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Targeting recognition surfaces on natural proteins with peptidic foldamers

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Abstract

Molecules intended to antagonize protein–protein interactions or augment polypeptide-based signaling must bind tightly to large and specific surfaces on target proteins. Some types of unnatural oligomers with discrete folding propensities (‘foldamers’) have recently been shown to display this capability. This review covers important recent advances among several classes of foldamers, including α -peptides with secondary structures stabilized by covalent bonds, D- α -peptides, α/β -peptides and oligo-oxopiperazines. Recent advances in this area have involved enhancing membrane permeability to provide access to intracellular protein targets, improving pharmacokinetics and duration of action *in vivo*, and developing strategies appropriate for targeting large and irregularly-shaped protein surfaces.

Introduction

Inhibiting specific protein–protein interactions or augmenting polypeptide-based signaling can be effective for the treatment of diverse human diseases. Poly-L- α -peptides, including engineered antibodies, polypeptide hormones and hormone analogues, have been very successful in this regard, but such molecules can be challenging to produce, susceptible to proteolytic degradation *in vivo* and subject to adverse immunological responses. These realities have inspired exploration of oligomers based on unnatural amide-based backbones that display discrete conformational propensities, collectively designated ‘foldamers’, as an alternative source of ligands specific for large surfaces on target proteins. Significant progress toward these goals has been reported in the past few years and serves as the focus of this brief review.

α -Peptides with buttressed secondary structures

Individual α -helices play important roles in many protein–protein interfaces [1,2], but isolated α -helices are generally not very stable conformationally. Strategies to fortify short α -helical conformations via side chain crosslinking have therefore received considerable attention. Although the α -amino acid-derived backbone is retained, this work seems

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Conflict of interest statement

JWC and SHG are co-inventors on a patent application covering several of the α/β -peptides discussed in this review. SHG is a co-inventor on several other foldamer patent applications. SHG is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of α/β -peptides.

pertinent to our consideration of ‘foldamer’ approaches. Many crosslinking chemistries have been evaluated [3]; perhaps most popular now is crosslinking of unnatural alkene side chains via olefin metathesis, as first described by Blackwell and Grubbs [4]. Schafmeister and Verdine subsequently employed α,α -disubstituted alkene residues and purely hydrocarbon crosslinkers (Figure 1) [5]. These ‘stapled’ helical α -peptides have been designed to target a variety of protein–protein interactions, and some cross cell membranes to reach intracellular targets [6–8]. In a recent example, a hydrocarbon-crosslinked peptide derived from B cell lymphoma 9 (BCL9), designed to disrupt the BCL9- β -catenin complex (a transcriptional regulator in the Wnt signaling pathway), suppressed tumor growth in two Wnt-driven mouse models of cancer [9•]. Peptides with more than one crosslink generally provide modest benefits relative to those with a single crosslink [10,11]. Recently, Grossmann *et al.* showed that hydrocarbon crosslinking can stabilize an irregular (non-helical) secondary structure, which opens new possibilities for application of this design strategy [12•].

At present, it is not clear how a hydrocarbon crosslink can confer membrane permeability. The high hydrophobicity of such appendages presumably favors peptide-membrane association. Such peptides may enter cells through passive diffusion or endocytosis [6,13•]. However, the incorporation of a hydrocarbon crosslink does not guarantee membrane permeability [7,14,15•,16•]. Furthermore, some of these peptides display problematic properties including low solubility and inactivation by serum [7,8,17,18•]. The crosslink can make direct contacts with a target protein, which may alter recognition properties and selectivity relative to the non-stapled prototype peptide [19,20].

Arora *et al.* have pioneered an alternative crosslinking strategy for α -helix stabilization, the hydrogen bond surrogate (HBS) approach (Figure 1). Here, an $i,i+4$ backbone hydrogen bond is replaced by a carbon–carbon bond generated via olefin metathesis [21,22], leading to substantially enhanced helix stability and less exposure of the crosslinker to the environment relative to side chain-to-side chain crosslinks [22,23]. Relative to side chain crosslinking, the HBS approach has the advantage that side chain functionality is not altered. Recent applications of the HBS approach include a design based on hypoxia-inducible factor 1 α (HIF-1 α), a protein that affects expression of certain oncogenes [24]. One such HBS peptide bound co-activator protein p300 and blocked its interaction with HIF-1 α , inhibiting transcription of target genes. A related HBS peptide targeting p300 was shown to suppress tumor growth *in vivo* [25•], which represents a very significant advance. A different HBS design, derived from the guanine nucleotide exchange factor Sos, inhibited the Ras-Sos interaction in living cells [26], which shows that the HBS strategy has generality in terms of achieving cell-permeability.

