Published in final edited form as:

J Am Chem Soc. 2015 September 9; 137(35): 11365–11375. doi:10.1021/jacs.5b05896.

α/β -Peptide Foldamers Targeting Intracellular Protein-Protein Interactions with Activity in Living Cells

James W. Checco[†], Erinna F. Lee^{‡,§}, Marco Evangelista[‡], Nerida J. Sleebs[‡], Kelly Rogers^{‡,§}, Anne Pettikiriachchi[‡], Nadia J. Kershaw^{‡,§}, Geoffrey A. Eddinger[†], David G. Belair[∞], Julia L. Wilson[†], Chelcie H. Eller^{||}, Ronald T. Raines^{†,||}, William L. Murphy^{∞,▽}, Brian J. Smith[○], Samuel H. Gellman^{†,*}, and W. Douglas Fairlie^{‡,*}

[†]Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, 53706, United States

[‡]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, 3052, Australia

[§]Department of Medical Biology, University of Melbourne, Parkville, Victoria, 3010, Australia

[∞]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, Wisconsin 53706, United States

^{||}Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin, 53706, United States

[▽]Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, Madison, Wisconsin, 53706, United States

[○]Department of Chemistry, La Trobe Institute of Molecular Science, Melbourne, Victoria, 3086, Australia

Abstract

Peptides can be developed as effective antagonists of protein-protein interactions, but conventional peptides (i.e., oligomers of L- α -amino acids) suffer from significant limitations in vivo. Short half-lives due to rapid proteolytic degradation and an inability to cross cell membranes often preclude biological applications of peptides. Oligomers that contain both α - and β -amino acid residues (“ α/β -peptides”) manifest decreased susceptibility to proteolytic degradation, and when properly designed these unnatural oligomers can mimic the protein-recognition properties of analogous “ α -peptides”. This report documents an extension of the α/β -peptide approach to target intracellular protein-protein interactions. Specifically, we have generated α/β -peptides based on a “stapled” Bim BH3 α -peptide, which contains a hydrocarbon crosslink to enhance α -helix stability. We show that a stapled α/β -peptide can structurally and functionally mimic the parent stapled α -peptide in its ability to enter certain types of cells and block protein-protein interactions associated with apoptotic signaling. However, the α/β -peptide is nearly 100-fold more resistant to

*Corresponding Authors: gellman@chem.wisc.edu, fairlie@wehi.edu.au.

Supporting Information. Additional details for experimental protocols, Supplementary Figures S1-S6, and Supplementary Table S1. This material is available free of charge via the Internet at <http://pubs.acs.org>

The authors declare the following competing financial interest(s): S.H.G. is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of α/β -peptides.

proteolysis than is the parent α -peptide. These results show that backbone modification, a strategy that has received relatively little attention in terms of peptide engineering for biomedical applications, can be combined with more commonly deployed peripheral modifications such as side chain crosslinking to produce synergistic benefits.

INTRODUCTION

Misregulation of protein-protein interactions is often associated with disease states, and compounds that selectively modulate such interactions may be used as therapeutic agents. Small molecules, the traditional choice for drug compounds, are often ineffective at targeting protein-protein interactions because of the large protein contact surfaces involved in many of these associations.¹ In contrast, medium-length peptides can be developed to bind with high affinity and selectivity to large surfaces on proteins. However, *in vivo* applications of peptides are often severely limited because of their rapid degradation by proteolytic enzymes.² Furthermore, because most peptides do not spontaneously cross cell membranes, intracellular protein-protein interactions are typically not viable targets for peptide antagonists.

Oligomers that contain both α - and β -amino acid residues (" α/β -peptides") can mimic natural α -helices and modulate helix-mediated protein-protein interactions.^{3,4} The unnatural backbone of the α/β -peptide reduces susceptibility to protease degradation relative to peptides that consist only of α -amino acid residues (" α -peptides").⁵ Our prior work has shown that α/β -peptides can function as antagonists in cell-based systems.^{4,6,7} Recently, we reported that α/β -peptides can also show prolonged activity *in vivo* relative to the parent α -peptides, highlighting the potential of these compounds for therapeutic use.⁸ To date, the design of biologically active α/β -peptides has been limited to protein-protein interactions that occur at the cell surface.

α -Helical secondary structures play an important role in many protein-protein interactions.⁹ We have previously used α -helical BH3 domains from Bcl-2 family proteins as a model system for exploring the effects of $\alpha \rightarrow \beta$ -amino acid residue substitutions on the recognition of a helical ligand by partner proteins.^{3,10,11} BH3 domains are short (~20-residues) α -helical segments that mediate interactions between pro- and anti-apoptotic Bcl-2 family proteins.¹² These domains bind to long, complementary grooves displayed by anti-apoptotic family members such as Bcl-x_L, Mcl-1 and Bcl-2. Binding of members of the BH3-only sub-class (e.g., Bim, Puma, Bad) to anti-apoptotic partners results in the initiation of apoptosis in damaged, redundant, or potentially dangerous cells. These interactions displace pro-apoptotic proteins such as Bax, Bak or "activator" BH3-only proteins from sequestration by the anti-apoptotic family members. This release of pro-apoptotic factors triggers mitochondrial membrane permeabilization, cytochrome *c* release, and caspase activation. The survival versus death decision is finely controlled by the balance of pro- and anti-apoptotic Bcl-2 family members within a cell. Several types of cancer cells rely on overexpression of certain anti-apoptotic Bcl-2 family proteins as a mechanism to evade cell death. This observation has engendered speculation that antagonism of anti-apoptotic Bcl-2 family proteins could be beneficial for cancer treatment.¹³ Indeed, several small molecule

Bcl-2 protein antagonists have recently shown promising results in cancer patient samples and clinical trials.¹⁴

Other groups have sought to improve the properties of short peptides similar to BH3 domain-derived α -peptides by introducing side-chain crosslinks that are intended to stabilize the binding conformation (an α -helix). A variety of different strategies have been employed¹⁷, including lactam crosslinking via amino acid residue side chains^{18,19}, alkylation of cysteine residues with crosslinking groups²⁰, and alkene crosslinking using olefin metathesis.²¹ Use of a hydrocarbon crosslink, formed by ring-closing metathesis of two α,α -disubstituted pentenyl-containing amino acids (S5) at i and $i+4$ positions (e.g., **α -1**, Figure 1), has been the most intensively studied strategy.^{16,22-24} These “stapled” α -helical (“SAH”) peptides display increased helicity and decreased susceptibility to protease action relative to conventional α -peptide analogues. In some exceptional cases the stapled peptide manifests cellular permeability that is not observed for related α -peptides containing only natural residues. The mechanism for cellular entry of stapled helical peptides is not understood, but it has been suggested that the hydrophobic crosslinker allows association of the peptide with the cellular membrane, with subsequent cell entry proceeding via energy-dependent endocytosis.²³ Some stapled analogues of BH3 domains that enter cells can initiate apoptosis by antagonizing the actions of anti-apoptotic Bcl-2 proteins.^{16,23}

