not required, library diversities $>10^{12}$ can be generated⁶⁹. Due to the proposed correlation between library diversity and the affinity of

selected ligands, this large increase in library diversity over typical viral or cell surface display systems provides a distinct advantage.

Ribosome display^{74,75} capitalizes on the fact that it is possible to stall the *in vitro* translation of a polypeptide so that the ribosome remains assembled and attached to the mRNA transcript and the nascent translated polypeptide. This mRNA-ribosome-polypeptide ternary complex serves to link genotype to phenotype and can be panned against a target to isolate sequences of interest. The ternary complex can then be eluted and dissociated with EDTA, allowing for the isolation of the original mRNA transcript.

Alternatively, RNA display⁷⁶ links phenotype to genotype by connecting an mRNA sequence directly to the peptide it encodes. This linkage is accomplished by chemically attaching the antibiotic puromycin to the 3' end of the RNA via a DNA linker. As the mRNA is being translated, the ribosome will stall once it reaches the DNA linker, allowing puromycin to enter the ribosomal A site, where the ribosome catalyzes covalent attachment to the recently translated polypeptide. This peptide-RNA complex can then be subjected to panning against a specific target.

While *in vitro* display techniques that link the peptide phenotype to an RNA genotype overcome many of the limitations of phage display, the instability of RNA molecules along with other technical challenges fundamental to these techniques has limited their application to a relatively small number of expert labs. To address these challenges, techniques that link the library peptides directly to their encoding DNA have recently been developed.

CIS display (Isogenica) exploits the unique activity of RepA, a bacterial plasmid DNA-replication initiation protein⁷⁷. RepA is a cis-acting protein that tightly binds to the origin of replication (*ori*) on the plasmid from which it was expressed. A stretch of DNA between the sequence that encodes RepA and the *ori* known as the CIS element contains a rho-dependent transcriptional terminator that is thought to stall the RNA polymerase during transcription of RepA. The current model holds that this delay allows the newly synthesized RepA protein emerging from the ribosome to interact with the CIS element, which subsequently directs RepA to the *ori* DNA. Peptide libraries can be fused to the N-terminus of RepA, thereby creating a link between phenotype and the DNA genotype. Like other *in vitro* techniques, CIS display has the capability to accommodate peptide libraries much larger than those possible for phage display. In one example⁷⁷, a library of $>10^{12}$ randomized 18-mer peptides was constructed and was used to isolate sequences that bound to disparate targets. In a similar approach, DNA sequences encoding randomized peptide libraries are fused to the bacteriophage P2A gene. P2A is an endonuclease involved in the rolling circle replication of bacteriophage P2 DNA. P2A becomes covalently attached to the same DNA molecule from which it was expressed, linking phenotype to genotype. This technique has been used in a pilot study to select single-chain antibodies from a 10^7 -member library and may be suitable for screening much larger libraries⁷⁸.

D-peptide Inhibitors of HIV Entry

Here we describe the history of our potent D-peptide inhibitors of HIV entry, developed in the Kim and Kay laboratories. Initially, mirror-image polyvalent phage display was used to screen naïve peptide libraries of various lengths and geometries for binding to an HIV N-trimer pocket mimic (IQN17)¹⁸. Pocket-specific binding was only observed in disulfide-constrained 10-mer sequences (CX₁₀C) containing an EWXWL consensus sequence. An initial group of ~10 winning sequences were validated by measuring their binding to the desired target and several negative control targets (mutated or missing pockets) to demonstrate pocket-specific binding.

