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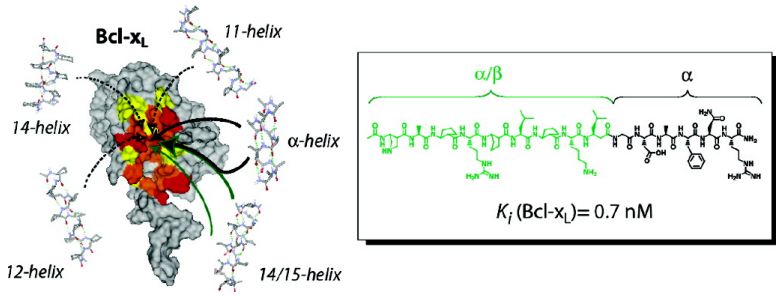

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Chimeric ($\alpha/\beta + \alpha$)-Peptide Ligands for the BH3-Recognition Cleft of Bcl-x_L: Critical Role of the Molecular Scaffold in Protein Surface Recognition

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Molecules that bind to specific protein surface sites are of fundamental interest, from the perpective of molecular recognition, and practical interest, from the perspective of medicine. Such molecules should disrupt specific protein−protein interactions, which are frequently associated with human diseases. Traditional "small molecule" approaches, very successful for enzyme inhibition, have been less productive for generation of protein−protein interaction antagonists,[1] although some recent achievements are very impressive.[2] We and others are interested in the prospect that unnatural oligomers with discrete folding propensities ("foldamers") might provide a rational and general basis for development of molecules that block protein−protein interactions.[3] Here we explore this possibility in the context of Bcl-x_L/BH3 domain interactions, a system that is attractive for fundamental studies because considerable structural information is available.[4]

Interactions within the Bcl-2 protein family control the fate of a cell in response to cytotoxic stimuli. In many cancers, anti-apoptotic Bcl-2 proteins such as Bcl-x_L are overexpressed and protect malignant cells from death (apoptosis) by direct interaction with pro-apoptotic proteins such as Bak and Bad.[5] Thus, inhibitors of the Bcl-x_L/Bak interaction could be therapeutically useful. A 16-residue peptide from the BH3 domain of Bak binds as an α-helix to a hydrophobic groove on Bcl-x_L, burying four hydrophobic Bak side chains (Val-74, Leu-78, Ile-81, and Ile-84).[6] Many small molecule ligands for the BH3-recognition domain have been described. Most have only modest activity (K_i values in competition binding assays typically >1 μM),[7] perhaps because of the large surfaces buried in the Bcl-x_L/Bak 16-mer complex; however, a potent small molecule has very recently been reported.[2c] Numerous medium-length α-peptides (16−32 residues) have shown high affinity for Bcl-x_L.[8] Foldamers that mimic the α-helical display of Bak side chains might be a good source of Bcl-x_L/Bak interaction antagonists.[3c] Foldamers can be proteolytically stable,[9] an advantage relative to α-peptide inhibitors.[9] We describe foldamers containing both α- and β-amino acid residues that compete effectively with the Bak 16-mer for binding to Bcl-x_L; the development of these antagonists illustrates principles that may be general for foldamer-based approaches to inhibitors of protein−protein interactions.

Our initial efforts focused on β-peptides as potential ligands for the BH3-recognition cleft of Bcl-x_L, because it is possible to design β-peptides that will adopt specific helical conformations, the 12- or the 14-helix, and display side chains in predictable arrangements.[10] Of these two β-peptide scaffolds, the 12-helix appears to be a better structural match to the α-helix.[3b,10] After evaluating >100 12-helical β-peptide sequences, however, we could obtain only very weak Bcl-x_L ligands ($IC_{50} > 500$ μM in a competition fluorescence polarization (FP) assay that employs a fluorescently labeled derivative of the Bak 16-mer as the ligand to be displaced).[11] We then examined ~60 14-helical designs, but again we could obtain only weak Bcl-x_L ligands. In both classes, the best ligands were shown via HSQC NMR analysis to bind to the BH3-recognition cleft on Bcl-x_L. Our inability to generate high-affinity β-peptide ligands led us to suspect that neither the 12-helix nor the 14-helix has a shape that is sufficiently complementary to the cleft on Bcl-x_L.