Here we describe α/β -peptide analogues of **α -1** (previously reported in the literature as **BimSAHB**^{15,16}), a crosslinked α -helical α -peptide derived from the Bim BH3 domain. The “stapled” α/β -peptide mimics the activity of the crosslinked parent α -peptide in cell-based experiments. However, the crosslinked α/β -peptide manifests greater resistance to proteolytic degradation than does the analogous crosslinked α -peptide. These results show that properly designed α/β -peptides can recapitulate favorable characteristics of stapled peptides, such as protein recognition, conformational stability, and membrane permeability, while providing additional benefits. Such compounds may be useful as chemical probes for studying interactions among Bcl-2 family proteins within cells or as starting points for the development of future therapeutic agents.

RESULTS

Design of α/β -peptide mimics of **α -1**

We recently reported α/β -peptide analogues of the Bim BH3 domain that contain cyclic β -amino acid residues (ACPC and sAPC, Figure 1B) and bind to both Bcl-x_L and Mcl-1 with high affinity.¹¹ These α/β -peptides are significantly less susceptible to proteolytic degradation relative to α/β -peptides that contain only flexible, acyclic β -residues. Based on this precedent, we designed **α/β -1** (Figure 1), an analogue of **α -1** that contains three $\alpha \rightarrow$ cyclic β -residue substitutions (Trp2 $\rightarrow$ ACPC, Glu6 $\rightarrow$ sAPC, Ala16 $\rightarrow$ ACPC) along with the side chain crosslink. We also synthesized linear “unstapled” analogues of **α -1** and **α/β -1** (**α -1-LIN** and **α/β -1-LIN**, respectively) to evaluate the effect of the covalent crosslink on protein affinity and cellular activity. As a further basis of comparison, we prepared **α/β -2**, an analogue of **α/β -1-LIN** that contains two hydrophobic ACPC cyclic β -residues in place of the S5 residues.

Protein binding by SPR

We used a surface plasmon resonance (SPR) competition assay to evaluate the affinity of the Bim-derived α - and α/β -peptides to three key anti-apoptotic proteins: Bcl-x_L, Mcl-1, and Bcl-2 (Table 1). α -1, α/β -1, α -1-LIN, and α/β -1-LIN all bound to Mcl-1 as tightly as did the parent α -Bim sequence. α/β -1 also bound to Bcl-x_L and Bcl-2 similarly to α -Bim, while α -1, α -1-LIN, and α/β -1-LIN generally bound one or both of these anti-apoptotic proteins somewhat more weakly than did α -Bim (~5- to 50-fold decline in affinity, depending on the combination). Interestingly, α/β -2, an analogue of α/β -1-LIN that contains the cyclic β -residue ACPC in place of the S5 residues, retained relatively high affinity for Mcl-1 but displayed reduced affinity for Bcl-x_L and Bcl-2 compared to α/β -1-LIN. Overall, these data imply that α/β -1 mimics the protein recognition profiles of both α -Bim and α -1, which suggests that the incorporation of cyclic β -amino acid residues into this sequence, in place of α -residues, does not negatively impact the affinity for anti-apoptotic proteins. Indeed, for some anti-apoptotic proteins, the interaction of α/β -1 is stronger than the interaction of α -1.

Structural analysis in solution by circular dichroism

To gain insight into the impact of the hydrocarbon crosslink on oligomer conformation in solution, we examined the far-UV circular dichroism (CD) spectra of Bim-derived α - and α/β -peptides (Figure 2). Consistent with previous reports,^{15,16} α -Bim showed little evidence of helicity in aqueous solution, while the stapled peptide α -1 showed a strong α -helical signature in the far-UV CD, with minima at ~208 and 222 nm. Interestingly, α -1-LIN, the “unstapled” analogue of α -1, also showed a pronounced α -helical CD signature, although the minima were less intense than those of α -1. This result suggests that incorporation of the α,α -disubstituted S5 residue alone is sufficient to promote a helical conformation, although not to the extent observed with an intact crosslink. α/β -1 and α/β -1-LIN both showed a single minimum at ~209 nm, consistent with the signal observed for α/β -peptides that adopt an α -helix-like conformation in solution.²⁵ As seen for the α -1-LIN versus α -1 comparison, α/β -1-LIN displayed a less intense helical signature relative to cyclized analogue α/β -1. We note that the CD signal intensity for all of the peptides containing the S5 residue, with or without cyclization, was concentration-dependent in the range 5-50 μ M (Figure S2), which suggests that these peptides aggregate under these conditions. Hence, the relative differences in the signal intensities for the linear versus crosslinked peptides could reflect slightly altered propensity to aggregate.

Crystal structures of α/β -1 and α/β -1-LIN in complex with Bcl-2

To gain atomic-resolution insight regarding the impact of the β -amino acid residues and the hydrocarbon crosslink on binding to an anti-apoptotic partner, we solved X-ray crystal structures of α/β -1 (PDB: 5AGW) and α/β -1-LIN (PDB: 5AGX) in complex with Bcl-2 (Figure 3, Table S1).

Both α/β -peptides crystallized with Bcl-2 under similar conditions and produced similar crystal forms. No structure is available for the α -Bim:Bcl-2 complex for comparison, but both α/β -1 and α/β -1-LIN engage Bcl-2 *via* the large hydrophobic groove (formed by helices α 3, α 4, and the N-terminal segment of α 5) that is known to be the interaction site on

Bcl-2 for BH3 peptide ligands (Figure 3A, B); comparable grooves are evident in structures of other anti-apoptotic proteins.²⁶⁻²⁹ The four conserved hydrophobic residues (h1-h4) on the buried face of the α/β -peptides project into the groove on Bcl-2 in a manner similar to that in which equivalent residues on α -Bim and α -1 interact with the equivalent groove on Bcl-x_L (Figure 3C). The salt bridge between Asp12 of the α/β -peptides and Arg146 from the BH1 domain of Bcl-2 matches a salt-bridge observed in most reported structures of BH3 domains bound to anti-apoptotic proteins. As anticipated, the β -amino acid residues of α/β -1 and α/β -1-LIN are aligned as a “stripe” along the solvent-exposed face of the helix formed by each α/β -peptide, although the long sAPC side-chain projects close to the α 3 helix of Bcl-2 and makes a hydrogen bond with Glu114 in the α/β -1:Bcl-2 complex structure. The hydrocarbon crosslink in the α/β -1:Bcl-2 complex overlays almost perfectly with the crosslink in the structure of α -1 bound to Bcl-x_L (PDB:2YQ6).¹⁹ Neither crosslink makes any contacts with the anti-apoptotic protein. In the α/β -1-LIN structure the S5 sidechains project out to the solvent.

