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Folding and function in α/β-peptides: Targets and therapeutic applications

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Abstract

Combining natural α-amino acid residues and unnatural β-amino acid residues in a single chain leads to heterogeneous-backbone oligomers called α/β-peptides. Despite their unnatural backbones, α/β-peptides can manifest a variety of folding patterns and biological functions reminiscent of natural peptides and proteins. Moreover, incorporation of β-residues can impart useful properties to the oligomer such as improved stability to degradation by protease enzymes. α/β-Peptides have been developed that engage diverse biological targets, including proteins involved in apoptotic signaling, HIV-cell fusion, hormone signaling, and angiogenesis. For some systems, promising results obtained *in vitro* have paved the way for demonstrated activity *in vivo*, where α/β-peptides show equal potency and improved duration of effect compared to α-peptide counterparts.

Introduction

Protein–protein binding interactions are involved in myriad cellular functions, including those associated with many diseases. Agents that modulate binding interactions between proteins have tremendous therapeutic potential; however, the structural properties of protein–protein interfaces can make the development of inhibitors challenging.[1] Peptides and proteins have proven a promising alternative to small-molecule scaffolds for some targets. While the number of peptide-based drugs is large and growing, they suffer from drawbacks to clinical use such as rapid degradation by proteases in serum.[2] A broad field of research has demonstrated that α-peptides are just one of many oligomeric backbones capable of complex folding behavior and that their unnatural-backbone analogues can adopt similar folds while resisting enzymatic degradation. Efforts to develop unnatural oligomers with folding patterns reminiscent of peptides and proteins (“foldamers”) have recently expanded into the realm of biological function.[3-6] Among the many backbones explored in this context, chains that blend α-amino acid residues with β-amino acid residues (α/β-peptides) have emerged as an important class of heterogeneous-backbone foldamers.[7-10] The present review surveys published research from the last ~5 years on α/β-peptides of

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moderate length that show biological function resulting from binding to a natural protein receptor (Table 1).

β-Residues are among the most common building blocks in foldamer research, and a number of interesting biological activities have been reported for appropriately designed β-peptides.[3-6] Although β-residues are structurally diverse, those found in the bioactive α/β-peptides covered in this review fall into one of two categories (Figure 1): β^3 or β^{cyc}. β^3-Residues are backbone-homologated variants of α-residues. In α/β-peptides, β^3-residues offer the advantage of retaining protein-like side chain functionality at the potential cost of increased backbone flexibility due to an additional rotatable bond. In β^{cyc}-residues (e.g., ACBC, ACC, ACPC, APC), the central backbone torsion is constrained by a carbocyclic or heterocyclic ring. This offers the advantage of exerting control over folding behavior of the β-residue based on selection of ring size and stereochemistry.

Apoptotic Signaling in the Bcl-2 Protein Family

A network of binding interactions among B-cell lymphoma-2 (Bcl-2) family proteins regulates the life and death of cells.[11] Anti-apoptotic Bcl-2 proteins, commonly overexpressed in tumor cells, exert their biological effect by binding to an amphiphilic α-helical BH3 domain on pro-apoptotic effectors, and the inhibition of this interaction has been an active target for development of cancer therapies.[12] The BH3 domain is an important early example where α/β-peptide foldamer mimicry of a bioactive α-helix led to inhibition of a biologically important protein–protein binding interaction. While the oldest of the systems covered in this review, work on the BH3 domain helps define two paradigms for the development of bioactive α/β-peptides.

Pioneering structural studies elucidated several helical folding patterns available to α/β-peptides with an alternating 1:1 backbone repeat,[13,14] and one of these scaffolds was used to develop an α/β-peptide BH3 domain mimic.[15] Structure-guided introduction of side chains on the foldamer scaffold based on the known structure of the Bcl-2 family effector protein Bak bound to anti-apoptotic Bcl-x_L resulted in an α/β-peptide that bound Bcl-x_L, albeit with affinity much weaker than the prototype α-peptide BH3 domain. Replacement of the C-terminal half of the alternating α/β-peptide with a pure α-peptide backbone resulted in a chimeric oligomer with tight binding affinity for Bcl-x_L as well as the ability to induce release of mitochondrial cytochrome C in cancer cell lysates. Unfortunately, the α-residue rich portion of the chimera proved highly susceptible to proteolytic degradation.

