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Extending Foldamer Design beyond α-Helix Mimicry: α/β-Peptide Inhibitors of Vascular Endothelial Growth Factor Signaling

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Supporting Information

ABSTRACT: Diverse strategies have been explored to mimic the surface displayed by an α-helical segment of a protein, with the goal of creating inhibitors of helix-mediated protein−protein interactions. Many recognition surfaces on proteins, however, are topologically more complex and less regular than a single α-helix. We describe efforts to develop peptidic foldamers that bind to the irregular receptor-recognition surface of vascular endothelial growth factor (VEGF). Our approach begins with a 19-residue α-peptide previously reported by Fairbrother et al. (*Biochemistry* **1998**, 37, 17754) to bind to this surface on VEGF. Systematic evaluation of $\alpha \rightarrow \beta$ replacements throughout this 19-mer sequence enabled us to identify homologues that contain up to ~30% β residues, retain significant affinity for VEGF, and display substantial resistance to proteolysis. These α/β-peptides can block VEGF-stimulated proliferation of human umbilical vein endothelial cells.

Interactions between specific pairs of proteins are biomedically attractive targets for disruption.[1] Pairings in which one partner contributes a relatively small surface area, e.g., a short extended segment or helix, can be blocked in some cases by small-molecule inhibitors,[2] but interactions that feature large surfaces on each partner generally require engineered proteins or large peptides for effective inhibition.[3] Development of inhibitor design strategies that transcend conventional polypeptides represents a fundamental challenge in terms of molecular recognition; such strategies might ultimately generate alternatives to protein-based therapeutic agents. Many creative approaches have been reported for antagonizing protein−protein interactions in which one partner contributes a single α-helix to the interface, based on mimicry of the critical α-helix with a foldamer (i.e., an oligomer that displays a specific conformational propensity).[4,5] Here we take the first step toward applying a foldamer-based strategy to inhibition of an interaction that involves a polypeptide surface with less regularity than an α-helix.

Binding of vascular endothelial growth factor (VEGF) to the cell-surface receptor tyrosine kinases VEGFR1 and VEGFR2 plays an important role in angiogenesis,[6] and agents that block these interactions are used to treat cancer and wet macular degeneration.[7] There is considerable overlap between the surfaces on the VEGF homodimer that make contact with each of these receptors.[8] On VEGFR1, the contact surface is centered on domain 2 of the extracellular portion, and a crystal structure of the complex between $VEGF_{8\text{-}109}$ and $VEGFR1_{D2}$ reveals that ~820 Å^2 is buried at the receptor-recognition surface of the VEGF dimer (Figure 1A). This complex appears

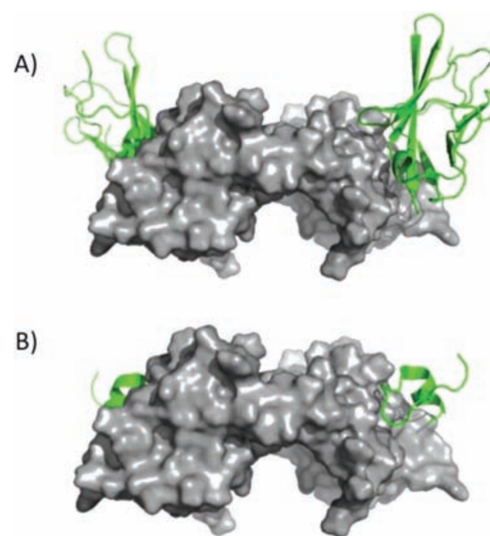

Figure 1. Previously reported high-resolution structures of the $VEGF_{9\text{-}108}$ homodimer (gray) bound to (A) domain 2 of VEGFR1 ($VEGFR1_{D2}$ is green; PDB: 1QTY) or (B) peptide v107 (v107 is green; PDB: 1KAT).

to be representative of many protein−protein interactions in that the binding surface on each partner is relatively flat and dominated by hydrophobic side chains.[1,8b] The receptor-recognition surfaces on the VEGF homodimer are targeted by therapeutic proteins bevacizumab, ranibizumab, and aflibercept, which act by antagonizing VEGF signal transduction.[7] The VEGF system provides an excellent opportunity to evaluate design strategies intended to generate protein−protein interaction antagonists that are different from and complementary to engineered proteins.