Cytochrome *c* release assay (permeabilized cells)

To establish whether α/β -1 could neutralize anti-apoptotic protein function in a cellular milieu, we tested whether this stapled α/β -peptide could induce cytochrome *c* release from mitochondria (a hallmark of cell death induction). These studies were conducted in wild-type mouse embryonic fibroblasts (MEFs) in which the plasma membrane was permeabilized by treatment with digitonin (Figure 4). This permeabilization approach is usually required to enable BH3-derived peptides to reach the proteins that regulate apoptosis [*seems as though we need a reference here*]. Mouse embryonic fibroblasts express both Bcl-x_L and Mcl-1, and both proteins contribute to MEF cell survival.³⁰ Hence, BH3 peptides must antagonize both Bcl-x_L and Mcl-1 to induce apoptosis. Consistent with its binding profile for these anti-apoptotic proteins, α/β -1 induced movement of cytochrome *c* from the pellet fraction (which contains the mitochondria) to the soluble cytosolic fraction in permeabilized MEFs. Comparable behavior was observed with the α -Bim peptide, which is comprised solely of α residues (Figure 4). Slightly less cytochrome *c* release was observed with α/β -2 relative to α/β -1, consistent with the difference in affinity for Bcl-x_L between these two α/β -peptides (Table 1). No cytochrome *c* release was seen with a negative control Bim-derived α -peptide (**Bim4E**) in which key interacting residues (h1-h4) were mutated to glutamic acid, or in permeabilized MEFs derived from embryos deficient in Bax and Bak, the essential mediators of apoptosis. Combined, these data suggest that α/β -1 can neutralize Bcl-x_L and Mcl-1 in permeabilized cells, leading to the activation of Bax and/or Bak.

Cell killing assays

Previous studies have shown that some cell types, particularly haematological cancer cell lines, are either killed or are sensitized to apoptosis by treatment with stapled BH3 domain α -peptides, but that other cell lines (e.g., fibroblasts, HCT-116 colon cancer) are resistant to these stapled peptides.^{16,19,23,31} We therefore screened a panel of various cell lines to identify those cells that could be used in detailed functional analyses. The crosslinked α/β -peptide α/β -1 was compared to crosslinked the α -peptide α -1 at a high concentration (10 μ M) in a CellTiter-Glo assay (Promega), which measures intracellular ATP levels as an

indicator of cell viability (Figure 5). Consistent with previous reports with α -1, we saw essentially no effect of α/β -1 or α -1 on non-cancerous MEFs (Figure 5),¹⁶ even though α/β -1 can engage the apoptotic network when the MEF outer membrane has been permeabilized (Figure 4). Additionally, no significant cell death was induced by α/β -1 or α -1 with any of the adherent cancer cell lines tested, including those derived from mammary (MDA-MB-231, MDA-MB-468), colon (LIM-2405, SW480), or lung (A549) tumors. In contrast, α/β -1 potently impacted U937 lymphoma cell viability, similar to the effect previously observed with α -1 on these cells.¹⁶ Overall, these data indicate that α/β -1 mimics the highly selective cell-killing profile of α -1, suggesting that the non-natural α/β backbone supports mimicry of the complex biological activity of the stapled α -peptide α -1.

To gain insight into the relative potency of α/β -1 versus α -1 toward U937 cells, and to establish whether the loss of cell viability observed following treatment was due to activation of the apoptotic cascade or to some non-specific effect (e.g., rupture of the plasma membrane), we performed titrations of α -1 or α/β -1 in the presence or absence of the broad-spectrum caspase inhibitor Q-VD-OPh (Figure 6). Following just 2 hours of treatment with crosslinked oligomer in the absence of caspase inhibitor, a significant reduction in cell viability was observed for cells treated with either α -1 or α/β -1. This loss in viability was attenuated by Q-VD-OPh for both the stapled α -peptide and the stapled α/β -peptide, suggesting that the cell killing was due to activation of the intrinsic cell death pathways (in which caspases play a critical role) rather than a non-specific effect. Additional caspase-dependent killing was seen after 6 hours of treatment for both oligomers; however, after treatment with peptides for 24 hours most of the cell death observed was not inhibited by Q-VD-OPh. α -1 was slightly more potent than α/β -1 ($EC_{50} = \sim 2 \mu M$ for α -1 versus $\sim 4 \mu M$ for α/β -1 at 6 hours), but there appeared to be slightly more caspase-independent killing (not blocked by Q-VD-OPh) at the earlier time points for α -1 compared to α/β -1.

Recent reports^{31,32} suggest that the hydrocarbon crosslink *per se* may not be required for cell entry of some stapled peptides, but rather that the presence of the two S5 residues alone (no cyclization via ring-closing metathesis) may be sufficient to facilitate cell entry and initiation of apoptosis. To evaluate the importance of the hydrocarbon crosslink in our system, we tested the linear peptides α -1-LIN and α/β -1-LIN for killing activity toward U937 cells. Consistent with an earlier report using α -1-LIN,³¹ both linear oligomers were effective at reducing U937 cell viability in a caspase-dependent manner (Figure 6). Indeed, at the 6 hour time point there was no statistically significant difference in the killing activity of the linear versus the cyclized peptides. In contrast, the α/β -peptide that contained ACPC residues in place of the hydrocarbon crosslink (α/β -2) had no significant effect on cell viability, even after 24 hours.

We next used another marker of apoptosis, propidium iodide (PI) staining of DNA combined with fluorescent microscopy, to monitor cell viability and morphology over time (Figure 7). U937 cells were treated with $5 \mu M$ of α/β -1 or α -1 and imaged every 30 minutes. The results closely matched the CellTiter-Glo assay data, although cell killing appeared to proceed with slightly different kinetics, presumably due to the differences in experimental readout between the two assays. These studies revealed a dependence on caspase activity for cell killing by both α/β -1 and α -1, particularly at the earlier time points, as indicated by the

delay in cell death for conditions containing Q-VD-OPh (Figure 7A). These observations are fully consistent with those based on CellTiter-Glo assays. Close examination of the images reveals that even after just 1 hour of treatment, classical features of apoptotic cell death such as membrane blebbing start to appear. These features are present in the majority of cells within 2 hours (Figure 7B), but are not seen in the presence of caspase inhibitor Q-VD-OPh. Significant PI staining (i.e., to >50% of cells) was slightly delayed in cells treated with α/β -1 compared to α -1, consistent with the CellTiter-Glo results, suggesting α -1 killed more rapidly than did α/β -1 (Figure 7A). The majority of cells treated with either α/β -1 or α -1, however, were dead (PI⁺) within 2-6 hours, and this cell death was accompanied by the prodigious release of small apoptotic bodies, another hallmark of apoptosis induction.