D-Peptides

Peptides comprised of D- α -amino acid residues (Figure 1) are dramatically less susceptible to proteolytic degradation relative to L-peptides [27,28]. However, rational design of D-peptides to target a particular protein surface is challenging [29,30]. Mirror-image phage display, a clever strategy developed by Kim *et al.* [31], enables the discovery of D-peptides that target a specific protein surface [32]. Phage-based peptide libraries are screened for binding to the enantiomer of the protein target (a D-protein); the enantiomer of an L-peptide

identified in this way must bind to the native L-protein. D-Peptide ligands for gp41 [33–35], MDM2 [36,37], PD-L1 [38••], and vascular endothelial growth factor (VEGF) (Figure 2a) [39••] have been described. An anti-VEGF D-peptide displayed a longer *in vivo* half-life and lower immunogenicity in mice relative to the enantiomer [40•]. A potential limitation of mirror-image phage display is the requirement for chemical synthesis of the D-protein target, which becomes increasingly difficult as size increases. D-Peptides are competent as haptens, that is, antibodies against D-peptides can be raised if T cell help is provided [41•]; thus, it remains unclear whether long-term administration of D-peptides or D-proteins could provoke an immune response. This concern applies to all oligomers with unnatural backbones.

β-Peptides

Some of the earliest research on peptidic foldamers focused on oligomers of β-amino acids, or 'β-peptides' (Figure 1) [42–44]. Although β-peptides can adopt specific helical conformations, and they are highly resistant to protease degradation, this foldamer class has not proven to be generally effective in terms of α-helix mimicry [44,45]. Helical conformations available to β-peptides, such as the 12-helix or 14-helix, do not recapitulate the three-dimensional side chain display patterns observed for α-helices. This mismatch was recently highlighted in the context of efforts to mimic the type 2 diabetes drug exenatide, a potent α-peptide agonist of the glucagon-like peptide-1 receptor (GLP-1R) [46]. A long α-helix formed in the C-terminal portion of exenatide makes extensive contacts with the extracellular domain of GLP-1R [47]. Replacement of this α-helix-forming segment with a β-peptide segment caused a dramatic loss in agonist potency [48].

α/β-Peptides

When the proportion and distribution of β residues are properly chosen, oligomers containing both α-amino acid and β-amino acid residues ('α/β-peptides', Figure 1) can adopt a helical conformation that very closely resembles the canonical α-helix formed by pure α-peptide backbones [44]. Many α/β-peptides containing 25–33% β residues, with ααβ, αααβ or ααβαααβ backbone patterns and lengths up to 38 residues (10 helical turns), have been demonstrated by crystallography to form α-helix-mimetic secondary structures [44,49–53]. This family of α/β-peptides appears to be unique among known foldamers in the ability to display large numbers of side chains in a manner that closely mimics side chain presentation by a long α-helix. However, this level of mimicry comes at a potential cost in terms of susceptibility to degradation by proteases, because these oligomers are comprised of mostly α residues. Evaluation of diverse 18–38-mer α/β-peptides with aggressive enzymes such as proteinase K indicates that even spacing of 25–33% β residues across a sequence typically provides substantial protection from degradation, often 100-fold increase in half-life relative to the analogous α-peptide [44,49–51,54]. α/β-Peptides with higher β residue densities should be even more resistant to proteolysis, but the ability to mimic an α-helical conformation seems to diminish at β residue proportions above 33% [52].

Two types of β residues have been employed so far in generating α-helix-mimetic α/β-peptides: (i) β³ residues, which bear a side chain on Cβ (adjacent to nitrogen), typically the

native side chain; and (ii) cyclic β residues in which C α and C β are incorporated into a five-membered ring (see Figures 2c and 3b). The cyclic β residues stabilize an α -helix-like conformation relative to the more flexible β^3 residues, but the side chain diversity available for cyclic β residues is limited at present. Mimicry of α -helical BH3 domains, which bind to complementary clefts on anti-apoptotic proteins in the Bcl-2 family, has been a useful model system for establishing general design guidelines [50,54], and recently this system has been used to evaluate the impact of $\beta^3 \rightarrow$ cyclic β replacement [55]. At sites that do not make contact with the target protein, multiple $\beta^3 \rightarrow$ cyclic β replacements enhance affinity for Bcl-2 family proteins. At sites that directly engage the partner protein surface, $\beta^3 \rightarrow$ cyclic β replacement can lead to substantial selectivity among Bcl-2 family members. In addition, $\beta^3 \rightarrow$ cyclic β replacement enhances resistance to proteolysis, as previously observed in other systems [49,53].

