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STRUCTURAL BASIS OF Bcl-x_L RECOGNITION BY A BH3-MIMETIC α/β -PEPTIDE GENERATED VIA SEQUENCE-BASED DESIGN

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Abstract

The crystal structure of a complex between the pro-survival protein Bcl-x_L and an α/β -peptide 21-mer is described. The α/β -peptide contains six β -amino acid residues distributed periodically throughout the sequence and adopts an α -helix-like conformation that mimics the bioactive shape of the Puma BH3 domain. The α/β -peptide forms all of the noncovalent contacts that have previously been identified as necessary for recognition of the pro-survival protein by an authentic BH3 domain. Comparison of our α/β -peptide:Bcl-x_L structure with structures of complexes between native BH3 domains and Bcl-2 family proteins reveals how subtle adjustments, including variations in helix radius and helix bowing, allow the α/β -peptide to engage Bcl-x_L with high affinity. Geometric comparisons of the BH3-mimetic α/β -peptide with α/β -peptides in helix-bundle assemblies provide insight on the conformational plasticity of backbones that contain combinations of α - and β -amino acid residues. The BH3-mimetic α/β -peptide displays pro-survival protein-binding preferences distinct from those of Puma BH3 itself, even though these two oligomers have identical side chain sequences. Our results suggest origins for this backbone-dependent change in selectivity.

Keywords

apoptosis; BH3 domain; foldamer; peptides; peptidomimetics

INTRODUCTION

Disruption of protein-protein interactions is challenging because the protein surfaces involved are typically expansive ^[1]. Synthetic peptides may be better suited to this task than are small molecules, but susceptibility to proteolysis limits the prospects for long-term biomedical utility ^[2]. These considerations have led to exploration of protein-protein interaction antagonism by “foldamers”, unnatural oligomers with well-defined conformational propensities ^[3, 4]. Unnatural backbones are less susceptible to enzymatic

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degradation than is the α -peptide backbone, and the predictability of foldamer shape offers the prospect of mimicking the recognition surface on one of the interacting proteins. Most studies to date have focused on isolated α -helices that mediate protein associations [5, 6]. Oligomers designed *de novo*, such as β -peptides, oligo-phenyl compounds, peptoids or acylated oligoamines, have been evaluated as α -helix mimics [7-11], but the efficacy of these clever approaches can be limited because the unnatural backbones do not precisely reproduce the side chain projection pattern of an α -helix [12, 13]. As an alternative, one can partially modify the backbone of an active α -peptide sequence with the goal of retaining the recognition properties of the original α -helix while diminishing susceptibility to proteolysis. This latter strategy has been dubbed “sequence-based design” [14]. Here we provide new insights on the efficacy of a sequence-based design strategy, including the crystal structure of an α -helix-mimetic oligomer containing both α - and β -amino acid residues (an “ α/β -peptide”) bound to a target protein.

Apoptotic cell death is initiated when the α -helical BH3 domains of pro-apoptotic Bcl-2 family members (e.g., Puma) engage a hydrophobic cleft on their pro-survival counterparts (e.g., Bcl-x_L) [15]. Small molecules that mimic BH3 domains are gaining attention as potential anti-cancer drugs since they can induce apoptosis in tumors dependent on Bcl-2 proteins for survival [16]. BH3 domain mimicry remains an active field of research because not all pro-survival proteins have yet been effectively targeted with small molecules. Moreover, most BH3 domains and compounds that mimic BH3 domains target multiple pro-survival Bcl-2 family proteins, and it would be valuable to develop highly selective ligands as research tools and, ultimately, to reduce clinical side-effects that arise from broad targeting of this family.

Beyond the biomedical significance of these protein-protein interactions, the recognition of BH3 domains by pro-survival Bcl-2 family members represents an excellent system for exploration of principles that govern α -helix mimicry. An extensive structural database of complexes between authentic BH3 domains and pro-survival proteins is available for comparison [17-25], and critical aspects of BH3 domain sequence-function relationships have been elucidated [22, 23, 26-30]. The value of BH3 domain mimicry as a test-bed for design guidelines that can be extended to other helix-recognition challenges is manifested in the use of this system to evaluate foldamer-based strategies [12-14, 24, 31-34] as well as complementary approaches based on α -helix stabilization via cross-linking [35, 36].