Validated D-peptides inhibited HIV entry (lab strain HXB2) with IC_{50} values ranging from 11–270 μM ¹⁸. A co-crystal structure of one of the higher affinity D-peptides (D10-p1) in complex with IQN17 shows that D10-p1 contains two short left handed α -helical segments flanking a turn imposed by the disulfide constraint. The binding interface between the hydrophobic pocket of IQN17 and D10-p1 is mediated by residues in the C-terminal α -helix, with residues in the EWXWL consensus motif making the largest contributions. Comparison of the D10-p1/IQN17 crystal structure to the native post-fusion gp41 structure¹⁷ reveals that critical residues for binding in D10-p1 are very similar in chemical character to those of the natural C-peptide ligand (primarily W628, W631, and I635), but adopt distinct conformations due to their opposite chirality (Fig. 2).

Due to library diversity limitations, the first-generation library only surveyed about one in a million possible sequences¹⁸. The identification of a strong EWXWL consensus sequence allowed us to fix these 4 residues to produce a “constrained” library with only 6 randomized residues ($\sim 10^9$ possible sequences). Panning this library produced ~ 4 -fold more potent inhibitors⁷⁹.

Surprisingly, an 8-mer (CX₈C) was also among the winning sequences. Since 8-mers were not part of the library design and likely arose from rare replication errors, their relative success suggested that the 8-mer geometry might provide a better pocket-binding solution. Our crystal structure of the first identified 8-mer, PIE1 (Pocket-specific Inhibitor of Entry), bound to IQN17 reveals that the key pocket binding residues (WXWL) adopt nearly identical positions within the pocket as seen with D10-p1, leading to very similar binding interfaces despite PIE1's reduced length⁷⁹. The key difference between PIE1 and D10-p1 is a more compact D-peptide structure with a tighter hydrophobic core devoid of water. PIE1 has a D-Pro at position 8 that likely aids making the tighter turn necessary for circularization forced by the shorter disulfide constrained loop⁷⁹.

To completely explore 8-mer sequence space, a new library was generated with the core consensus sequence WXWL fixed (CX₄WXWLC). While screening this library using traditional solid-phase phage display, we observed that polyvalency made it difficult to distinguish modest (μM) and tight (nM) binders. Solid-phase target presentation is advantageous for selection of weak initial binders from a naïve library, but problematic for identifying strong binders in a sea of modest binders since all binders are strongly retained on the high-density target surface. Moving the binding reaction into solution (solution-phase phage display) reduces inter-target avidity and allows additional selection pressure by reducing target concentration through rounds of panning⁸⁰. Despite reduced inter-molecular avidity, solution-phase phage were still found to have dramatically higher binding affinities in the context of the panning than expected based on K_D values of the derived D-peptides, likely due to intra-molecular avidity on the trimeric target. To overcome this barrier, an L-peptide version of PIE2⁷⁹ (identified during earlier rounds of solution-phase phage display) was employed as a soluble competitor for subsequent rounds of panning. Increased selection pressure was applied by escalating PIE2 concentrations, leading to the discovery of PIE7, which is ~ 15 -fold more potent than D10-p1 ($IC_{50} = 620$ nM, HXB2 strain).

Our co-crystal structure of PIE7 in complex with IQN17 suggested that further gains in binding affinity could be made through optimization of the residues outside the disulfide bond, which make significant gp41 contacts⁷⁹. Initially, these four “flanking” residues outside the disulfide bond (Gly-Ala on the N-terminus and Ala-Ala on the C-terminus) were not varied due to library cloning restrictions. We redesigned the phage display vector to relocate the cloning sites and allow randomization of the flank residues. After four rounds, PIE12 (HP-[PIE7 core]-EL) was identified with ~ 20 -fold improved potency over PIE7. The PIE12/IQN17 crystal structure reveals that PIE12's improved binding is likely due to ring-

stacking interactions of D-His1 and D-Pro2 with the pocket residue Trp571 and burial of an additional 50 Å² hydrophobic of surface area by DLeu15⁷⁰. Beyond the changes in the flanking regions, the central core structure is unchanged from PIE7.