Bim BH3 domain-mimetic α/β -peptides were recently used to evaluate the compatibility of this foldamer class with a side chain crosslinking strategy that has been developed with α -peptides [56]. A hydrocarbon-cross-linked α/β -peptide was shown to match a reported cross-linked α -peptide analogue [57] in terms of affinity for several Bcl-2 family partners, and both crosslinked peptides induced apoptosis in U937 lymphoma cells, but not other types of cancer cells [56]. The most significant functional difference involved enzymatic cleavage: the crosslinked α/β -peptide was ~ 100 -fold more resistant to proteolysis than was the crosslinked α -peptide. α/β -Peptide mimicry is also compatible with the HBS approach, as recently demonstrated by Arora *et al.* [58]. This group showed that HBS α/β -peptide analogues of the N-terminal segment of p53 could be more conformationally defined than the parent HBS α -peptide.

Beyond isolated α -helices

The helix-into-cleft motif illustrated by BH3 domain binding to an anti-apoptotic Bcl-2 family protein is architecturally simple relative to most protein-protein interactions, which frequently involve specific but irregularly-shaped surfaces on both partners. Although the use of periodic $\alpha \rightarrow \beta$ substitution can be effective for generating protease-resistant α -helix mimics, this approach is less successful when the poly- α -peptide backbone is not helical [59]. A strategy of potentially broad scope based on side-by-side helix pairs has recently been demonstrated by Checco *et al.* [60••]. This work was inspired by the large number of peptides derived from the 'Z-domain', a three-helix mini-protein scaffold, that have been optimized via phage display to bind specifically to diverse protein partners [61–63]. Target proteins are engaged by a broad surface generated by two of the three Z-domain helices; the third helix stabilizes the helical tertiary structure of the Z-domain-derived peptide. This 'structural' helix can be eliminated via careful engineering to generate a two-helix 'mini-Z-domain' (40 residues) in which the helix-loop-helix tertiary structure necessary to create the target-binding surface is reinforced by an inter-helical disulfide [64,65]. Checco *et al.* developed an α/β mini-Z-domain scaffold containing unnatural residues (β and Aib) on the opposite side of the tertiary structure relative to the target-recognition surface (Figure 2b) [60••]. Z-domain-derived peptides that bind to VEGF, tumor necrosis factor- α (TNF α) or IgG identified by other groups were used to select 'contact' residues to be displayed by the target-recognition surface on the α/β mini-Z scaffold. This approach generated α/β mini-Z-

domains that could bind strongly and selectively to each of the three protein targets. The unnatural subunits within α/β mini-Z-domains conferred considerable protease resistance relative to a full-length Z-domain or an all- α mini-Z-domain.

Peptide hormone analogues

G protein-coupled receptors (GPCRs) in the B-family are activated by long polypeptide hormones such as glucagon-like peptide-1 (GLP-1), parathyroid hormone (PTH), and vasoactive intestinal peptide (VIP). Several peptide drugs that target these GPCRs have been approved (e.g. exenatide, liraglutide, teriparatide, teduglutide). Foldamer-based analogues of the natural peptide agonists might be attractive because such analogues should be less susceptible to proteolytic cleavage and therefore display improved pharmacokinetics (longer *in vivo* half-life). However, precedents show that replacing even a small part of a natural polypeptide hormone with non-natural segments can drastically diminish activity. The negative consequences of partial β -peptide replacement in an exenatide analogue were noted above [48]. Among PTH analogues it has been shown that a single $\alpha \rightarrow \beta$ replacement can be highly deleterious [66].

Three recent studies have demonstrated that multiple $\alpha \rightarrow \beta$ replacements at carefully selected positions can provide potent hormone analogues that maintain activity *in vivo*. Cheloha *et al.* evaluated α/β -peptide analogues of PTH(1–34) that contained exclusively β^3 residues (5–7 substitutions) in an $\alpha\alpha\alpha\beta$ pattern distributed over the C-terminal portion (Figure 3a) [67••]. All α/β -peptide analogues were agonists of PTHR1, with only minor variations in potency arising from variations in β^3 residue placement. These analogues were compared in terms of their affinities for two functionally distinct states of PTHR1, one in which the G_{α_s} protein is associated with the intracellular receptor surface (the ‘RG’ state), and another in which G_{α_s} is absent (‘R⁰’). These two receptor states presumably differ subtly in conformation [68]. Affinity for RG appears to correlate with agonist potency, while affinity for R⁰ seems to correlate with duration of action *in vivo* [69]. Most β^3 residue placements had little impact on RG affinity, but significant variations were observed in R⁰ affinity [67••]. Differences in relative RG vs. R⁰ affinity between two α/β analogues of PTH(1–34) appeared to correlate with pharmacodynamic differences *in vivo* (Figure 3c). This observation raises the intriguing possibility that $\alpha \rightarrow \beta$ replacements may offer a powerful and general way to modulate signaling via B-family GPCRs.