We have recently identified two new foldamer scaffolds, the 11-helix and 14/15-helix formed by α/β-peptides (oligomers with a 1:1 alternation of α- and β-amino acid residues along the backbone),[12] and we designed new Bcl-x_L ligand candidates based on these secondary structures. Both helices appear to be promoted by β-residues with a five-membered ring constraint.[12] α/β-Peptide designs based on the 11-helix were no more successful than the pure β-peptide designs ($IC_{50} > 500$ μM). In contrast, designs based on the 14/15-helix displayed significant activity in the FP assay, although these α/β-peptides were not as effective as α-peptides corresponding to natural BH3 domain sequences. For example, α/β-peptide 15-mer **1** displayed $IC_{50} = 40$ μM ($K_i = 1.5$ μM),[13] while $IC_{50} = 0.67$ μM ($K_i = 0.025$ μM) for the unlabeled Bak 16-mer (Figure 1).[14] In **1** and related α/β-peptide designs, Leu-6 is intended to play the role of the Leu residue conserved in all BH3 domains reported to date (e.g., Leu-78 of Bak).[6] We speculate that ACPC-3, β^3-homonorleucine-9 (β^3-hNle-9), and β^3-hPhe-13 of **1** also contribute to the hydrophobic surface required for binding to the BH3-recognition cleft of Bcl-x_L. Arg-4 and Asp-11 of **1** may be involved in electrostatic interactions with residues on the edge of the Bcl-x_L cleft, as proposed for analogous residues in the Bak 16-mer.[6]

We wondered whether the failure of **1** and related α/β-peptides to show greater efficacy in the FP assay reflects a local mismatch between some portion of the helical scaffold and the BH3-recognition cleft of Bcl-x_L. To test this hypothesis, we examined chimeric oligomers in which either the N-terminal portion or the C-terminal portion of **1** is replaced by an α-amino acid segment based on an α-peptide known to bind tightly to Bcl-x_L. For example, ($\alpha/\beta + \alpha$) oligomer **2** contains the first nine residues of α/β-peptide **1**, but the last six α-residues are related to the C-terminal segment of the Bak 16-mer, with Phe-13 of **2** intended to correspond to Ile-84 of Bak. In ($\alpha + \alpha/\beta$) oligomer **3**, the first nine residues correspond to positions 72−81 of Bak with Val-74 replaced by Leu; the final seven residues correspond to the C-terminal portion of α/β-peptide **1**. These complementary chimeric analogues of **1** show very different activities in the FP assay: for ($\alpha/\beta + \alpha$)

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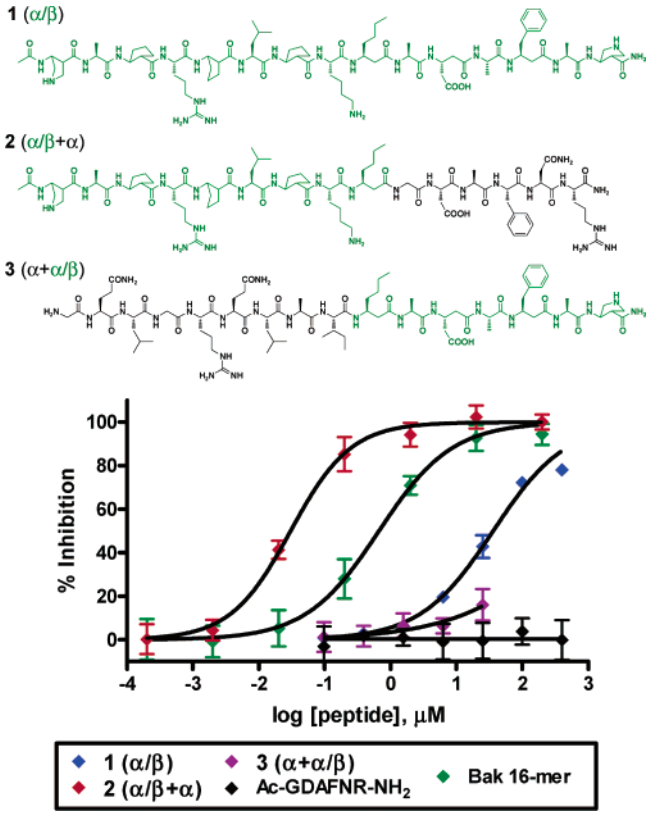

Figure 1. Competition FP data for binding to Bcl-x_L of **1**−**3**, Bak 16-mer, and control hexa-α-peptide Ac-GDAFNR-NH_2. A fluorophore-labeled Bak 16-mer peptide was used as the displaced fluorescent probe (see Supporting Information for details).

oligomer **2** IC_{50} = 0.059 μM (K_i = 0.0019 μM), while for (α + α/β) oligomer **3** IC_{50} > 700 μM (Figure 1). Thus, **2** is 10-fold more potent than the Bak 16-mer. This result suggests that the 14/15-helical α/β-peptide scaffold is well-suited to occupy a portion of the BH3-recognition cleft on Bcl-x_L, but that some other foldamer backbone, distinct from the β- and α/β-peptides we have examined thus far, will be necessary to replace the proteolytically susceptible α-peptide segment[15] for optimal fit to the remainder of this cleft.