In previous studies of BH3 mimicry by foldamers containing β -amino acid residues, we found that helices formed by purely β -amino acid backbones were inadequate as α -helix mimics, as were helices formed by α/β -peptides with a 1:1 α/β alternation along the backbone [12, 13]. Specifically, grafting a set of side chains known to be important for BH3 domain recognition onto these various foldamer helices led to oligomers with only weak affinity for Bcl-x_L. In contrast, promising preliminary results emerged from sequence-based designs in which a subset of the Puma BH3 domain α -amino acid residues were replaced with homologous β -amino acid residues (e.g., leucine $\rightarrow$ β^3 -homoleucine; Fig. 1) [14]. Such changes maintain the original sequence of side chains, but add a CH₂ unit to the backbone for each $\alpha \rightarrow \beta$ substitution.

Here we report the first co-crystal structure of an α/β -peptide generated via sequence-based design bound to a pro-survival protein. The data show that side chains from the α/β -peptide form all of the contacts that are believed to be crucial for tight binding to pro-survival proteins by authentic BH3 domains. Geometric analysis elucidates the way in which the elongated α/β -peptide backbone is able to recapitulate the multipoint interaction of the α -helical prototype.

RESULTS

Binding profile and biological activity of an α/β -peptide derived from the Puma BH3 sequence

In our preliminary studies ^[14] periodic replacement of α -residues in the BH3 domain of Puma (Sequence **1**, Fig. 1A) with β -residues bearing the natural side chain identified an α/β -peptide analogue, **2**, that bound tightly to Bcl-x_L. The $\alpha \rightarrow \beta$ replacements in **2** were introduced in an $\alpha\beta\alpha\alpha\beta$ pattern, which was tuned to the heptad repeat of an α -helix and leads to alignment of the β -residues in a 'stripe' along one side of the helix. The incorporation of the β -amino acid residues throughout the polypeptide confers significant resistance to proteolytic degradation ^[14]. The native Puma BH3 sequence is a promiscuous binder capable of engaging all pro-survival proteins with high affinity ^[37]. In contrast, surface plasmon resonance (SPR) data indicate that α/β -peptide **2** is more selective (Table 1). The unnatural oligomer matches Puma BH3 α -peptide **1** in binding to Bcl-x_L, Bcl-2 and Bcl-w, but the α/β -peptide has considerably lower affinity for Mcl-1 relative to the authentic BH3 domain. These findings are consistent with an earlier comparison of binding to Bcl-x_L vs. Mcl-1 via competition fluorescence polarization assays ^[14].

To determine whether α/β -peptide **2** can engage full-length pro-survival proteins in the complex environment provided by a cellular milieu, we tested the ability of **2** to elicit cytochrome *c* release from permeabilized mouse embryonic fibroblasts (MEFs). These experiments provide an important complement to binding assays performed with isolated recombinant proteins, which are C-terminally truncated (and N-terminally truncated in the case of Mcl-1). In addition, these studies provide insight into the potential pro-apoptotic activity of the foldamer, since release of cytochrome *c* from mitochondria is a critical step in apoptosis and requires antagonism of both Bcl-x_L and Mcl-1 in wild-type MEFs ^[38]. As expected, **2** was inactive when tested with permeabilized wild-type MEFs which express both Bcl-x_L and Mcl-1 (Fig. 1B), presumably because this α/β -peptide does not bind tightly to Mcl-1. In contrast, cytochrome *c* release was observed upon treatment of permeabilized *mcl-1*^{-/-} MEFs with **2**, consistent with strong binding of this α/β -peptide to Bcl-x_L (Mcl-1 antagonism is not required for cytochrome *c* release in these cells). For *bcl-x*^{-/-} MEFs, which depend exclusively on Mcl-1 for maintenance of outer mitochondrial membrane integrity, no cytochrome *c* release was observed upon treatment with α/β -peptide **2**. An identical activity profile was observed for the Bad BH3 domain (Fig. 1B), which is consistent with the similar protein-binding profile displayed by this α -peptide and α/β -peptide **2**. In contrast, the Puma BH3 domain itself, **1**, releases cytochrome *c* in all cell types, which is consistent with strong binding of this α -peptide to both Bcl-x_L and Mcl-1 (Table 1) ^[37]. These data highlight the functional consequences of the altered binding profile of α/β -peptide Puma BH3 analogue **2** relative to the authentic Puma BH3 domain, **1**.