Cytochrome c release assay (unpermeabilized U937 cells)

To further assess whether cell killing by stapled α/β -1 occurred *via* the mitochondrial pathway, we examined unpermeabilized U937 cells for cytochrome *c* release 3 hours after treatment (Figure 8). Here we observed that most of the cytochrome *c* was present in the soluble fraction, which contrasted with the location of cytochrome *c* exclusively in the pellet after intact cells were treated with either α -Bim or α/β -2, neither of which is stapled. The absolute amount of cytochrome *c* detected in cells treated with α/β -1 was somewhat lower than the amount in cells subjected to the other treatments; this trend may reflect some degree of leakage through the plasma membrane of the dying cells after treatment with α/β -1.

Cellular uptake of fluorescein-labeled analogues by live-cell confocal microscopy

We examined the cellular uptake of fluorescein (Flu)-labeled derivatives of α -Bim, α -1, α/β -1, α -1-LIN, and α/β -1-LIN in both U937 and MEF cells by live-cell confocal microscopy (Figures 9 and 10, Figures S4 and S5). When U937 cells were treated with Flu-labeled peptides at 2 μ M for 3 hours under serum-free conditions, we observed intracellular green fluorescence for both **Flu- α -1** and **Flu- α/β -1** (Figure 9), consistent with the hypothesis that α -1 and α/β -1 can cross membranes spontaneously and gain access to targets within the cytoplasm of living cells. We observed similar intracellular fluorescence for **Flu- α -1-LIN** and **Flu- α/β -1-LIN**, which is consistent with our finding that both α -1-LIN and α/β -1-LIN can also initiate apoptosis in U937 cells. With all oligomers containing the S5 residue, whether subjected to cyclization or not, we also observed significant green fluorescence associated with the outer membrane of some cells, which we interpret as aggregated peptide. These aggregates remained strongly associated with the cell membrane even after multiple washes prior to imaging. U937 cells treated with **Flu- α -Bim**, which does not contain the S5 residue, displayed negligible green fluorescence inside cells or associated with the outer membrane, consistent with the hypothesis that α -Bim does not enter cells.

When MEFs were treated with fluorescein-labeled analogues of Bim-based oligomers the confocal microscopy images were similar to those seen with the U937 cells, with **Flu- α -1**, **Flu- α/β -1**, **Flu- α -1-LIN**, and **Flu- α/β -1-LIN** all appearing to enter the MEFs (Figure 10). These observations are consistent with a previous report showing that labeled α -1 can enter MEFs, despite the stapled peptide's inability to kill these cells.¹⁶ However, the fluorescent staining pattern appeared somewhat more punctate in the MEFs than was observed with the U937 cells. This result suggests that although the stapled and unstapled α - and α/β -peptides

can enter MEFs, these peptides may be unable to move into the cytoplasm in this cell type, in contrast to U937 cells.

Protease susceptibility

We evaluated a subset of the peptides for susceptibility to degradation by proteinase K, a generic and aggressive protease (Table 1, Figure S1). In this assay, the linear α -peptide **α -Bim** was very rapidly degraded, showing a half-life of 0.27 minutes. **α -1**, an α -peptide that contains the hydrocarbon crosslink, showed modestly diminished susceptibility to degradation by proteinase K, with a half-life of 1.6 minutes under the assay conditions. The ~6-fold increase in half-life for **α -1** relative to **α -Bim** is consistent with previous reports in terms of the extent to which a hydrocarbon crosslink confers resistance to proteolytic destruction.^{23,33} This modest resistance to proteolysis presumably arises from the presence of the α,α -disubstituted residues and the crosslinking segment, both of which favor a helical conformation. **α/β -1**, which contains three cyclic β -amino acid residues in addition to the crosslink, showed a large improvement in resistance to proteolysis relative to **α -1**, with a half-life of 150 minutes (>90-fold increase in relative to the stapled peptide **α -1** and >500-fold increase relative to **α -Bim**). Thus, the incorporation of β -amino acid residues can be used to develop an α/β -peptide analogue that mimics the complex functions of a stapled α -peptide (apoptosis initiation in certain cell types, in the case of **α -1**) while dramatically reducing susceptibility to protease degradation.

DISCUSSION

We have previously shown that properly designed α/β -peptides, in which the peptidic backbone deviates from that of conventional peptides and proteins, are able to mimic analogous α -peptides structurally and functionally in terms of selective recognition of target proteins *in vitro*.^{3,4,7} Furthermore, recent studies have shown that α/β -peptides can exhibit improved properties *in vivo* relative to analogous α -peptides, likely due to a decreased susceptibility of the unnatural α/β backbone to proteolytic destruction.⁸ However, the inability of most α - and α/β -peptides to cross cellular membranes has limited these compounds to extracellular targets for cellular and *in vivo* applications. The remarkable ability of the so-called “stapled” BH3 domain peptides (first reported by Walensky et al.) to penetrate cell membranes and initiate apoptosis^{16,23} led us to investigate whether α/β -peptide analogues of a hydrocarbon-crosslinked BH3 α -peptide could retain these interesting and useful characteristics.

Several reports have highlighted the significant impact of primary sequence on the activity of stapled α -peptides in living cells. Small changes in sequence or charge can significantly alter the ability of these peptides to enter cells, which indicates that the presence of a hydrocarbon crosslinker does not ensure membrane transit.^{19,24,34} In light of these results, it was of interest to determine whether the replacement of a subset of α -amino acid residues with β -amino acid residues in a known, active cell-penetrating stapled peptide (**α -1**, often referred to in prior reports as **BimSAHB**^{16,23}) would alter the ability of the resulting α/β -peptides to cross cell membranes and initiate apoptosis.