Shortly after the work on chimeric α/β-peptide BH3 mimetics, an alternate design approach was developed. Starting from the BH3 domain of Bcl-2 family member Puma, a series of α/β-peptide variants were examined in which the side chain sequence of the natural ligand was displayed on all possible α/β^3-peptide backbones with an ααβααβ repeat.[16] This “sequence-based” residue replacement strategy was inspired by findings that such modification can generate α/β-peptides with complex helix-bundle folding patterns.[17] Screening seven oligomers based on the Puma BH3 domain yielded an α/β-peptide with Bcl-x_L binding affinity indistinguishable from the natural α-peptide and significantly improved proteolytic stability. Later structural studies confirmed the binding mode of the Puma-based

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α/β-peptide with Bcl-x_L was identical to that of natural BH3 domains.[18] An important difference between the Puma α-peptide and its α/β-peptide mimic was that the unnatural backbone lost the capacity to bind tightly to the anti-poptotic Bcl-2 family protein Mcl-1; however, structure-guided side-chain modification was able to restore this function.[19]

A key finding related to the α/β-peptide Puma BH3 domain mimics was the importance of the pattern of backbone alteration. Two α/β-peptides with identical side-chain sequences but different patterns of β^3-residue incorporation bound to Bcl-x_L with 10^5-fold different association affinities.[16] This observation was explored though a comprehensive screen of all possible α/β-peptides resulting from $\alpha \rightarrow \beta^3$ residue replacement in the Bim BH3 domain with an αααβ, ααβααβ, or ααβ repeat.[20] The results underscored the idea of backbone modification as a means for controlling binding selectivity. The Bim BH3 domain binds promiscuously to anti-apoptotic Bcl-2 family members, including Bcl-x_L and Mcl-1. Effective α/β-Peptide Bim BH3 mimics were found for both Bcl-x_L and Mcl-1; however, the optimal pattern of $\alpha \rightarrow \beta^3$ residue replacement for high affinity binding differed for the two targets.

HIV Cell Entry via the Viral Protein gp41

The rapid success of sequence-based design as a strategy to generate α/β-peptide mimics of BH3 domains suggested it might be applicable to other prototype α-peptide sequences. Another system in which the concept was explored was a protein–protein interaction involved in cell entry by human immunodeficiency virus (HIV). As an enveloped virus, HIV must deliver its genetic material through the fusion of the viral and host cell membranes.[21] This process is mediated by gp41, a trimeric viral protein anchored to the HIV membrane via its C-terminus. In the course of host cell recognition by the virus, the N-terminus of gp41 inserts into the plasma membrane, anchoring the virion to the cell. A structural rearrangement ensues in which three helical gp41 C-terminal heptad repeat (CHR) domains pack against a trimer of helices in the gp41 N-terminal heptad repeat (NHR) region. The resulting antiparallel six-helix bundle fuses the viral and host plasma membranes by inducing close physical proximity. The CHR/NHR interaction has been successfully targeted clinically for HIV treatment with the 36-residue α-peptide entry inhibitor enfuvirtude, a fragment of the gp41 CHR domain.[22]

The first attempts at developing α/β-peptide inhibitors of gp41-mediated HIV cell entry were guided by success in the Bcl-2 family proteins described above; sequence-guided $\alpha \rightarrow \beta^3$ residue replacement was carried out in a known CHR α-peptide ligand. These efforts were not fruitful, however, leading to a best-case α/β-peptide affinity for the gp41 CHR binding cleft 10^4-fold weaker than the prototype α-peptide.[23] A solution to the problem of low affinity was found through the strategic rigidification of the α/β-peptide backbone by replacing a subset of β^3-residues with cyclically constrained analogues. This approach was inspired by earlier work on α/β-peptide helix bundles, in which it was found that β^{cyc}-residues based on a *trans*-substituted five-membered ring (ACPC, APC) supported similar helical folds as β^3 residues while considerably stabilizing the quaternary structure.[24] $\beta^3 \rightarrow \beta^{cyc}$ substitution in the CHR α/β-peptide mimics had a similar stabilizing effect.[23] The best rigidified variant showed excellent structural homology to the native CHR domain,

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comparable antiviral activity in cell-based infectivity assays, and improved stability to degradation by proteases (Figure 2). While it is tempting to suggest an entropic effect resulting from a rigidified backbone, later work revealed that gp41 CHR mimetic α/β-peptides lacking β^{cyc}-residues but capable of forming an array of salt bridges involving solvent-exposed β^3-residues were equally potent.[25] Recent insights into the folding thermodynamics of protein-like tertiary structures with α/β-peptide backbones suggest the relationship between folding energetics among α-, β^3-, and β^{cyc}-residues is more complex than previously appreciated.[26]