Structure of Puma-derived α/β -peptide bound to Bcl-x_L

We sought high-resolution structural data to understand why the Puma-derived α/β -peptide **2** displays a binding pattern different from that of the Puma BH3 domain itself, a variation that presumably underlies the different biological behaviours of these two molecules. As in previous studies, crystallization trials were performed using a "loop-deletion" construct of Bcl-x_L that has proven useful for obtaining structures of complexes with BH3 peptides ^[24, 25]. Initial trials with **2** and Bcl-x_L yielded crystals that diffracted poorly (> 6 Å). As a consequence we tested related α/β -peptides and found that crystals of Bcl-x_L bound to α/β -peptide **3**, a truncated analogue of **2**, diffracted to a resolution of 2.4 Å (Table 2). Relative to **2**, α/β -peptide **3** is missing three residues at the N-terminus and two residues at the C-terminus. Control experiments showed that the binding profiles among anti-apoptotic proteins of **2** and **3** are similar (Table 1), although **3** displays a more substantial decrease in

affinity for Mcl-1 relative to the other proteins, compared to **2**. These observations are consistent with previous reports showing that additional residues at the termini of BH3 peptides can influence affinity, perhaps as a result of increased helicity^[19]. Most importantly with regard to permeabilized MEF experiments, **3** qualitatively mirrors **2** in terms of high affinity for Bcl-x_L and low affinity for Mcl-1, and this parallel likely underlies the comparable behavior of these two α/β -peptides in terms of cytochrome *c* release.

The crystal structure (PDB: 2YJ1) confirms that **3** engages the BH3-recognition groove of Bcl-x_L predominantly formed by helices $\alpha 2$ - $\alpha 5$ (Fig. 2A). Comparison of structures of Bcl-x_L bound to various BH3 peptides with the unliganded Bcl-x_L reveals that peptide binding requires a widening of the binding groove, typically involving movement of helices $\alpha 3$ and $\alpha 4$. This is also the case with the binding of α/β peptide **3**. One point of difference noted in the various BH3:Bcl-x_L structures, however, is the relative orientation of residues Tyr101 and Phe105; in some complexes (e.g., with Bim BH3; PDB: 3FDL) Tyr101 projects into the groove and Phe105 into solvent, whereas in others (e.g., with Beclin BH3; PDB: 2P1L) the reverse orientation is observed^[16]. The **3**:Bcl-x_L structure is similar to Bim BH3:Bcl-x_L in this respect, and overall the structures of Bcl-x_L in both these complexes are highly similar (RMSD in the superposition of C α atoms over residues 1-194 = 0.76 Å)

Previous mutagenesis and structural studies have shown that key residues in BH3 sequences for binding pro-survival proteins include the four conserved hydrophobic residues (h1-h4, Fig. 1A) and the aspartate residue (position h3+2, Fig. 1A) which is conserved on nearly all BH3 domains^[23, 26, 29, 30, 39]. An overlay of the **3**:Bcl-x_L complex with known BH3:pro-survival protein crystal structures (e.g., Bim BH3:Bcl-x_L and Puma BH3:Mcl-1; Figs. 2A,B) reveals striking mimicry of the α -helix by the α/β -peptide. Most notably, the arrangement of the four signature hydrophobic side chains, h1-h4, of a natural BH3 domain is accurately recapitulated (Fig. 2C), and the critical salt bridge between Arg139 on Bcl-x_L and the conserved Asp (residue 14 in **3**) on the outward-facing surface of the BH3 helix is preserved (Fig. 2D). All of the β -amino acids align in a “stripe” along the solvent exposed face of the peptide, as they were designed to do. Moreover, the hydrogen bonding between backbone carbonyl O and amide H(N) atoms of the α/β -peptide maintains the *i* to *i*+4 interaction pattern of regular alpha helices along the length of the peptide. Hence, despite incorporation of 6 β -amino acids residues within the 21-mer, the mode of Bcl-x_L binding by **3** is remarkably similar to that of natural BH3 peptides.