To evaluate the impact of $\alpha \rightarrow \beta$ -residue substitution on the activity of the α -peptide **α -1**, we designed **α/β -1** using an $\alpha \rightarrow \beta$ substitution pattern previously shown to be effective for mimicry of the Bim BH3 domain.¹¹ **α/β -1**, along with linear analogue **α/β -1-LIN** and an analogue bearing ACPC residues in place of the S5 residues (**α/β -2**), were able to bind tightly to anti-apoptotic proteins (Table 1), indicating that the $\alpha \rightarrow \beta$ substitutions did not dramatically alter protein recognition (although **α/β -2** displayed reduced affinity for Bcl-x_L and Bcl-2 relative to **α/β -1** and **α/β -1-LIN**). **α/β -1** and **α/β -2** both displayed activity similar to the prototype α -peptide **α -Bim** in promoting Bax/Bak-dependent cytochrome *c* release in membrane-permeabilized MEFs (Figure 4), indicating that each of these α/β -peptides can engage the apoptotic machinery in a cellular environment if the membrane barrier is removed.

The crystal structures of **α/β -1** and **α/β -1-LIN** bound to Bcl-2 demonstrate that these α/β -peptides engage the BH3-recognition groove of the anti-apoptotic protein, making all of the expected side-chain contacts to Bcl-2 (Figure 3). Overall, the co-crystal structures indicate that both **α/β -1** and **α/β -1-LIN** recapitulate the classic BH3 binding mode, and that incorporation of the S5 and cyclic β -amino acid residues does not dramatically alter the structure or binding mode of the resulting α/β -peptides relative to a classical α -helical BH3 domain. To our knowledge, these are the first crystal structures of α/β -peptides containing the recently reported cyclic β -amino acid residue sAPC¹¹ and the first structures of an α/β -peptide bound to Bcl-2.

When tested against a broad panel of non-cancerous and cancerous cell lines, **α/β -1** showed the same profile of activity as **α -1**, efficiently killing U937 lymphoma cells while having negligible impact on the viability of the other cell lines tested (Figure 5). This very selective activity is consistent with the previous reports of **α -1**^{16,19,31} and may be due to the high reliance on Mcl-1 for survival that is characteristic of acute myeloid leukemia cells such as U937.³⁵ Both **α/β -1** and **α -1** bind to Mcl-1 with high affinity (Table 1). Live-cell confocal microscopy indicated that fluorescein-labeled analogues of **α/β -1**, **α/β -1-LIN**, and **α -1-LIN** were each able to enter U937 cells in a manner similar to the entry of fluorescein-labeled **α -1** (Figures 9, S4, and S5). Our detailed viability studies indicated that the ability of **α/β -1** to kill U937 cells was dose-dependent, attenuated by caspase inhibitor Q-VD-OPh, and resulted in significant cell membrane blebbing and release of “apoptotic bodies”, all consistent with the induction of apoptosis rather than an off-target cytotoxic effect (Figures 6 and 7). Furthermore, the release of cytochrome *c* from the mitochondria of unpermeabilized U937 cells is consistent with initiation of the apoptotic pathway by **α/β -1** (Figure 8). These data demonstrating the on-target effects of **α/β -1** are in line with previous studies showing that stapled BH3 peptides, including **α -1**, are able to initiate apoptosis in certain cell types. Interestingly, at the 24 hour time point, the level of cell death that could be inhibited by Q-VD-OPh was significantly less than at the earlier time-points. This observation could indicate that the caspase inhibitor was “overwhelmed” due to the sustained apoptosis activation in the cells. An alternative explanation is that prolonged exposure of cells to the stapled (or non-stapled but S5-containing peptides) results in non-specific toxicity. This latter hypothesis is consistent with a recent report³⁶ showing that stapled helical α -peptides based on the N-terminal segment of p53 kill cells in a p53-

independent manner because these peptides cause disruption of the plasma membrane, which leads to non-specific cell death at high concentrations ($>10\ \mu\text{M}$). Further studies would be required to establish whether either, or potentially both, of these explanations account for the apparent non-caspase-dependent death after long-term exposure of U937 cells to the peptides discussed here.

Neither α -1 nor α/β -1 demonstrated any significant killing activity toward MEFs, an observation that is consistent with previous reports regarding the effects of stapled BH3 peptides on these cells.^{16,19} These results are somewhat surprising given that: 1) our binding assays showed that α/β -1 and α -1 can bind tightly to both Mcl-1 and Bcl-x_L (the critical anti-apoptotic proteins in MEFs) (Table 1), 2) assays using permeabilized cells demonstrated that α/β -1 can induce cytochrome *c* release in MEFs (Figure 4), and 3) live-cell imaging demonstrated that fluorescein-labeled analogues of both α -1 and α/β -1 can enter these cells (Figure 10). One explanation for this lack of activity could be that the peptides do not reach the cytosol of MEF cells, and instead remain trapped within intracellular vesicles. This possibility is consistent with the punctate staining pattern revealed by our live-cell imaging of MEF cells. However, it is unclear why these peptides are able to enter the cytoplasm of U937 cells; the unique features that allow U937 cytoplasm entry merit further investigation. It is also unclear from our study why neither α -1 nor α/β -1 caused toxicity toward other cancer cell lines tested (this behavior has been previously noted for α -1³¹). One possibility is that the peptides become trapped within vesicles in these cells. A second possibility is that these peptides are not taken up via endocytosis in these cell types. A third possibility is that the peptides do not bind sufficiently tightly to the critical anti-apoptotic protein (e.g., Bcl-2) in these cell types.

Recent reports have highlighted examples of linear (“unstapled”) analogues of stapled α -peptides that contain the S5 residue and manifest cell-killing activity.^{31,32} The experiments reported here are consistent with these precedents; we observe that the linear α -peptide α -1-LIN and the linear α/β -peptide α/β -1-LIN are able to enter cells (Figures 9 and 10) and initiate apoptosis in U937 cells (Figure 6). These results, together with the similar IC50 values displayed by pairs of stapled and unstapled analogues (i.e., by α -1 and α -1-LIN, and by α/β -1 and α/β -1-LIN) and the co-crystal structures of complexes formed by α/β -1 or α/β -1-LIN with Bcl-2, suggest that the S5 residue is a critical determinant of biochemical and cellular activity, whether or not a covalent crosslink is formed. The role of this α,α -disubstituted α -amino acid residue could arise from the high intrinsic hydrophobicity and/or from the strong helix-promoting properties that are well-known for residues of this type, such as 2-aminoisobutyric acid (Aib). Indeed, our CD data (Figure 2) show that the presence of two S5 residues leads to a significant enhancement in α -peptide helicity, even in the absence of a crosslink. The lack of cellular activity for α/β -2, which does not contain S5 residues, raises the possibility that some aspect of the α,α -disubstituted unit is important for cell entry. However, an alternative explanation for the lack of α/β -2 activity is the low affinity of this α/β -peptide for some anti-apoptotic proteins (Table 1), which is also reflected in this α/β -peptide's decreased ability to induce cytochrome *c* release in permeabilized MEFs relative to α/β -1 (Figure 4).