G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are responsible for transducing signals from extracellular stimuli across the membrane and into the cell. GPCRs are a diverse protein class (~800 exist in humans); this diversity is reflected in the structures of their ligands, which include small molecules, peptides (as small as 3 residues), and small proteins (<60 residues).[27] GPCRs are important in medicinal chemistry, representing the target of action of the majority of FDA-approved drugs.[28] The difficulty identifying small molecules that modulate the function of GPCRs that natively bind large peptide ligands has motivated efforts to create peptide-based therapeutics for these targets.[29] Replacement of α-residues in short peptide GPCR ligands with unnatural building blocks has a rich history in peptidomimetics research, and β-residues have found use in several such studies.[10] A body of more recent work, summarized below, highlights the potential of α/β-peptides to mimic larger GPCR peptide ligands.

The 36-residue hormone neuropeptide Y (NPY) is among the most abundant peptides in the brain and plays diverse biological roles, including regulation of food intake and mood.[30] NPY acts through binding interactions with four Y-family GPCRs: Y_1R, Y_2R, Y_4R, and Y_5R. Because of the varying functions of these different receptor subtypes, synthetic ligands with high affinity and specificity are valuable as both tools to elucidate biological roles of the receptors as well as potential therapeutic leads. Recent efforts have shown α/β-peptide analogues of NPY address an important and previously unmet need for potent ligands that selectively activate Y_4R.[31] Pioneering research in the area demonstrated that incorporation of the β^{cyc}-residue ACC into a 12-residue fragment from NPY generated an α/β-peptide a with an altered profile of binding affinity among the receptor subtypes.[32] Building on this work, a recent study reported a thorough survey of β^{cyc} residues differing in ring size and stereochemistry incorporated at two positions in the NPY sequence.[31] Remarkably, a single $\alpha \rightarrow \beta^{cyc}$ substitution (ACBC) resulted in an α/β-peptide with potent and selective Y_4R agonist activity in cell-based assays.

Parathyroid hormone (PTH) is a protein GPCR ligand that helps to regulate blood calcium levels. The 34-residue N-terminal peptide from PTH ($PTH_{1\text{-}34}$) has the same activity as the full length protein and is a clinically used drug for the treatment of osteoporosis.[33] Structural studies on the interaction of PTH with its receptor (PTH1R) suggest that residues 15-34 fold to form an α-helix that binds with high affinity to the GPCR extracellular domain, while the N-terminal region contacts the transmembrane domain and is responsible for signaling.[34] Sequence-guided $\alpha \rightarrow \beta^3$ residue replacement in an ααaβ pattern generated

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an α/β-peptide PTH_{1-34} analogue with comparable receptor binding affinity and agonist activity in cell-based assays (Figure 3).[35] A particularly significant result of this work was the demonstration that α/β-peptide mimicry of the α-peptide prototype translated from *in vitro* experiments to an *in vivo* context. Mice treated with PTH_{1-34} or an α/β-peptide analogue showed identical initial spikes in blood calcium levels; however, a dramatic difference was observed in the duration of the effect. The α/β-peptide was able to sustain increased blood calcium several hours after levels in mice treated with the α-peptide had returned to baseline. Quantification of peptide in serum suggested that this effect results from enhanced stability of the α/β-peptide to proteolytic degradation.

The 30-residue hormone glucagon-like peptide 1 (GLP-1) stimulates glucose-dependent insulin release through interaction with GPCRs, and several peptide-based GLP-1 mimics are clinically approved drugs for the treatment of diabetes.[36] A drawback to GLP-1 in clinical applications is its rapid degradation in serum, motivating a recent study examining the ability of α/β-peptide analogues to combine efficacy and improved biostability.[37] Sequence-based $\alpha \rightarrow \beta^3$ substitutions, quite successful in the PTH analogues described above, were not tolerated in GLP-1; incorporation of just three β^3-residues in the 30mer abolished agonist activity entirely. The use of β^{cyc}-residues proved much more effective; receptor binding affinity was retained in an analogue with five $\alpha \rightarrow \beta^{cyc}$ replacements in an ααaβ pattern. Interestingly, the 12-residue N-terminal segment of GLP-1 proved quite sensitive to backbone alteration; however, this problem was solved by the judicious use of two α-aminoisobutyric acid residues to block known proteolytic cleavage sites near the N-terminus. The optimized α/β-peptide GLP-1 mimic showed agonist activity identical to the α-peptide hormone in cell-based assays and significantly improved *in vitro* proteolytic stability. These results were maintained *in vivo*, where mice treated with the more proteolytically stable α/β-peptide showed improved glucose tolerance when challenged several hours after dosing compared to mice treated with the α-peptide. Paralleling observations for the PTH mimics, improved stability to proteolytic degradation translated into a more sustained effect *in vivo*.