Basis for pro-survival protein selectivity of α/β Puma

Selectivity between Bcl-x_L and Mcl-1 can be manipulated by modifying the hydrophobic residues within the BH3:pro-survival protein binding interface^[26, 29]. However, since, the side chains in α/β -peptide **3** (and presumably **2**) that form key contacts with the pro-survival protein are identical to the key side chains from α -peptide **1**, the data presented here raise the possibility that differences in the solvent-exposed side chains could account for the different selectivity profile of α/β -peptides **2** and **3** compared to the native Puma BH3 domain. Indeed, overlaying the structures of the α/β -peptide:Bcl-x_L and Puma BH3:Mcl-1 complexes reveals a potential side chain clash between His233 on Mcl-1 and Arg6 on the α/β -peptide (Supplementary Fig. 1). Accordingly, we undertook mutational studies to probe this possibility (Table 1). Initially, Arg6 on full-length α/β -peptide **2** was mutated to alanine (creating **4**, Fig. 1A) to shorten the side-chain and thereby reduce the likelihood of a steric clash with residues on Mcl-1. This mutation resulted in only a minor (2.4-fold) decrease in IC₅₀ compared to α/β -peptide **2**. We interpreted this result to indicate a residual clash between Ala6 of α/β -peptide **4** and His233 of Mcl-1, or other nearby residues that project into the BH3-binding groove, such as Arg229. We therefore examined the ability of α/β -peptide mutant **4** to bind to an Mcl-1 mutant with residues His233 and Arg229 switched to Ala (generating “Mcl-1mut”). This approach led to a nearly 9-fold lower IC₅₀ relative to

association of **2** with wild-type Mcl-1 (Table 1), supporting our hypothesis that unfavourable interactions involving solvent-exposed side chains play a role in the relatively weak binding of α/β -peptide **2** to Mcl-1.

Geometric analysis of the α/β -peptide that mimics the Puma BH3 domain

How does the α/β -peptide so accurately mimic the α -helical conformation and side chain display of the native Puma BH3 domain despite the presence of approximately one additional backbone carbon per helical turn? Previous work has shown that α/β -peptides derived from sequence-based design can manifest subtle structural differences as compared to the prototype α -helix [40-43]. The crystal structure of α/β -peptide **3** bound to Bcl-x_L provides the opportunity for direct comparison with the structures of numerous analogous natural (all- α -amino acid) BH3 domains bound to pro-survival proteins, as well as with other helical α/β -peptides.

The amino acid sequence of **3** conforms to an $\alpha\alpha\beta\alpha\alpha\beta$ heptad repeat. Hence, for accurate mimicry of an all- α -amino acid residue BH3 domain, each heptad would need to “adjust” somewhat to accommodate the two additional methylene groups contributed by the two β -amino acid residues. Comparison of the helical conformation of **3** to a selection of BH3 α -helices from reported structures of complexes with pro-survival proteins reveals that this structural accommodation is achieved through significant differences in the key geometric parameters, including helix radius measured at each amino acid residue (i.e., the distance from the helix axis to the C α atom of each residue) the helix rise per residue and phase yield per residue (see Fig. 3A for a graphical definition of these parameters). Specifically, the helix adopted by α/β -peptide **3** expands slightly, particularly at each β -amino acid residue, where there is a significant ($p < 0.002$) increase in the helix radius compared to the radii of α -helical BH3 peptides (2.50 Å vs. 2.31 Å). Indeed, the overall radius of the α/β -peptide helix is increased relative to the α -helix: even at the α -amino acid residues in the α/β -peptide helix, the radius is slightly greater than for the typical BH3 domain α -helix (2.44 Å vs. 2.31 Å, $p < 0.001$). The phase yield per residue, too, differs between the α/β -peptide helix and BH3 domain α -helices. This parameter for α -helical BH3 domains (average = 98.6°) is very close to the value of 100° for an ideal α -helix. In the helix formed by α/β -peptide **3**, however, the phase yield per residue is significantly ($p < 0.001$) smaller for each β -residue (average = 93.2°), and dramatically larger for each β -residue (average = 121°). Similarly, there is a slight decrease in the rise for each α -amino acid residue in the α/β -peptide helix (average = 1.40 Å) relative to the α -residues in an α -helical BH3 domain (average = 1.49 Å), whereas this value increases for each β -residue (average = 1.75 Å). The “give-and-take” in these key parameters results in an overall geometry of the helix formed by α/β -peptide **3** that almost exactly matches that of an α -helical BH3 domain: phase yield per $\alpha\alpha\beta\alpha\alpha\beta$ heptad = 708° vs. 700° for all- α heptad; helical rise per heptad = 10.4 Å for both $\alpha\alpha\beta\alpha\alpha\beta$ and all- α . Moreover, very similar values for these geometric parameters are observed in helices formed by a range of α/β -peptides, based on leucine zipper sequences, with varying backbone patterns ($\alpha\alpha\beta\alpha\alpha\beta$, $\alpha\alpha\beta$ or $\alpha\alpha\alpha\beta$; Supplementary Table 1), which suggests that these parameters are general for helical secondary structures adopted by α/β -peptides, rather than being limited to α/β -peptides based on BH3 sequences [40-42].