Several control studies were conducted with (α/β + α) oligomer **2** and related compounds. The hexa-α-peptide corresponding to the C-terminal segment of **2** (Ac-GDAFNR-NH_2) at 500 μM displayed no interaction with Bcl-x_L in the FP assay (Figure 1). Thus, the α-peptide segment of chimeric oligomer **2** is probably not the dominant contributor to Bcl-x_L binding affinity.[16]

Binding of **2** to ^{15}N-labeled Bcl-x_L was examined via [^{1}H,^{15}N]-HSQC NMR measurements (Figure 2). Most of the Bcl-x_L amide N−H cross-peaks were significantly shifted upon addition of 50 μM **2** to 100 μM Bcl-x_L (Figure 2A). The pattern of shifts and resonance broadening induced by addition of **2** are comparable to the effects induced by addition of the Bak 16-mer α-peptide (Figure 2B).

A fluorescein-labeled derivative of **2** (**Flu-2**) was prepared to compare binding to Bcl-x_L with binding to unrelated proteins. Direct FP titration of 50 nM **Flu-2** with protein indicates K_d = 0.014 μM for **Flu-2** binding to Bcl-x_L (Figure 3). In contrast, no binding to bovine γ-globulin (BGG) could be detected at 500 μM BGG, and the onset of binding to bovine serum albumin (BSA) occurred above 10 μM BSA (Figure 3). Thus, binding of **Flu-2** to BGG or BSA is at least 10^3-fold weaker than binding to Bcl-x_L. Both BGG and BSA are promiscuous receptors for hydrophobic ligands;[17] the failure of **Flu-2** to bind tightly to either of these proteins suggests

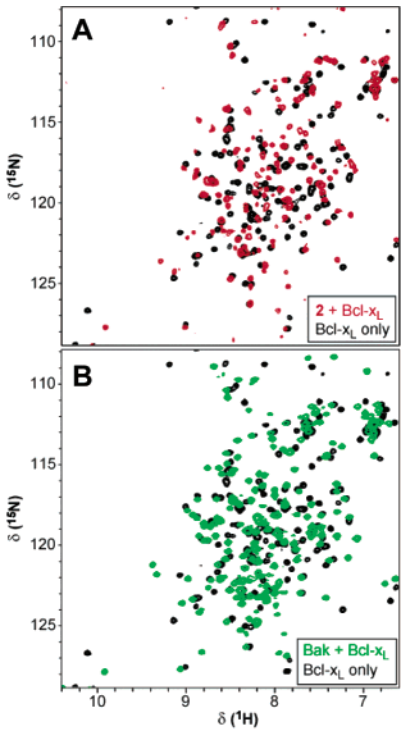

Figure 2. HSQC NMR binding assays. (A) Overlay of ^{15}N-Bcl-x_L spectra in the presence (red) and absence (black) of (α/β + α)-peptide **2**. (B) Overlay of ^{15}N-Bcl-x_L spectra in the presence (green) and absence (black) of the Bak 16-mer.

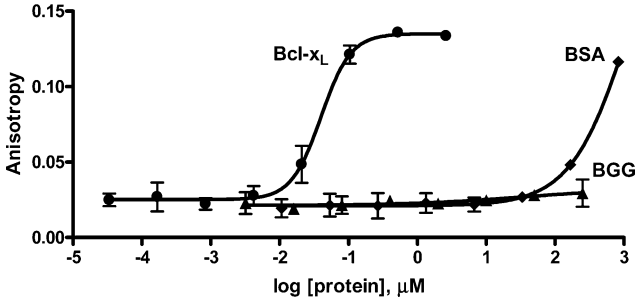

Figure 3. Direct FP titration of fluorescein-labeled (α/β + α)-peptide **Flu-2** with Bcl-x_L, BSA, and BGG.

that the affinity of **2** for Bcl-x_L is not simply the result of its hydrophobicity, but instead reflects complementarity to the BH3-recognition cleft. As a further test of such complementarity, we compared (α/β + α) oligomer **4** and its enantiomer in the competition FP assay (Figure 4). Oligomer **4** is an isomer of **2** in which β^3-hNle-9 has been replaced by β^3-hLeu; this small change leads to slightly improved affinity for Bcl-x_L (IC_{50} = 0.029 μM, K_i = 0.0007 μM for **4**). The enantiomer of **4**, however, displays very low affinity for Bcl-x_L (IC_{50} > 1000 μM).