Johnson *et al.* examined α/β -peptide analogues of GLP-1 [70••]. Initial efforts focused on $\alpha \rightarrow \beta^3$ replacements; however, in stark contrast to trends among PTH(1–34) analogues, even a few $\alpha \rightarrow \beta^3$ replacements near the C-terminus ($\alpha\alpha\alpha\beta$ pattern) were highly deleterious in terms of potency at GLP-1R. Activity could be rescued by using ring-constrained β residues. An analogue containing five cyclic β residues along with two Aib residues (Figure 3a) displayed high resistance to cleavage by dipeptidyl peptidase-4 or neprilysin, the two proteases that rapidly degrade GLP-1 *in vivo*. In mice, this α/β -peptide mimicked GLP-1 in fostering regulation of blood glucose levels (Figure 3d), but the α/β -peptide displayed extended duration of action relative to GLP-1 itself, presumably because the α/β -peptide resists proteolysis.

Olson *et al.* described α/β -peptide analogues of VIP that are selective for one or the other of the two receptors, VIPR1 and VIPR2, that are activated by the peptide hormone itself [71••]. These analogues were evaluated in a mouse model for the neurodegeneration associated with Parkinson's Disease. The VIPR2-selective α/β -peptide analogue displayed particularly favorable effects in terms of protecting neurons in the substantia nigra region of the brain and altering the animals' immunological/ inflammatory profile.

Immune response to α/β -peptides

Foldamers that engage target protein surfaces *in vivo* and are intended for diagnostic or therapeutic applications should ideally not be susceptible to immunological recognition. Polypeptides are 'perceived' via two immunological mechanisms, one based on antibodies and B cell receptors, the other based on major histocompatibility complexes (MHCs) and T cell receptors (TCRs). MHCs bind relatively short peptides generated by proteolytic cleavage of proteins, for presentation at the cell surface, and MHC-peptide complexes are interrogated by TCRs. Several groups have reported that MHC I epitopes containing a single $\alpha \rightarrow \beta^3$ replacement can be recognized by a TCR that recognizes the homologous α -peptide bound to the MHC [72–74]. However, Cheloha *et al.* observed that MHC I epitopes containing two or three $\alpha \rightarrow \beta^3$ replacements ($\alpha\alpha\alpha\beta$ or $\alpha\alpha\beta\alpha\alpha\beta$ pattern) bound weakly to an MHC I that recognizes the homologous α -peptide, and that these α/β -peptides did not elicit TCR-based signaling [41•]. In addition, antibodies raised against an α -peptide did not cross-react substantially with α/β -peptides containing $\alpha \rightarrow \beta^3$ replacements in the $\alpha\alpha\alpha\beta$ or $\alpha\alpha\beta\alpha\alpha\beta$ pattern. These α/β -peptides and the enantiomeric α -peptide (D-peptide) were competent as haptens when conjugated to a carrier protein, but the resulting antibodies did not cross-react strongly with the original L- α -peptide. Overall, these findings are encouraging with regard to *in vivo* applications of α/β -peptides, but whether these observations represent general trends will not be clear until additional systems have been examined.

More dramatic excursions from the α -peptide backbone

Arora *et al.* have developed oxopiperazine helix mimetics (OHMs) for modulating helix-mediated protein–protein interactions [75]. In a particularly impressive recent example, OHMs intended to mimic the α -helical domain of HIF-1 α were shown to target the transcriptional co-activator p300 (Figure 4a) [76••]. These oligomers were active *in vivo*, downregulating hypoxia-induced gene expression and reducing tumor volume in mice bearing MDA-MB-231 xenografts. A subsequent study showed that the Rosetta software can be used to design OHMs that display high affinity and specificity for protein targets [77••].