An additional geometric parameter, the radius of curvature, indicates the extent to which the helix deviates from being perfectly straight. In general, BH3-domain α -helices are very straight (radius of curvature >100 Å; Fig. 3B). However, 3 of the 13 BH3 domain α -helices analyzed above (PDB 2BZW, 3I1H, and chain C of 3BL2), display somewhat more bent structures, since the radius of curvature is significantly less than 100 Å. In each of these three cases the α -helix curves *toward* the cleft of the pro-survival protein. This sense of curvature seems to fit a general pattern noted long ago: the axis of amphipathic α -helices on the surface of a protein tends to distort away from the solvent [44]. The helix formed by α/β -

peptide **3** is not straight; the radii of curvature are 56.9 Å and 63.0 Å for the two independent molecules of **3** in the crystal structure. However, unlike the distortion seen for some BH3 domain α -helices, the distortion of the α/β -peptide helix is not toward but rather away from the stripe of β -residue methylene groups, which is located on the solvent exposed face of the helix (Fig. 3B). Previously reported structures of helical α/β -peptides, based on leucine zipper sequences, showed very large radii of curvature (average ~ 200 Å), which indicates that α/β -peptide helices can be quite straight [40-42]. Overall, the available structural data suggest that α/β -peptide helices are similar to α -helices in that some degree of bending can occur to accommodate a binding partner.

DISCUSSION

BH3 domains are relatively simple folding motifs that provide a robust and well-characterized platform for evaluating α -helix mimicry by unnatural oligomers. BH3 domains share the defining $\Phi A/GXXLXX\Phi A/GDE\Phi$ motif (where Φ represents a hydrophobic residue and X any amino acid), yet they display distinct binding profiles for pro-survival Bcl-2 family members. The underlying basis for the partner selectivities of natural BH3 sequences is still not well understood; however, various approaches have led to unnatural α -peptide sequences that display unique binding profiles for pro-survival proteins [26, 29]. Such peptides have been suggested as alternatives to small molecules as BH3-mimetic drugs, particularly if these peptides could resist proteolysis in a biological milieu, although the oral bioavailability of small molecules is a profound advantage relative to peptides. Proteolytic resistance can be conferred via placement of cross-links within an α -peptide sequence [35, 36]. Alternatively, resistance to proteolysis can be achieved via substitution of some α -amino acid residues with analogous β -residues, if the additional backbone carbon atoms introduced along with the α -residues do not distort the binding epitope [14, 43]. High-resolution structural data of the type reported here are essential if we are to develop a general understanding of α -helix mimicry by α/β -peptides, and ultimately learn how to design potent inhibitors of helix-recognition events associated with human pathologies.