One major motivation for the design of α/β -peptide analogues of α -helical α -peptides is to decrease susceptibility to proteolysis. Stapled α -helical α -peptides containing two S5 residues crosslinked by olefin metathesis are less prone to protease degradation relative to analogous non-stapled α -peptides containing only natural residues, presumably because of enhanced helicity conferred by introduction of the α,α -disubstituted S5 residues and the covalent crosslink.²²⁻²⁴ The data presented here show that incorporating a small number of β -amino acid residues into the stapled α -peptide **α -1** (to generate **α/β -1**) leads to a much more pronounced protection from proteolysis than does introduction of the S5 α -residues and the staple alone (Table 1). Overall, crosslinked α/β -peptides may be better suited for eventual *in vivo* applications than are crosslinked α -peptides. One caveat to these studies is that the incorporation of the S5 residue appears to result in a tendency for both α - and α/β -peptides to aggregate, as judged by CD experiments. This conclusion is consistent with previous reports on α -peptides containing S5 residues.³⁷ Susceptibility to proteolysis is likely to be influenced by staple incorporation, by β -residue incorporation and by aggregation, and it is not possible from our data to isolate the effect exerted by each of these factors. Nevertheless, the increase in protease resistance we observed for **α/β -1** versus **α -1** is consistent with many prior reports from our groups involving $\alpha \rightarrow \beta$ substitutions.^{3-7,10,11} Other significant limitations of the stapling strategy, which we observed both for α -peptides and α/β -peptides, including low aqueous solubility, inactivation in the presence of serum, and activity toward only limited cell types, remain to be addressed.²⁴

CONCLUSIONS

We have described the design of an α/β -peptide analogue of a “stapled” α -helical α -peptide that structurally and functionally mimics the original stapled α -peptide and can kill one type of cancer cell by engaging anti-apoptotic protein targets. This report marks the first demonstration that an intracellular protein-protein interaction can be modulated by an α/β -peptide. Because α/β -peptides are less susceptible to proteolytic degradation than are their α -peptide analogues, stapled α/β -peptides may be useful for probing the functional roles of specific protein-protein interactions in living cells. Ultimately such α/β -peptides might serve as starting points for the development of therapeutic agents. The results presented here show that backbone modification via periodic $\alpha \rightarrow \beta$ -residue replacement can deliver oligomers that are highly resistant to proteolysis while retaining other desirable properties of the original α -peptide, particularly cell-permeability and specific protein recognition.

EXPERIMENTAL METHODS

Peptide and α/β -peptide synthesis and purification

α -Peptides and α/β -peptides were synthesized by microwave-assisted solid phase peptide synthesis on NovaPEG Rink Amide resin, as previously described.³⁸ Full details on peptide labeling, the metathesis reaction, and purification can be found in the Supporting Information.

Recombinant protein expression and purification

Recombinant Bcl-2 anti-apoptotic proteins with N- and/or C-terminal truncations for binding studies (Bcl-2 C22, Bcl-x_L C24, Mcl-1 N170 C23) were expressed and purified as described previously for hexahistidine tagged proteins (in the case of Bcl-2)³⁹ or GST tagged proteins (in the case of Bcl-x_L and Mcl-1).²⁸ The human Bcl-2 construct (Bcl-2 C32 Loop) used for crystallization was identical to that reported previously for structural studies.⁴⁰ This construct has the large unstructured loop between residues 35-91 replaced with residues 35-50 of Bcl-x_L. The C-terminal 32 residues corresponding to the hydrophobic transmembrane domain were also deleted to improve protein solubility. The protein was expressed as a GST fusion protein from a pGEX6P3 vector, and purified using a combination of glutathione-affinity chromatography followed by gel-filtration chromatography.

Surface plasmon resonance

Solution competition assays were performed using a Biacore 3000 instrument exactly as described previously.⁴¹

Circular dichroism (CD)

CD measurements were carried out in purified water using an Aviv Model 420 Circular Dichroism Spectrometer. Spectra were collected in a quartz cuvette (0.1 cm or 1 cm) at 20°C with a wavelength step size of 1 nm and a 10 second averaging time.

Crystallography

Crystals were obtained by mixing Bcl-2 C32 Loop with each peptide at a molar ratio of 1:1.3 and then concentrating the sample to 10 mg/mL. Crystallization trials were performed at the Bio21 Collaborative Crystallization Centre. All crystals were grown by the sitting drop method at room temperature. For the α/β -1:Bcl-2 structure, the crystals were grown in 20 mM CaCl₂, 0.1 M sodium MOPS pH 7.0, 10% (v/v) 2-propanol. For the α/β -1-LIN:Bcl-2, the crystals were grown in 0.1 M CaCl₂, 0.1 M MES pH 6.5, 20% (v/v) ethanol. Prior to cryo-cooling in liquid N₂, crystals were equilibrated into cryoprotectant consisting of reservoir solution containing 15% (v/v) ethylene glycol. Crystals were mounted directly from the drop and plunge-cooled in liquid N₂.

Crystal diffraction data collection and structure determination

Diffraction data were collected at 100 K at the Australian Synchrotron MX2 beamline (Victoria, Australia) (wavelength for both structures was 0.9537 Å). The diffraction data were integrated and scaled with XDS.⁴² The structure was obtained by molecular replacement with PHASER⁴³ using the crystal structure of the Bcl-2:Bax BH3 complex (PDB code 2XA0)²⁹ with the BH3 peptide removed as the search model. Multiple rounds of building in COOT⁴⁴ and refinement in PHENIX⁴⁵ led to the final model.

Cytochrome c release assays

Cytochrome *c* release assays performed with digitonin-permeabilized MEFs were performed as described previously.⁴¹ For the cytochrome *c* release assay with the unpermeabilized

U937 cells, 2×10^6 cells were treated with peptides (10 μ M) for 3 hours at 37°C before pelleting by centrifugation. Cell pellets were then resuspended in digitonin-containing lysis buffer (20 mM HEPES pH 7.2, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.05% (w/v) digitonin (Calbiochem) supplemented with protease inhibitors (Roche)) for 45 minutes at 30°C to permeabilize the plasma membrane, then pelleted again. The supernatant was retained (soluble fraction) and the pellet resuspended in cell-lysis buffer (20 mM Tris pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol supplemented with protease inhibitors (Roche)) and incubated on ice for 1 hour before centrifugation. This supernatant was then retained as the pellet fraction. Both soluble and pellet fractions were subsequently analyzed by Western blotting using an anti-cytochrome *c* antibody (clone 7H8.2C12; BD Biosciences).