In this study we explore the impact of α-to-β-amino acid residue substitution on the VEGF affinity of a peptide ligand previously identified via phage display and demonstrated to

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bind to the receptor-recognition region of the $VEGF_{9\text{-}108}$ homodimer. In prior work we have identified oligomers of α- and β-amino acid residues ("α/β-peptides") that very effectively mimic the structure and recognition properties of individual α-helices,[9] but targeting the receptor-recognition surface on VEGF constitutes a more complex topological challenge than mimicking an isolated helix. The α/β-peptide approach could be useful in a biomedical context because these oligomers can display substantial resistance to proteolysis.[9]

The 19-mer peptide designated v114 (Figures 1B and 2) is the tightest-binding ligand for $VEGF_{9\text{-}108}$ found via phage-display by Fairbrother et al., based on inhibition of binding to extracellular domains 1−3 of VEGFR2.[10] The next-best ligand, v107, binds ~3-fold less avidly to the $VEGF_{9\text{-}108}$ homodimer. Peptides v107 and v114 both contain a single disulfide (C5−C15) and differ only at positions 1−4, 8, and 9 (Figure 2). An NMR structure of the v107-$VEGF_{9-108}$ complex (Figure 1B) confirms that this peptide binds to the receptor-recognition surface.[11] Among the six sites of sequence variation between v107 and v114, only one (Ala8 in v107) contributes a side chain to the v107-VEGF interface. We have described a competition fluorescence polarization (FP) assay involving homodimeric $VEGF_{165}$ and a fluorescently labeled derivative of v114 as the tracer.[12] This assay identifies molecules that bind to the receptor-recognition surface of VEGF; K_i = 0.60 μM for v107, and K_i = 0.070 μM for v114.

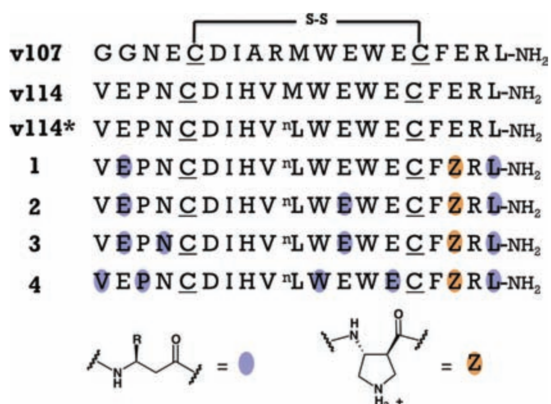

Figure 2. Sequences of α- and α/β-peptides. The side chain is defined by the conventional single-letter code for α-amino acids (^{n}L denotes norleucine). Blue circles indicate β^3-residues, and tan circles indicate the cyclic β-residue shown (Z). All peptides have a disulfide link between the two Cys residues (underlined).

The present study began with an alanine scan[13] of v114, which revealed that the side chains of residues V9, M10, W11, W13, and F16 play dominant roles in the binding of v114 to $VEGF_{165}$ (K_i > 20-fold higher than for v114 itself for Ala replacement at each site). These results are consistent with the reported impact of alanine mutations at the corresponding sites of v107 and with contacts observed in the v107-$VEGF_{9\text{-}108}$ structure.[11] Thus, the VEGF-v114 complex structure appears to be comparable to the VEGF-v107 complex structure previously elucidated via NMR.

We next conducted a "β^3 scan"[14] in order to try to identify positions in v114 that might tolerate $\alpha\rightarrow\beta^3$ replacement. We used the M10→norleucine variant (designated v114*), because this variation has little impact on binding to VEGF but precludes adventitious sulfoxide formation. Sequential replacement of α- residues with β^3 homologues is complementary to single site replacement with alanine: the latter removes the side chain while the former alters the backbone but maintains the side chain. At many sites either change caused only a modest decline in affinity for VEGF (≤5-fold increase in K_i; Figure 3).