Angiogenesis

Angiogenesis, the growth of new blood vessels, is fundamental to normal physiology but also plays a role in disease.[38] A crucial contributor to angiogenesis is the protein vascular endothelial growth factor (VEGF), which stimulates blood vessel growth through a signaling cascade that begins with binding to a receptor tyrosine kinase. Inhibitors of angiogenesis, including antibodies that block the interaction between VEGF and its receptors, have found clinical use in the treatment of cancer and diseases of the eye.[38] Efforts to develop smaller agents that block VEGF/VEGFR signaling led to the identification by phage display of a 19-residue α-peptide that blocks angiogenesis by engaging the receptor-binding region of VEGF.[39] In recent work, this sequence was used as a starting point to design an α/β-peptide mimic.[40] A combination of alanine scanning and "β^3 scanning" (individual sequence-guided $\alpha \rightarrow \beta^3$ substitutions) identified sites in the prototype sequence tolerant to backbone and/or side chain modification. Combining several of the most effective substitutions generated an α/β-peptide analogue with ~1/3 unnatural backbone content. The α/β-peptide bound VEGF with much lower affinity than the α-peptide prototype; however, it

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showed markedly improved proteolytic stability and retained some activity in cell-based assays.

While anti-VEGF therapies have shown great promise in the treatment of cancer, their utility is limited in many cases by rapid development of resistance.[41] The protein galectin-1 (Gal-1) has been identified as participating in one compensatory pathway that enables sustained angiogenesis in the face of VEGF-targeted treatment.[42] Interestingly, much earlier work identified a 33-residue α-peptide of *de novo* design (anginex) that inhibits tumor cell proliferation *in vitro* and *in vivo* through a mechanism involving Gal-1 as its primary target.[43,44] From the standpoint of α/β-peptide mimicry, anginex poses the challenge of having a multi-stranded β-sheet as its bioactive conformation. Every other example of an α/β-peptide engaging a biological target covered in this review involves the mimicry of helix or loop structure in the native ligand. Sheets have proven less straightforward than helices for mimicry by α/β-peptides, but some approaches for sequence-guided α→β residue replacement have been reported.[45] Scanning a stripe of $\alpha \rightarrow \beta^3$ residue replacement along the sheet of anginex led to two analogues that showed dose-dependent inhibition of endothelial tumor cell proliferation comparable to the parent α-peptide.[46]

A promising class of α-peptide ligands for diverse protein targets, termed affibodies, have been developed through phage-display on a 58-residue three-helix bacterial protein scaffold.[47] Later work showed the three-helix parent protein could be truncated to a shorter 38-residue two-helix variant without compromising activity.[48] Phage-derived sequences based on both the parent scaffold and truncated version have been shown effective at targeting VEGFR *in vivo* for tumor imaging applications.[49] Sequence-guided $\alpha \rightarrow \beta^3$ residue replacement in the two-helix VEGF-binding α-peptide, along with incorporation of β^{cyc}-residues and Aib, generated an α/β-peptide mimic with identical affinity for VEGF (Figure 4). Comparison of the crystal structures of each ligand in complex with VEGF showed the details of molecular recognition were unchanged between the α-peptide and α/β-peptide.[50] The α/β-peptide showed improved stability to proteolytic degradation *in vitro* and was able to inhibit VEGF-induced proliferation in cell-based assays. The potential generality of the affibody scaffold as a source of α/β-peptide ligands for diverse targets was demonstrated through the design of α/β-peptide analogues of previously reported phage-derived sequences for immunoglobulin G and tumor necrosis factor-α.

Conclusions

α/β-Peptides are a class of molecules with significant therapeutic potential, especially in cases where the biological target is an extended protein surface. While early studies on mixed backbones containing β-residues focused heavily on the mimicry of natural protein structure, more recent work has shifted toward the functional mimicry of bioactive α-peptide sequences. These studies have shown that α/β-peptides can be generated with biological properties identical to a prototype α-peptide, but with increased stability to degradation by proteases in serum. Examples of α/β-peptides with long *in vivo* lifetimes represent a significant milestone toward improving bioavailability, a major limitation of current α-peptide pharmaceuticals. Although questions of potential toxicity and immunogenicity

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remain areas for further investigation, α/β-peptides already show tremendous promise as therapeutic agents and appear to be on a trajectory toward potential clinical use.