Crosslinking and the Resistance Capacitor

After battling the confounding effects of avidity throughout our phage display screens, we hoped to re-introduce avidity to boost the potency of our D-peptides. Our D-peptide/N-trimer crystal structures reveal the precise relationship between neighboring D-peptides binding to the three symmetry-related pockets. Using this information we used discrete polyethylene glycol (PEG) crosslinkers to generate dimeric and trimeric D-peptides⁷⁹, which showed dramatically improved antiviral potency (up to 2000-fold) over monomeric D-peptides^{70,79}. PIE12-trimer, our lead inhibitor, is ~30-fold more potent than Fuzeon and inhibits a diverse panel of the most common circulating HIV strain subtypes worldwide in the high pM – low nM range⁷⁰.

Interestingly, we encountered a limit to the potency gains that could be achieved by monomer affinity optimization and crosslinking. We hypothesized that this potency plateau was imposed by the limited time window available for inhibitor binding (target is only available in the transient pre-hairpin intermediate) and the inhibitor association rate (limited by diffusion), as previously observed for the pre-hairpin intermediate inhibitor 5-helix⁸¹. Although this potency limit would prevent us from designing more potent inhibitors, we hypothesized that “over-engineering” our inhibitors (i.e., continuing to improve inhibitor binding despite a lack of corresponding improvement in potency) would endow them with a reserve of binding energy that would stall the development of resistance mutations. We predict that this “resistance capacitor” would also greatly delay the emergence of resistance by eliminating the selective advantage conferred by these mutations (i.e., severing the link between affinity and potency). Only a profoundly disruptive mutation would escape the resistance capacitor. In support of this hypothesis, we were only able to identify high-level PIE12-trimer resistance after 65 weeks of viral passaging in the presence of inhibitor, compared to ~3 weeks for Fuzeon⁷⁰. As predicted, PIE12-trimer was also able to absorb the impact of earlier-generation D-peptide resistance mutations.

Protease-resistant peptides face other pharmacokinetic challenges

Reduction of peptide susceptibility to proteases increases peptide longevity, but another major threat to serum half-life is rapid clearance via renal filtration. For globular proteins, the glomerular filtration size limit is ~70 kDa. Although albumin is slightly smaller, it avoids filtration because of electrostatic repulsion from the highly negatively charged glomerular basement membrane. Albumin is the smallest major unfiltered protein, efficiently circulating in the bloodstream with a half-life of approximately 19 days in humans⁸². The small size of peptide therapeutics means that an additional level of design is required to reduce renal filtration and realize the full benefits of protease resistance. Several common PK optimization strategies suitable for peptides are briefly described below.

Polyethylene glycol (PEG) is a hydrophilic polymer commonly used for protein conjugation. Adding PEG to a protein has been one of the most clinically successful strategies for improving pharmacokinetics⁸³. Early studies on the effects of PEG size on biodistribution revealed that good serum retention is achieved between 40–60 kDa, while exceeding this range resulted in increased uptake and accumulation within the reticuloendothelial system⁸⁴. Thus, the PEGylation field has largely adopted the strategy of adding ~40 kDa of PEG weight to peptide and small protein therapeutics. PEG is extensively hydrated such that its hydrodynamic radius is much larger than expected from its molecular weight. Furthermore, distributing the weight of the PEG polymer in a branched geometry improves half-life and

reduces steric interference⁸⁵. PEG conjugation can also be reversible (e.g. an ester linkage), creating a circulating depot from which the therapeutic is cleaved over time (e.g., in case drug activity is adversely affected by PEG conjugation)^{86,87}. Limitations of PEGylation include steric interference with binding, long-lived accumulation in renal tubule cells, viscosity, and polydispersity. An alternative approach uses a hydroxyethyl starch polymer (HESylation) to reduce renal filtration⁸⁸.

Albumin binding (covalent or non-covalent) is another recently validated approach for prolonging serum half-life (reviewed by⁸⁹). Promising albumin-binding strategies include covalent albumin-peptide conjugation, as well as reversible binding to circulating albumin via albumin-binding peptides, small molecules, or fatty acids^{89–91}. As an example, albumin conjugation of an HIV C-peptide inhibitor (either *in vitro* or *in vivo*) dramatically improves serum half-life⁹², as does cholesterol conjugation to a lesser extent, presumably via weak reversible interactions with albumin and/or cell membranes⁹³.