The contrast in binding preferences between α/β -peptide **2** and authentic Puma BH3 domain **1** (Table 1) is striking given that these two oligomers bear an identical side chain sequence. The different binding selectivities of **1** and **2** become even more intriguing in light of our structural data, which show that the BH3-defining hydrophobic side chains, as projected from the helical conformation of α/β -peptide **3**, engage the appropriate binding pockets within the BH3-binding groove of Bcl-x_L. Indeed, the α/β -peptide side chains in this complex very accurately mimic analogous side chains from native BH3 domains. Similarly, the conserved aspartic acid side chain in **3** forms the salt bridge that is seen in all BH3:pro-survival complexes. Our mutagenesis studies indicate that the difference in pro-survival protein binding preferences between α -peptide **1** and α/β -peptide **2** may result in part from a steric clash involving surface-exposed residues on the α/β -peptide with side-chains of residues lining the binding groove of Mcl-1.

The mimicry of the key binding interactions from an authentic BH3 domain by the side chains of α/β -peptide **3**, despite the extra backbone methylene carbon contributed by each β -residue, appears to depend on subtle adjustments in the α/β -peptide backbone. Notable features include a slight expansion of the helix radius, particularly at each β -residue, together with variations in the helix phase yield and rise, which allow the side chain positions to remain in register with contact points on Bcl-x_L. Similar helix expansion and variation in the helix geometry are observed in a number of recent structures of α/β -peptide helices that form coiled-coil assemblies [40-42] (Supplementary Table 1). Hence, these structural alterations appear to be a common feature by which mimicry of the α -helical conformation is achieved by backbones that contain both α - and β -residues. A significant

bowing of the α/β -peptide helix was observed in the crystal structure of the **3**:Bcl-x_L complex; this feature could also contribute to the three-dimensional arrangement of key side chains that is necessary for tight binding to Bcl-x_L.

Previously we reported the structure of a BH3-mimetic α/β -peptide foldamer bound to Bcl-x_L (PDB: 3FDM) [12], but in that case the α/β -peptide was quite different from **2** and **3**. The earlier α/β -peptide was generated via *de novo* structure-based design; this 15-mer was chimeric, featuring 1:1 α/β alternation in the N-terminal portion, and purely α -residues in the final six positions. Many of the β -amino acid residues in this earlier structure did not bear a proteinogenic side chain but instead contained a five-membered ring constraint. The helix formed by this earlier α/β -peptide is even less regular than the helix documented here for α/β -peptide **3**. In particular, a pronounced bulging of the previous helix was observed in the α/β segment, with the radius expanding by as much as 0.7 Å relative to an α -helix; this bulging was mostly localized at the α -residues. The structure of the **3**:Bcl-x_L complex shows that the $\alpha\beta\alpha\alpha\beta$ backbone can more accurately mimic an α -helix than does the 1:1 α/β backbone in the previous structure. This observation presumably explains why we found it impossible to identify oligomers containing a pure 1:1 α/β backbone that bind tightly to pro-survival proteins.

The results described here provide high-resolution structural insight regarding the translation of an α -helical message into a new molecular dialect. This recasting provides the benefit of diminished susceptibility to proteases with retention of tight binding to multiple Bcl-2-family pro-survival proteins [14]. Affinity for Mcl-1 is diminished in the current α/β -peptide design, relative to the authentic Puma BH3 domain, but structural data suggest that unfavorable interactions involving a solvent-exposed residue on the α/β -peptide may provide at least a partial explanation for the weak binding, and biochemical data support this conclusion. Since the approach that led to α/β -peptides **2** and **3** is operationally simple (periodic replacement of α -residues with homologous β^3 -residues) and can be readily accomplished *via* conventional solid-phase peptide synthesis [14], our structural elucidation of high-fidelity α -helix mimicry by **3** should encourage broader exploration of the sequence-based design strategy [43].

EXPERIMENTAL SECTION

Synthesis of foldamers and peptides

α/β -Peptides were synthesised via solid-phase methods on NovaPEG Rink Amide resin as described previously [14]. All α -peptides were synthesised on solid phase using a Symphony automated synthesizer (Protein technologies). Synthetic cycles were completed with a standard coupling time of 30 min using 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU, 5 equivalents) with DMF as the solvent. Five equivalents of Fmoc amino acid were used for each coupling cycle. Deprotection steps used 20% piperidine in DMF for 5 min, the solution was drained, and then 20% piperidine in DMF was added and the solution was mixed for an additional 15 min. Following a final deprotection step, acetyl caps were added to the peptides using acetic anhydride. Peptides were cleaved from resin by treatment with standard TFA / scavenger cocktails and precipitated from diethyl ether. Crude material was purified by preparative HPLC. The purity and identity of the final products were confirmed by analytical HPLC and MALDI-MS, respectively.