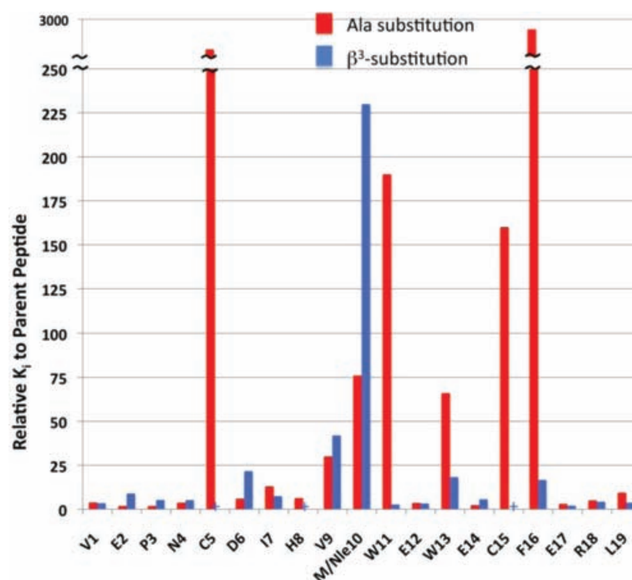

Figure 3. Relative inhibition constants normalized to those of parent peptides (v114 (K_i = 0.070 μM) for alanine substitutions; v114* (K_i = 0.060 μM) for β^3-substitutions). Alanine substitution data are shown as red bars, and β^3-substitution data are shown as blue bars. β^3-substitution was not made at either Cys residue..

However, some of the sites at which alanine substitution caused a profound loss of affinity were more tolerant of $\alpha\rightarrow\beta^3$ replacement. In the most dramatic case, W11→A caused a nearly 200-fold rise in K_i, while $\alpha\rightarrow\beta^3$ replacement at this position caused only a ~3-fold rise in K_i.

Previous α-helix-mimetic efforts showed that replacement of instrinsically flexible β^3 residues with ring-constrained β residues can improve affinity for a target,[9b] because the ring locally preorganizes the backbone for helical folding. The VEGF-bound conformation of v107 displays a short α-helix at the C-terminus (residues 13−19),[11] and it is likely that v114* contains an analogous α-helix. We therefore examined v114* homologues in which E14, E17, and R18 were individually replaced by the cationic cyclic residue (Z) shown in Figure 2. In each case, $\alpha\rightarrow\beta^3$ and $\alpha\rightarrow$cyclic β replacements had very similar impact on binding to VEGF, indicating that β residue preorganization at a single site had little effect on affinity.

The overall goal of $\alpha\rightarrow\beta$ replacements is to maintain affinity for VEGF while decreasing susceptibility to proteolysis,[9] and we therefore examined v114* derivatives containing multiple β residues (Table 1). Although regular patterns of $\alpha\rightarrow\beta$ replacement (such as $\alpha\alpha\beta\alpha\alpha\alpha\beta$ and $\alpha\alpha\alpha\beta$) enable mimicry of α-helices,[9,15] the irregular conformation of VEGF-bound v107 discouraged a pattern-based approach in this case. We evaluated several triple-replacement α/β-peptides, focusing on positions that seemed to tolerate individual $\alpha\rightarrow\beta$ replacement. Some triple replacements abolished binding to VEGF (K_i > 10 μM), even if the corresponding single-replacements were well tolerated (e.g., $\alpha\rightarrow\beta^3$ replacement at I7, E12, and E14), while others were more successful. α/β-Peptide **1**, with $\alpha\rightarrow\beta^3$ at E2 and L19 and $\alpha\rightarrow$cyclic β (Z) at E17, retained considerable affinity for VEGF (K_i = 0.26 μM, vs 0.06 μM for v114*). A

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Table 1. Binding and Proteolysis Data

oligomer	K_i (μM)[a]	$t_{1/2}$ (min)[b]
v107	0.60	–
v114	0.070	–
v114*	0.060	1.6
1	0.26	–
2	0.74	–
3	1.6	24
4	4.6	300

[a] K_i values determined by competitive FP assay. [b] Half-life of α- and α/β-peptides (50 μM) in the presence of proteinase K (10 μg/mL) in TBS, pH 7.5, with 5% DMSO.

fourth $\alpha\rightarrow\beta^3$ replacement, at E12 (α/β-peptide **2**), was reasonably well tolerated (K_i = 0.74 μM), as was a fifth $\alpha\rightarrow\beta^3$ replacement, at N4 (α/β-peptide **3**; K_i = 1.6 μM). Although **2** and **3** lost affinity for VEGF relative to prototype α-peptide v114* (12- and 27-fold, respectively), their behavior is nonetheless significant, given that the $\alpha\rightarrow\beta$ replacement strategy has not previously been applied to a non-helical prototype.