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Highlights

- α/β-Peptides blend natural α-amino acid residues with unnatural β-residues
- α/β-Peptides are more resistant than α-peptides to degradation by protease enzymes
- α/β-Peptide mimics have been reported for diverse bioactive α-peptides
- Biological targets include viral-cell fusion, GPCRs, and angiogenesis
- Relative to α-peptides, α/β-peptides can show potent, sustained activity *in vivo*

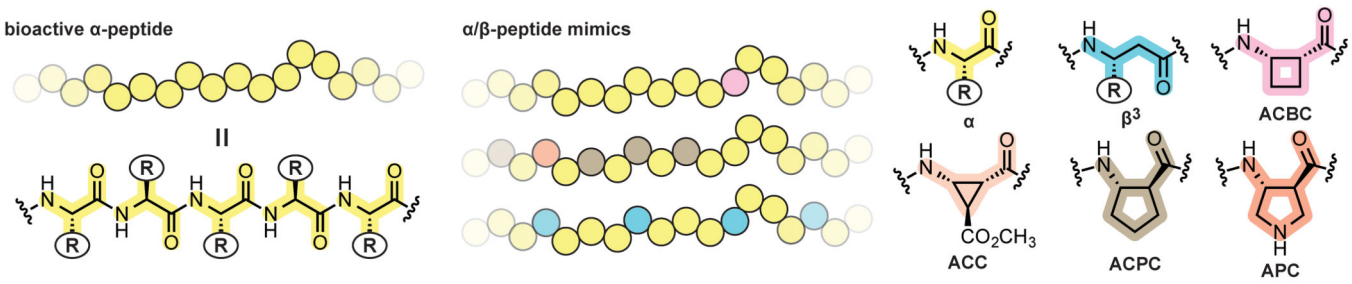

Figure 1.