Recombinant proteins

Expression and purification of the loop-deleted form of human Bcl-x_L (Δ 27-82, Δ C24) for crystallisation, and Bcl-2 Δ C22, Bcl-x_L Δ C24, Bcl-w C29S/A128E Δ C29, and human/

mouse chimeric Mcl-1 (Δ N170, Δ C23) for Biacore studies was performed as described previously [24, 26, 37].

Binding affinity measurements

Solution competition assays were performed using a Biacore 3000 instrument as described previously [37]. Briefly, pro-survival proteins (10 nM) were incubated with varying concentrations of peptide for at least 2 hours in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4) prior to injection at 20 μ L/min onto a CM5 sensor chip on which either a wild-type BimBH3 peptide or an inert BimBH3 mutant peptide (Bim4E) was immobilized. Specific binding of the pro-survival protein to the surface in the presence and absence of peptides was quantified by subtracting the signal obtained on the Bim mutant channel from that obtained on the wild-type Bim channel. The ability of the peptides to prevent protein binding to immobilized BimBH3 was expressed as the IC₅₀, calculated by nonlinear curve fitting of the data with Kaleidagraph (Synergy Software).

Cytochrome c release assay

Mouse embryonic fibroblasts (wild-type, *mcl-1*^{-/-} and *bcl-x*^{-/-}) ($\sim 2 \times 10^6$ cells) were permeabilized in digitonin-containing permeabilization solution (0.05% (w/v) digitonin (Calbiochem) in 20 mM HEPES pH 7.2, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose), supplemented with protease inhibitors (Roche), for 10 minutes on ice. The mitochondria-containing crude lysates were incubated with peptide (10 μ M) at 30° C for 1 hour before pelleting. The supernatant was retained as the soluble fraction while the pellet, which contained intact mitochondria, was solubilized in lysis buffer (1% (v/v) Triton-X-100 in 20 mM Tris-pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol) supplemented with protease inhibitors (Roche). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The cytochrome *c* was detected with anti-cytochrome *c* antibody (7H8.2C12, BD Pharmingen). The BimBH3 peptide that binds all pro-survival proteins with high affinity was included as a positive control.

Crystallisation

We employed a “loop-deleted” form of human Bcl-x_L (Δ 27-82 and without membrane anchor), which forms an α 1 domain-swapped dimer yet retains BH3 domain binding activity [24, 25]. Crystals of Bcl-x_L and **3** were obtained by mixing at a molar ratio of 1:1.3 then concentrating the sample to 10 mg/ml. Crystals were grown by the sitting drop method at room temperature in 0.05 M cadmium sulphate, 1.0 M sodium acetate, 0.1 M HEPES, pH 7.5. Prior to flash freezing in liquid N₂, crystals were equilibrated into cryoprotectant consisting of reservoir solution containing increasing concentrations of ethylene glycol to a final concentration of 15%.

Crystal data collection and structure determination

Diffraction data were collected at the Australian Synchrotron MX2 beamline. The diffraction data were integrated and scaled with HKL2000 [45]. The structure was obtained by molecular replacement with PHASER [46-48] using the structure of Bcl-x_L from the BeclinBH3/Bcl-x_L complex (PDB: 2P1L) (i.e., with the Beclin BH3 peptide removed) as a search model. Several rounds of building in COOT [49] and refinement in REFMAC5 [50] and PHENIX [51] led to the final model, with the folamer structure built into the density *de novo*.

Helix geometry analysis

Analysis of the helix geometry was performed using the PS program [52]. The PS program determines the parametric equations of a circle that defines the helix axis by minimizing the variance in the distance from the axis to atoms in the polypeptide chain. Here the variance of the distance of the carbonyl carbon atoms to the helix axis was minimized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Puma-mimetic α/β -peptides bind pro-survival proteins and elicit cytochrome c release
A. Peptides used in this study; β^3 -residues highlighted in blue. **B.** Consistent with their binding profiles, Puma-derived α/β -peptides **2** and **3** release cytochrome c from mitochondria of permeabilized *mcl-1*^{-/-} MEFs into the soluble fraction, but not from wild-type or *bcl-x*^{-/-} MEFs. In contrast, wild-type Puma releases cytochrome c from all MEFs. P: pellet fraction containing mitochondria, S: soluble fraction.