The folding of (α/β + α) oligomer **5** in CD_3OH was examined by 2D NMR;[18] **5** has two modifications relative to **2** (Ala-2 → Lys and Lys-8 → Ile), which moderately diminish binding to the BH3-recognition cleft of Bcl-x_L (IC_{50} = 0.40 μM). Good dispersion of ^{1}H resonances was observed for **5**, which allowed assignment of many NOEs between backbone protons. Numerous *i*,*i* + 3 NOEs were observed along the entire length of **5** (Figure 5). Of particular importance are the three α-residue $H_\alpha(i)$ → β-residue $H_\alpha(i + 3)$ NOEs in the α/β-peptide segment of **5**. This NOE pattern is predicted for the 14/15-helix but not for the 11-helix.[12] In contrast, α-residue $H_\alpha(i)$ → α-residue NH(i + 2) NOEs are predicted for the 11-helix but not for the 14/15-helix,[12] and none of these NOEs

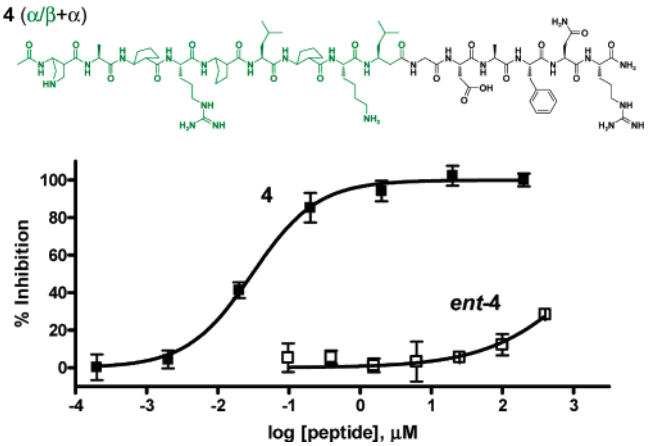

Figure 4. Competition FP binding assay for binding to Bcl-x_L of (α/β + α)-peptide **4** and its enantiomer.

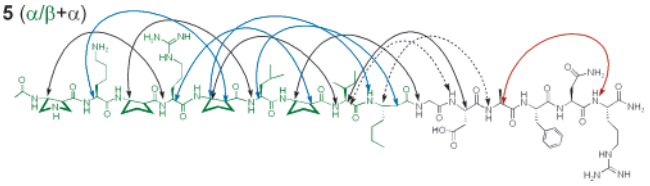

Figure 5. NOEs observed for oligomer **5** in CD_3OH. NOEs consistent with the 14/15-helix only (blue arcs), both the 11- and 14/15-helix (black solid arcs), and α-helix (red arcs). Ambiguously assigned NOEs are represented by dotted arcs.

is observed for **5**. Thus, the NMR data suggest that **5** has a substantial propensity to adopt the 14/15-helical secondary structure in its N-terminal region, a propensity that is likely to be manifested also by closely related molecules such as **2**. Interestingly, the *i*,*i* + 3 NOEs involving the C-terminal α-peptide region of **5** suggest that the 14/15-helical α/β-segment can nucleate helix formation in the short α-peptide segment.

Our results show that foldamer-based designs can provide tight-binding ligands for a large protein recognition site (K_i for **4** = 0.7 nM). The path from β- to α/β- to (α/β + α)-peptide ligands such as **2** leads us to conclude that foldamer-based strategies for disrupting protein−protein interactions will grow in scope and efficacy as the number of foldamer scaffolds with distinct shapes is increased. The tight binding of chimeric (α/β + α)-peptides to Bcl-x_L suggests that combining different foldamer scaffolds will be an effective and perhaps general strategy for protein ligand design.

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Supporting Information Available: Experimental details, peptide characterization data (HPLC, MS), and other results. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Rapid proteolysis of the α-peptide segment of **2** is observed upon treatment with Pronase, while the α/β segment remains intact for at least 24 h.

(16) The α/β-peptide oligomer corresponding to residues 1−9 of chimeric oligomer **4**, an isomer of **2** (see text), has an IC_{50} of 120 μM in the FP assay, suggesting that the α/β portions of **2** and **4** target the BH3 binding site and contribute substantially to binding.

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