Future Directions

Recombinant production of peptidomimetics

Although robust recombinant production of peptidomimetics is not yet possible, significant recent advances in synthetic biology may enable routine production of diverse peptidomimetic libraries in the near future. One promising approach is *in vitro* codon reprogramming for the synthesis of unnatural polymers. This approach relies on cell-free translation systems to reconstitute ribosomal peptide synthesis using a minimal set of purified protein components^{94–99}. By chemically or enzymatically charging tRNA molecules with novel amino acid analogues, the genetic code can be effectively reprogrammed *in vitro*. When these cell-free systems with genetic code modifications are used in conjunction with a display technology, peptides with novel amino acids can be screened for a desired property. For example, ribosome display was used in conjunction with *in vitro* codon reprogramming to isolate peptide sequences from an mRNA library that encoded an unnatural, selectable amino acid^{100–102}.

Along these lines, it has been demonstrated that tRNAs can be charged with a variety of amino acid analogues that will modify the peptide backbone, including α -hydroxy acids, N-methyl amino acids, α,α -disubstituted amino acids, β -amino acids, and D-amino acids¹⁰³. However, the efficiency of ribosomal incorporation of Ala/Phe analogues varies greatly from fairly robust (α -hydroxy acid and N-methyl) to weak (α,α -disubstituted amino acids) to undetectable (β - and D-amino acids)¹⁰³. Subsequent work has described the ability of the translation machinery to accommodate amino acid analogues with novel side chains and backbones¹⁰⁴.

In one example, seven codons were each reassigned to encode a unique α -hydroxy acid, and polymers as long as 12 consecutive α -hydroxy acids could be synthesized¹⁰⁵. In another report the incorporation efficiencies of 23 N-methyl amino acids, 19 of which bore naturally occurring side chains, were determined. 8 of these 19 N-methyl amino acids were incorporated at specific points in a polypeptide with >30% efficiency as compared to wild type. A peptide up to 10 residues long could be synthesized from three unique N-methyl amino acids¹⁰⁶.

While less success has been reported with ribosomal incorporation of D-amino acids, modifications to the ribosomal peptidyltransferase center and helix 89 of the 23S rRNA can relax the ribosome's natural substrate specificity, thereby enhancing the incorporation of D-amino acid residues into a growing polypeptide chain^{107,108}. Though these techniques have not yet been employed as such, in principle cell-free translation systems coupled with *in*

vitro display techniques could be used to screen libraries of polymers with novel backbones. As an advance in this direction, genetic code reprogramming has already been used in conjunction with mRNA display technology to generate mRNA-peptide fusions containing N-methyl amino acids¹⁰⁹.

Another approach to recombinantly produce peptidomimetics relies on the ability to expand the genetic code *in vivo* via the generation of evolved tRNA/aminoacyl-tRNA synthetase pairs. In these systems, the foreign tRNA functions as an amber suppressor, effectively allowing the amber nonsense codon to be reprogrammed to encode a nonnatural amino acid^{110,111}. It has been demonstrated that genetic code expansion can be used in conjunction with phage display to incorporate a nonnatural amino acid into a pIII fusion peptide¹¹². In the future, multiple codons could be reassigned, permitting the incorporation of multiple unnatural residues *in vivo*. Several advances have been made toward this end. In a recent publication describing a technique for rapid, genome-wide engineering, the authors show progress towards replacing all 314 TAG stop codons in *E. coli* with the TAA stop codon¹¹³. This type of genome manipulation could be used for the removal of redundancy from the genetic code, freeing up codons for potential reprogramming. In another approach involving evolved tRNA/aminoacyl-tRNA synthetase pairs, an evolved orthogonal ribosome able to read both 3- and 4-base codons was able to efficiently incorporate two different nonnatural amino acids into a single polypeptide chain *in vivo*^{111,114}. These approaches present tantalizing possibilities for the production of peptide libraries with unnatural side chains and backbones, but the technology is not yet sufficiently robust to allow for widespread application. Additional engineering of tRNA molecules, elongation factors, and the ribosome itself will likely be required for use with certain diverse peptidomimetics^{108,115,116}.