An α-peptide, example α/β-peptides, and their constituent residue structures. Replacing one or more α-amino acid residues in a biologically active α-peptide sequence with β-residues can lead to heterogeneous-backbone α/β-peptides with native-like function and improved biostability. Chemical structures and abbreviations are shown for a natural α-residue alongside those of the β-residues appearing in α/β-peptides covered in this review.

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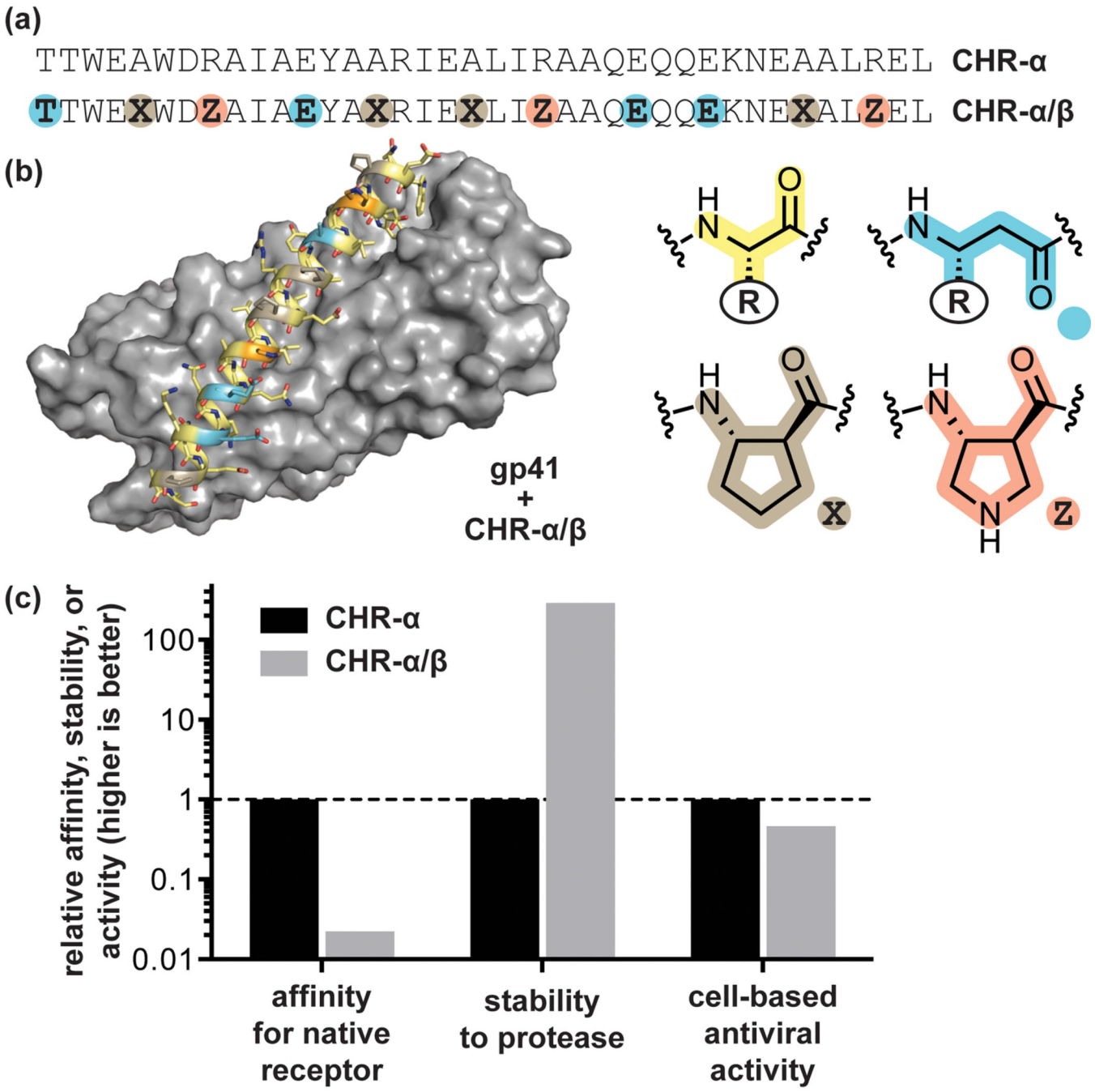