Cell killing assay

U937 and adherent cancer cells (MDA-MB-231, MDA-MB-468, LIM-2405, SW480 and A549) were maintained in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS). MEFs were maintained in FMA medium (DME KELSO medium supplemented with 10% (v/v) FBS, 250 μ M L-asparagine and 50 μ M 2-mercaptoethanol). Cell killing assays were performed similarly to those described previously for testing stapled peptides.¹⁶ U937 cells were aliquoted (1×10^4 cells in 50 μ L) into 96-well opaque plates and incubated with serial dilutions of peptides or DMSO ($\pm$ 50 μ M Q-VD-OPh) in serum-free RPMI medium for 2 hours, followed by addition of 50 μ L RPMI containing 20% FBS (final concentration FBS 10% (v/v) in 100 μ L). Cells were incubated for a further 0, 4 or 22 hours prior to addition of CellTiter-Glo chemiluminescence reagent (for a total time of 2, 6, or 24 hours incubation with peptide) and luminescence measured on Lumistar Optima (BMG Labtech) microplate reader. For assays using adherent cells, cells were plated in their respective media described above (1.5×10^3 cells/well) then 24 hours later were washed with the corresponding serum-free media prior to addition and incubation with the peptides, DMSO and QVD-OPh, as for the U937 cells.

Analysis of cell killing by live-cell imaging

U937 cells were plated in a black 96 well optical bottom plate (5×10^4 cells/well) together with DMSO, peptides (5 μ M) $\pm$ Q-VD-OPh (50 μ M) in phenol-red and serum-free RPMI medium (final volume 100 μ L) containing CellTracker Green dye (Life Technologies, C2925) and propidium iodide (2 μ g/ml). Cells were imaged every 30 minutes for 24 hours on a Zeiss Live Cell Observer, consisting of an Axiovert 200M inverted microscope, equipped with environmental control and CO₂, LED Colibri illumination, a 20 $\times$ /0.8 NA objective and AxioCam MRm camera. Zeiss filter set Ex 470/40 and Em 525/50 together with LED module 470 was used for CellTracker Green fluorescence and Ex 560/40 and 630/75 together with LED module 540-580 for propidium iodide fluorescence.

Analysis of cellular uptake by live-cell confocal microscopy

After treatment under the conditions indicated in the Figures, U937s and MEFs were washed and then imaged using an inverted Eclipse Ti-E A1R-Si laser scanning confocal microscope (Nikon) equipped with Plan Apo 10 $\times$ DIC L, Plan Apo VC 20 $\times$ DIC N2, and Plan Apo VC

60× Oil DIC N2 objectives, a 1.5× optical zoom, lasers for 405 nm (blue), 488 nm (green), and 561/594 nm (red) wavelengths, a dichroic mirror (405/488/561/640), filters for 450/50, 525/50, and 595/50 nm wavelengths, a galvano scanning head, and a computer running NIS Elements software. U937s were imaged using at 10× and at 60× (with 1.5× magnifier) and lasers for blue, green, and red. MEFs were imaged using 20× and 60× and lasers for blue, green, and red. Images were collected using 4× averaging, 6.4 ms dwell, and 1024×1024 image size. Images were brightness-adjusted uniformly across conditions at a given magnification using NIS Elements v4.1 software (Nikon).

Protease susceptibility assay

Stock solutions of ~100 μM peptide or α/β-peptide in water with 10% DMSO (for solubility) were prepared, as determined by UV-absorbance at 280 nm.⁴⁶ Solutions were then diluted in 10% DMSO/water to 50 μM and a solution of 50 μg/mL proteinase K in TBS (50 mM Tris pH 7.5, 200 mM NaCl) was added to give final concentrations = 40 μM peptide, 10 μg/mL proteinase K in 10 mM Tris, 40 mM NaCl, 8% DMSO. Protease reactions were allowed to proceed at room temperature and each reaction was run in duplicate. At each time point, 50 μL of the reaction was quenched with 125 μL of 1:1 water/acetonitrile + 1% trifluoroacetic acid. The resulting quenched solution (125 μL) was injected onto analytical HPLC. The amount of relative remaining oligomer at each time point was determined by integration of the peak at 220 nm in the resulting chromatogram. Half-lives were determined by plotting the amount of peptide remaining versus reaction time and fitting the data to an exponential decay model in GraphPad Prism 4.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIGMS (GM056414, to S.H.G.) and grants and fellowships from the NHMRC of Australia (Project Grant 1041936, to W.D.F., and Career Development Fellowship 1024620, to E.F.L.) and Cancer Council Victoria (Grant-in-aid 1057949). Infrastructure support from NHMRC IRISS grant #361646 and the Victorian State Government OIS grant is gratefully acknowledged. J.W.C. was supported in part by a Biotechnology Training Grant (NIH Grant T32 GM008349). Additional support was provided by NIH (HL093282, to W.L.M., and GM044783, to R.T.R.). In addition, W.L.M. acknowledges support from the University of Wisconsin–Madison Nanoscale Science and Engineering Center (DMR-0832760).

REFERENCES

- (1)(a). Wells JA, McClendon CL. *Nature*. 2007; 450:1001–1009. [PubMed: 18075579] (b) Arkin MR, Tang Y, Wells JA. *Chem. Biol.* 2014; 21:1102–1114. [PubMed: 25237857]
- (2). Vlieghe P, Lisowski V, Martinez J, Khrestchatsky M. *Drug Discovery Today*. 2010; 15:40–56. [PubMed: 19879957]
- (3). Horne WS, Boersma MD, Windsor MA, Gellman SH. *Angew. Chem., Int. Ed.* 2008; 47:2853–2856.
- (4). Horne WS, Johnson LM, Ketas TJ, Klasse PJ, Lu M, Moore JP, Gellman SH. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:14751–14756. [PubMed: 19706443]
- (5). Johnson LM, Gellman SH. *Methods Enzymol.* 2013; 523:407–429. [PubMed: 23422441]
- (6). Haase HS, Peterson-Kaufman KJ, Levengood SKL, Checco JW, Murphy WL, Gellman SH. *J. Am. Chem. Soc.* 2012; 134:7652–7655. [PubMed: 22548447]