D-peptides present a unique opportunity for designing an artificial recombinant production system. Because of their symmetry relationship with natural peptides, an *in vitro* translation system composed of all opposite-chirality components (D-proteins and nucleotides containing L-ribose) would function equivalently to natural translation, when provided with mirror-image DNA substrates. Synthesis of all ribosomal components presents an enormous synthetic challenge, but recent advances in SPPS and peptide ligation may now make this approach feasible. A mirror-image *in vitro* translation system would provide a useful tool for D-peptide drug discovery and production, but may not be ideal for large-scale production, especially of complex D-proteins (e.g., those requiring chaperones or post-translational modifications). The ultimate goal is to produce D-peptides using a synthetic mirror-image organism, a strategy we dub the “D. coli” project. The key to this project is synthesizing the minimal set of RNAs and proteins necessary to allow enzymatic production of other larger components and ultimately all components needed for a self-replicating organism. It is also not yet clear how to “start up” such an organism^{117,118}.

Cost and toxicity of peptidomimetics

In addition to achieving their biological objectives, peptidomimics will need to overcome concerns about cost and toxicity to succeed as therapeutics. Currently there are no FDA-approved fully peptidomimetic peptides, so information on their *in vivo* toxicity is extremely limited. Initial data from two D-peptides that have advanced to clinical trials (Genzyme's Delmitide¹¹⁹ and Allelix's ALX40-4C¹²⁰) showed that both D-peptides (one orally administered, one systemically delivered) were well tolerated in humans. Further comfort is provided by over a dozen approved D-amino acid-containing peptides, as well as two approved β -amino acid-containing peptides¹²¹. These data suggest that these amino acids are not intrinsically toxic, but more rigorous animal toxicology studies on different classes of fully protease-resistant peptides will be required for a definitive determination. Such studies will also determine whether these peptidomimetics induce significant immunogenicity upon

chronic administration. Finally, the cost of D-, β -, and other uncommon amino acids is currently significantly higher than the corresponding common L-amino acids, largely because of their current status as specialty reagents. However, we anticipate the cost of these amino acids will drop dramatically as they are adopted in high-volume production of therapeutic peptides, as has already occurred with several D-amino acids in large-scale peptide production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

HIV entry pathway. HIV Env is composed of surface (gp120, green) and transmembrane (gp41, blue) subunits. Fusion is initiated by binding to CD4 and a chemokine coreceptor, which activates gp41 and induces formation of the prehairpin intermediate. In this intermediate, the gp41 N-terminal region forms a trimeric coiled coil (N-trimer, gray), which is separated from the C-peptide region (dark blue). This intermediate slowly collapses to form a trimer-of-hairpins structure that brings the viral and cell membranes into close apposition, leading to fusion. C-peptide and D-peptide inhibitors bind to the N-trimer, preventing trimer-of-hairpins formation and membrane fusion.

Fig. 2. One pocket, two binding solutions. The gp41 pocket (from pdb code 3L35) is shown with A) the natural gp41 C-peptide (pdb code 1AIK) and B) D-peptide PIE12 (pdb code 3L35). Structures were aligned on the 17 pocket-forming residues from gp41 and rendered using Pymol.

Fig. 3.
Peptidomimetic structures.

Fig. 4. Mirror-image phage display. Phage bearing L-peptides are panned against a mirror-image protein (D-target). By symmetry, D-versions of binding peptides will bind to the natural L-targets.