Figure 2.

Design of α/β-peptide mimics of an α-peptide inhibitor of HIV-cell fusion. **(a)** Sequences of the prototype α-peptide inhibitor **CHR-α**, derived from the HIV protein gp41, and its α/β-peptide analogue **CHR-α/β**. **(b)** Crystal structure of **CHR-α/β** bound to an engineered variant of the HIV protein gp41 (PDB: 3O43). **(c)** Comparison of *in vitro* bioactivity data for **CHR-α** and **CHR-α/β**: relative association affinity for the engineered gp41 receptor, relative half-life to proteolytic degradation by the enzyme proteinase K, and relative activity in a cell-based HIV-1 infectivity assay. Source data are from reference [23].

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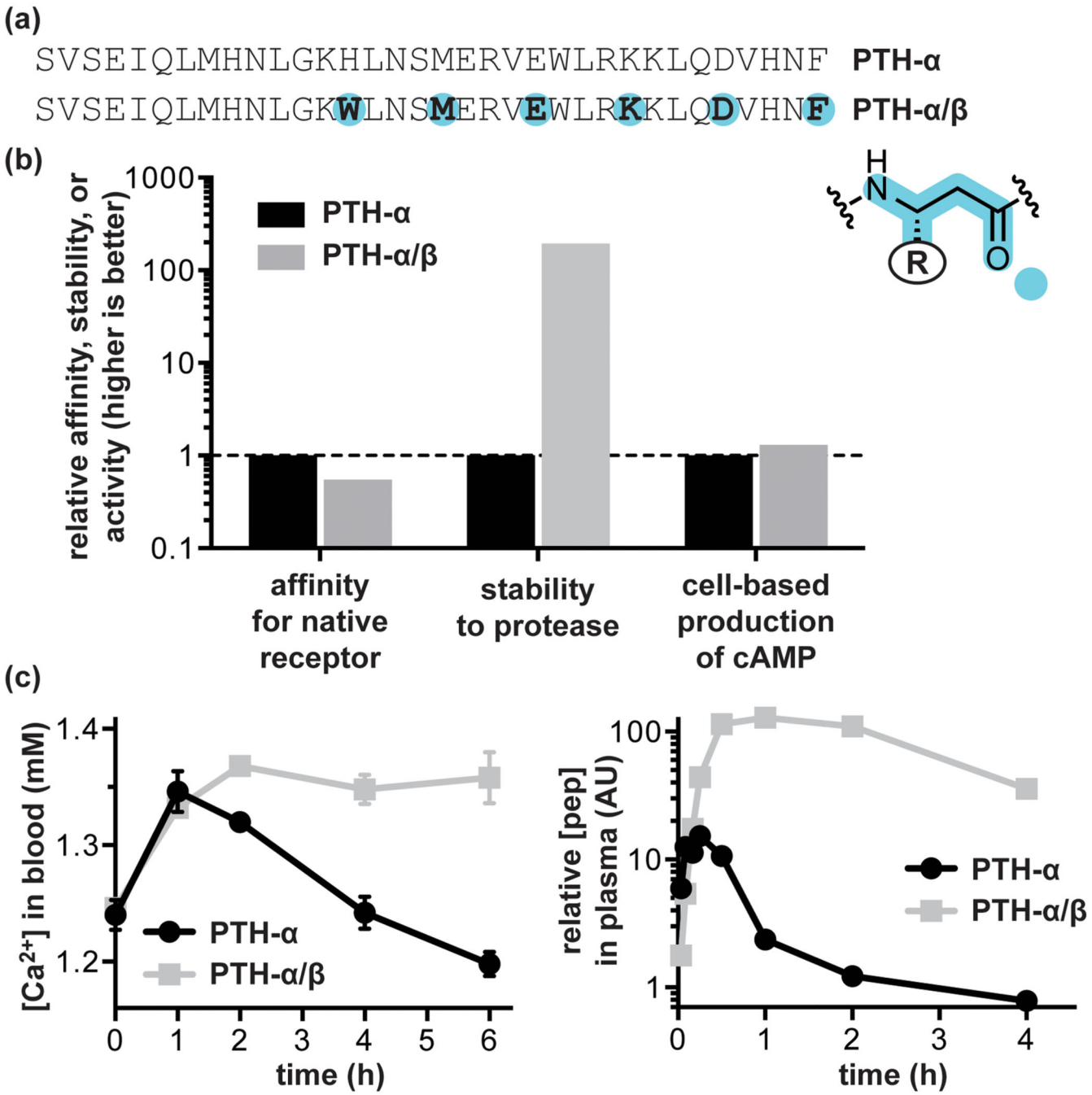

Figure 3.

Design of α/β-peptide mimics of the α-peptide GPCR ligand parathyroid hormone (PTH). **(a)** Sequences of the prototype α-peptide **PTH-α**, residues 1-34 of the native hormone, and its α/β-peptide mimic **PTH-α/β**. **(b)** Comparison of *in vitro* bioactivity data for **PTH-α** and **PTH-α/β**: relative association affinity for parathyroid hormone receptor-1, relative half-life to proteolytic degradation by the enzyme trypsin, and relative activity in a cell-based assay of cyclic adenosine monophosphate (cAMP) production. **(c)** Summary of key *in vivo* data obtained for **PTH-α** and **PTH-α/β** in mice: blood calcium levels after treatment with the

indicated peptide at a dose of 20 nmol/kg and relative concentration of peptide remaining in serum as a function of time after treatment. Source data are from reference [35].