Wilson *et al.* have reported significant recent advances in the development of aromatic oligoamide mimics of short α -helices involved in protein–protein recognition [78••]; this approach was pioneered by Hamilton *et al.* [79]. The Wilson group evaluated a small library of trimers containing N-alkylated para-aminobenzoate units; the three alkyl groups were intended to reproduce the display of three spatially consecutive side chains along the side of an α -helix (Figure 4b) [78••]. Examples that engage the p53-recognition cleft on HDM2 or

the BH3-recognition cleft on Mcl-1 were identified; activity in cell-based assays indicated that these aromatic oligoamides can cross cell membranes.

Limitations and challenges

Although recent progress in the development of foldamers that engage specific protein surfaces has been substantial, challenges and opportunities for innovation remain. Many different scaffolds can mimic short α -helices, such as the one formed at the N-terminus of p53 that is bound by HDM2; however, short α -helices can also be mimicked effectively by small organic molecules (such as the nutlins) [80], which suggests that foldamer approaches to this type of target must achieve outstanding performance to have a practical impact. In this regard the OHM strategy of Arora *et al.* seems particularly promising [75,76•,77•]. For longer α -helices, only the α/β -peptide approach has proven to be effective so far, to our knowledge. It would be exciting to find a purely unnatural backbone (no α residues) that can mimic the information encoded along the surface of a long α -helix.

Most protein–protein interfaces involve surfaces on each partner that are broader and less regular than a single α -helix. Engagement of these surfaces with molecules other than poly-L- α -peptides has so far been limited to two classes of unnatural oligomers, poly-D- α -peptides [39•] and two-helix α/β -peptides (Figure 2) [60•]. In both cases, identification of target-specific ligands can be accomplished indirectly via techniques such as phage display. For D-peptides, this approach requires access to the enantiomer of the protein target, which may be problematic for larger targets. For α/β mini-Z-domains, this approach involves grafting contact residues from a full-length Z-domain α -peptide. Despite a few successes in each case, the generality of these strategies remains to be established.

Ultimately, it seems likely that creative researchers will identify new foldamer-based designs with capabilities that are complementary or superior relative to poly-D- α -peptides or two-helix α/β -peptides in terms of targeting broad and irregular surfaces on proteins of biomedical interest. Progress of this type could emerge as chemists develop synthetic approaches to amino acids that represent sub-units for new families of peptidic foldamers. Such subunits could include γ -amino acids or δ -amino acids containing conformational constraints that lead to diverse but predictable conformational behavior. Facile preparative routes to new building blocks would support the fundamental structural studies necessary to establish folding ‘rules’ for peptidic backbones that contain these subunits. Thus, the successes that have recently been achieved with two classes of non-proteinogenic building block, D- α -amino acids and β -amino acids, provide strong motivation for expansion of basic research in the foldamer field.

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Figure 1.

Structures identifying some of the helix-stabilization and foldamer approaches described in the text. Structural elements that deviate from traditional peptides are highlighted in red.

Figure 2.

Foldamer strategies for targeting large and irregularly-shaped protein surfaces. **(a)** A D-peptide derived from mirror-image phage display (pink cartoon) in complex with VEGF (grey surface) (PDB: 4GLN) [39••]. **(b)** An α/β -peptide mini-Z-domain (yellow/green/orange cartoon) in complex with VEGF (grey surface) (PDB: 4WPB) [60••]. **(c)** Structures of a generic L- α residue (yellow), a generic D- α residue (pink), the Aib residue (green), and cyclic β residues ACPC and APC (orange). Colored circles correspond to residue types for the D-peptide and the α/β -peptide in panels A and B.

Figure 3.

α/β -Peptide mimicry of G protein-coupled receptor agonists. **(a)** Primary sequences of PTH(1-34), GLP-1(7-37) and biologically active α/β -peptide analogues. Colored circles indicate non-natural residues, as indicated in panel b. **(b)** Structures of a generic α residue, the Aib residue (green), a generic β^3 residue (blue), and cyclic β residues ACPC and APC (orange). **(c)** Effects of the indicated α -peptide or α/β -peptide (20 nmol/kg) on blood Ca²⁺ levels in mice [67••]. **(d)** Effects of the indicated α -peptide or α/β -peptide (1 mg/kg) on plasma glucose levels during a glucose tolerance test in mice [70••].

Figure 4. α -Helix-mimetic polyamides. **(a)** Cartoon representation of a portion of the HIF-1 α α -helix (left, PDB: 1L8C) and drawing of an OHM mimic of this helix (right) [76••]. Critical protein-contacting side chains are highlighted in red. **(b)** Cartoon representation of a portion of the p53 α -helix (left, PDB: 1YCR) and drawing of an aromatic oligoamide mimic (right) [78••]. Critical protein-contacting side chains are highlighted in red.