We used proteinase K to assess the impact of $\alpha\rightarrow\beta$ replacements on susceptibility to degradation.[9a,c,d,16] v114* was rapidly cleaved ($t_{1/2}$ = 1.6 min) by this aggressive protease under standard conditions, as expected for a medium-length α-peptide. α/β-Peptide **3** displays 15-fold greater stability ($t_{1/2}$ = 24 min). Mass spectroscopic analysis indicated that for both v114* and **3**, the principal cleavage occurs between ^{n}L10 and W11. Seeking greater resistance to proteolysis, we examined α/β-peptide **4**, containing six $\alpha\rightarrow\beta$ replacements, including W11. For **4**, $t_{1/2}$ = 300 min; thus, **4** is nearly 190-fold more resistant to proteinase K than is v114*, and **4** is >10-fold more resistant to proteolysis than is **3**. The affinity of **4** for VEGF is only modestly lower than the affinity of **3** (~3-fold).

Derivatives of α/β-peptides **3** and **4** bearing an N-terminal fluorescein unit were prepared, and their binding to VEGF was assessed via FP measurements, yielding K_d values of 0.18 and 1.4 μM, respectively. In each case, K_d is somewhat lower than K_i deduced from the competition FP assay, which may indicate that the fluorophore unit makes favorable contacts with VEGF upon α/β-peptide binding. We found that domain 2 of VEGFR1 displaces Flu-**3** from VEGF, which strongly supports the hypothesis that this α/β-peptide binds to the receptor-recognition surface on the VEGF dimer.

Antagonism of VEGF signaling can be evaluated with human umbilical vein endothelial cells (HUVECs), which are induced to proliferate when exposed to VEGF in culture.[17] It has been reported that v114 inhibits VEGF-induced HUVEC proliferation,[10] and we used this assay to assess the activity of α/β-peptides **3** and **4**. As a positive control, we used a potent, commercially available anti-VEGF antibody,[18] which displays full inhibition at 0.001 μM (Figure 4; the difference between 0.0001 and 0.001 μM antibody establishes the dynamic range of this assay). α-Peptide v114* at 30 μM displays essentially full inhibition of VEGF-stimulated HUVEC proliferation, consistent with published results for v114 itself.[10] α/β-Peptides **3** and **4** are less potent than v114*, as might be expected based on their reduced affinity for VEGF (~27- and ~77-fold higher K_i, respectively (Table 1); control studies indicate that v114* and α/β-peptides **3** and **4** are not toxic at the inhibitory concentrations). The ability of **3** and **4** to inhibit VEGF-stimulated HUVEC proliferation, albeit with modest potency,

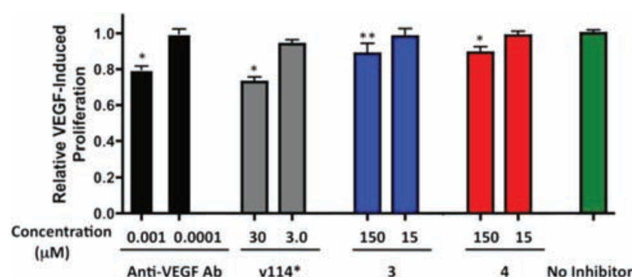

Figure 4. VEGF-induced proliferation of HUVECs as determined using the Click-iT EdU assay. All data are normalized to medium + VEGF controls run on each assay plate (green bar). Each inhibitor was tested above and below the measured K_i. *$p \leq 0.005$ vs no inhibitor control, **$p \leq 0.01$ vs no inhibitor control, as determined by a two-tailed t test. All samples contained 10 ng/mL VEGF. See ref 18 for description of the anti-VEGF antibody (black bars).

suggests that these α/β-peptides can block VEGF-mediated signal transduction via cell-surface receptors.

The results described here represent the first application of foldamer-based design principles to mimic the recognition behavior of a polypeptide that has a discrete but irregular conformation. The level of $\alpha\rightarrow\beta$ replacement in VEGF signaling antagonists **3** and **4** (~30%) is sufficient to cause significant decreases in protease susceptibility, relative to the prototype all-α peptide (v114*). This demonstration that a foldamer-based approach can be extended beyond mimicry of regular structures such as an α-helix is important because most protein–protein recognition surfaces are formed from irregular (and often discontinuous) backbone elements, as is true for the relevant surfaces on VEGF and its receptors. Appropriate preoganization of β-residues has been shown to enhance the binding properties of α-helix-mimetic α/β-peptides,[9b] and comparable benefits may be accessible in the context of more diverse peptide backbone conformations if a broader range of β-residue constraints becomes available.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1–5, supplementary Tables 1 and 2, experimental conditions, and peptide characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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