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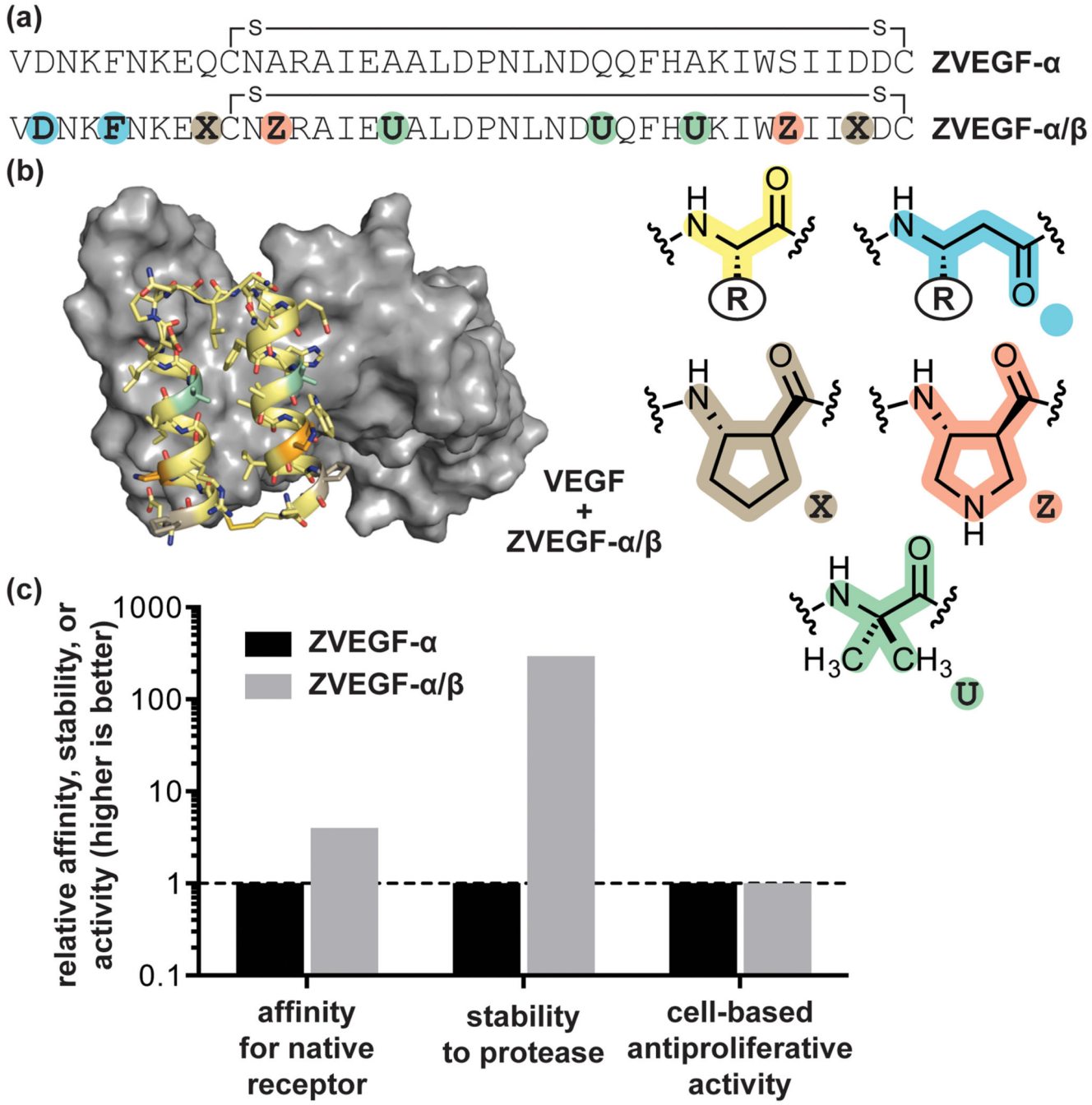

Figure 4.

Design of an α/β-peptide mimic of a phage-derived α-peptide that inhibits the interaction between VEGF and its receptors. **(a)** Sequences of the prototype VEGF-binding α-peptide **ZVEGF-α** and its α/β-peptide mimic **ZVEGF-α/β**. **(b)** Crystal structure of **ZVEGF-α/β** bound to VEGF (PDB: 4WPB). **(c)** Comparison of *in vitro* bioactivity data for **ZVEGF-α** and **ZVEGF-α/β**: relative association affinity for VEGF, relative half-life to proteolytic

degradation by the enzyme proteinase K, and relative activity in a cell-based assay of VEGF-induced proliferation. Source data are from reference [50].

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Table 1

Bioactive α/β-peptides with therapeutic potential.

α-Peptide Prototype	Peptide Length	β-Residue Content	Secondary Structure	Binding Partner	Function (Implication)	Ref
BH3 domains	26	27%	α-helix	Bcl-x_L, Mcl-1	Apoptosis regulation (cancer)	[15,16]
gp41 (CHR domain)	38	29%	α-helix	gp41 (NHR domain)	Viral cell entry (HIV)	[23]
Neuropeptide Y_{25-36}	12	17%	non-regular	Y_1R, Y_4R	Gastrointestinal regulation, vasoconstriction (various)	[31,32]
PTH_{1-34}	34	18%	α-helix	PTH1R	Calcium regulation (osteoporosis)	[35]
GLP-1	31	16%	α-helix	GLP1R	Glucose regulation (diabetes)	[37]
Phage-derived VEGF ligand	19	32%	non-regular[1]	VEGF	Angiogenesis (cancer, diseases of the eye)	[40]
Anginex	34	9%	β-sheet	Gal-1	Angiogenesis, (cancer, diseases of the eye)	[46]
Phage-derived VEGF ligand	39	15%[1]	helix-turn-helix[1]	VEGF	Angiogenesis, (cancer, diseases of the eye)	[50]

[1] Cyclized via a disulfide bond.

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