Fig. 2. A Puma-mimetic α/β -peptide binds Bcl-x_L similarly to natural BH3 domains

A. Overlay of 3:Bcl-x_L (PDB: 2YJ1) (navy:white) with Bim:Bcl-x_L (PDB:3FDL) (yellow:teal). **B.** Overlay of 3:Bcl-x_L (navy:white) with Puma:Mcl-1 (PDB:2ROC) (green:red). **C.** Overlay of α/β -peptide 3 (navy), the Puma BH3 domain (green) and the Bim BH3 domain (yellow). Key interacting hydrophobic residues (h1-h4) and the conserved aspartate from the α -peptides are mimicked by the α/β -peptide. **D.** Overlay of 3:Bcl-x_L (PDB: 2YJ1) (navy:white) with Bim:Bcl-x_L (PDB:3FDL) (yellow:teal) and Puma:Mcl-1 (PDB: 2ROC) (green:red). A key interaction for binding of BH3 domains to pro-survival proteins is a salt-bridge between the conserved aspartate at position 14 on the 3 BH3 sequence (Fig. 1A) and an arginine on the pro-survival protein. This is preserved in the α/β -peptide:Bcl-x_L complex.

Fig. 3. Helix expansion and bending contribute to α -helix mimicry with the $\alpha\alpha\beta\alpha\alpha\beta$ backbone
A. Graphical definitions of helix geometric parameters, including radius, rise and the angle ϕ , which represents the phase yield; the black dots on the helical spiral represent the end of one residue and the start of the next.
B. Geometric analysis of α -helices from a selection of reported BH3 peptide:pro-survival protein complex structures and the helix in Puma-mimetic α/β -peptide **3**. The designations (α) and (β) refer to measurements involving an α - or a β -amino acid residue, respectively, in α/β -peptide **3**.
C. The curvature of α/β -peptide **3** (lower panel) compared to the curvature the α -helical Puma BH3 domain when in complex with the A1 pro-survival protein (PDB: 2VOF) (upper panel); spheres in the lower panel indicate backbone methylene groups of the β^3 -homoamino acid residues.

Table 1

Binding of the Puma BH3 domain α -peptide **1** and α/β -peptides **2-4** to pro-survival proteins, as determined via surface plasmon resonance measurements. Values are IC₅₀ (standard error) in nM. ND: not determined.

	Bel-2	Bel-w	Bel-x_L	Mcl-1	Mcl-1mut
1	5 (1)	13 (7)	18 (6)	10 (1)	ND
2	7 (2)	42 (10)	18 (9)	480 (160)	140 (2)
3	50 (19)	190 (7)	26 (2)	8000 (140)	ND
4	ND	ND	ND	200 (10)	55 (4)

Table 2

X-ray data collection and refinement statistics

Data Collection	
Space group	$P2_12_12_1$
Cell Dimensions a, b, c (Å)	$a = 58.9, b = 71.4, c = 75.6$
Wavelength (Å)	0.953692
Resolution Range (Å)	50.00 - 2.24 (2.32 - 2.24)
R_{merge}	0.178 (0.521)
$I/\sigma(I)$	14.2 (3.3)
Completeness (%)	99.4 (97.1)
Redundancy	8.7 (4.8)
No. of copies / a.s.u.	2
Temperature	100 K
Refinement	
Resolution (Å)	45.44 - 2.24 (2.38 - 2.24)
No. reflections R_{work}	15803 (2404)
No. reflections R_{free}	787 (130)
R_{work}	0.19 (0.20)
R_{free}	0.25 (0.29)
No. atoms	
Protein	2312
Peptide	364
Solvent	56
R.m.s. deviations	
Bond lengths(Å)	0.008
Bond angles (°)	1.2
Chiral (Å ³)	0.08
Ramachandran*	
Preferred (number / %)	308 / 97.9
Allowed (number / %)	7 / 2.2

Values in parentheses represent statistics for highest resolution shell.

*
α-amino acids only