- (7). Checco JW, Kreidler DF, Thomas NC, Belair DG, Rettko NJ, Murphy WL, Forest KT, Gellman SH. *Proc. Natl. Acad. Sci. U. S. A.* 2015; 112:4552–4557. [PubMed: 25825775]
- (8)(a). Cheloha RW, Maeda A, Dean T, Gardella TJ, Gellman SH. *Nat. Biotechnol.* 2014; 32:653–655. [PubMed: 24929976] (b) Johnson LM, Barrick S, Hager MV, McFedries A, Homan EA, Rabaglia ME, Keller MP, Attie AD, Saghatelian A, Bisello A, Gellman SH. *J. Am. Chem. Soc.* 2014; 136:12848–12851. [PubMed: 25191938]
- (9)(a). Jochim AL, Arora PS. *Mol. BioSyst.* 2009; 5:924–926. [PubMed: 19668855] (b) Jochim AL, Arora PS. *ACS Chem. Biol.* 2010; 5:919–923. [PubMed: 20712375]
- (10). Boersma MD, Haase HS, Peterson-Kaufman KJ, Lee EF, Clarke OB, Colman PM, Smith BJ, Horne WS, Fairlie WD, Gellman SH. *J. Am. Chem. Soc.* 2012; 134:315–323. [PubMed: 22040025]
- (11). Peterson-Kaufman KJ, Haase HS, Boersma MD, Lee EF, Fairlie WD, Gellman SH. *ACS Chem. Biol.* 2015; 10:1667–1675. [PubMed: 25946900]
- (12)(a). Gross A, McDonnell JM, Korsmeyer SJ. *Genes Dev.* 1999; 13:1899–1911. [PubMed: 10444588] (b) Cory S, Huang DCS, Adams JM. *Oncogene.* 2003; 22:8590–8607. [PubMed: 14634621] (c) Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DCS. *Science.* 2007; 315:856–859. [PubMed: 17289999]
- (13)(a). Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettekheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. *Nature.* 2005; 435:677–681. [PubMed: 15902208] (b) Lessene G, Czabotar PE, Colman PM. *Nat. Rev. Drug Discovery.* 2008; 7:989–1000. [PubMed: 19043450]
- (14)(a). Rudin CM, Hann CL, Garon EB, de Oliveira MR, Bonomi PD, Camidge DR, Chu Q, Giaccone G, Khaira D, Ramalingam SS, Ranson MR, Dive C, McKeegan EM, Chyla BJ, Dowell BL, Chakravarty A, Nolan CE, Rudersdorf N, Busman TA, Mabry MH, Krivoshik AP, Humerickhouse RA, Shapiro GI, Gandhi L. *Clin. Cancer Res.* 2012; 18:3163–3169. [PubMed: 22496272] (b) Pan R, Hogdal LJ, Benito JM, Bucci D, Han L, Borthakur G, Cortes J, DeAngelo DJ, Debose L, Mu H, Dohner H, Gaidzik VI, Galinsky I, Golfman LS, Haferlach T, Harutyunyan KG, Hu J, Levenson JD, Marcucci G, Muschen M, Newman R, Park E, Ruvolo PP, Ruvolo V, Ryan J, Schindela S, Zweidler-McKay P, Stone RM, Kantarjian H, Andreeff M, Konopleva M, Letai AG. *Cancer Discovery.* 2014; 4:362–375. [PubMed: 24346116]
- (15). Walensky LD, Pitter K, Morash J, Oh KJ, Barbuto S, Fisher J, Smith E, Verdine GL, Korsmeyer S. *J. Mol. Cell.* 2006; 24:199–210.
- (16). LaBelle JL, Katz SG, Bird GH, Gavathiotis E, Stewart ML, Lawrence C, Fisher JK, Godes M, Pitter K, Kung AL, Walensky LD. *J. Clin. Invest.* 2012; 122:2018–2031. [PubMed: 22622039]
- (17). Lau YH, de Andrade P, Wu Y, Spring DR. *Chem. Soc. Rev.* 2015; 44:91–102. [PubMed: 25199043]
- (18)(a). Felix AM, Heimer EP, Wang CT, Lambros TJ, Fournier A, Mowles TF, Maines S, Campbell RM, Wegrzynski BB, Toome V, Fry D, Madison VS. *Int. J. Pept. Protein Res.* 1988; 32:441–454. [PubMed: 3149952] (b) Murage EN, Gao G, Bisello A, Ahn JM. *J. Med. Chem.* 2010; 53:6412–6420. [PubMed: 20687610]
- (19). Okamoto T, Zobel K, Fedorova A, Quan C, Yang H, Fairbrother WJ, Huang DCS, Smith BJ, Deshayes K, Czabotar PE. *ACS Chem. Biol.* 2013; 8:297–302. [PubMed: 23151250]
- (20). Jo H, Meinhardt N, Wu Y, Kulkarni S, Hu X, Low KE, Davies PL, DeGrado WF, Greenbaum DC. *J. Am. Chem. Soc.* 2012; 134:17704–17713. [PubMed: 22998171]
- (21)(a). Blackwell HE, Grubbs RH. *Angew. Chem., Int. Ed.* 1998; 37:3281–3284. (b) Blackwell HE, Sadowsky JD, Howard RJ, Sampson JN, Chao JA, Steinmetz WE, O'Leary DJ, Grubbs RH. *J. Org. Chem.* 2001; 66:5291–5302. [PubMed: 11485448] (c) Wang D, Liao W, Arora PS. *Angew. Chem., Int. Ed.* 2005; 44:6525–6529. (d) Patgiri A, Joy ST, Arora PS. *J. Am. Chem. Soc.* 2012; 134:11495–11502. [PubMed: 22715982]
- (22). Schafmeister CE, Po J, Verdine GL. *J. Am. Chem. Soc.* 2000; 122:5891–5892.

- (23). Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. *Science*. 2004; 305:1466–1470. [PubMed: 15353804]
- (24)(a). Walensky LD, Bird GH. *J. Med. Chem.* 2014; 57:6275–6288. [PubMed: 24601557] (b) Cromm PM, Spiegel J, Grossmann TN. *ACS Chem. Biol.* 2015; 10:1362–1375. [PubMed: 25798993]
- (25). Horne WS, Price JL, Keck JL, Gellman SH. *J. Am. Chem. Soc.* 2007; 129:4178–4180. [PubMed: 17362016]
- (26). Lee EF, Sadowsky JD, Smith BJ, Czabotar PE, Peterson-Kaufman KJ, Colman PM, Gellman SH, Fairlie WD. *Angew. Chem., Int. Ed.* 2009; 48:4318–4322.
- (27)(a). Lee EF, Smith BJ, Horne WS, Mayer KN, Evangelista M, Colman PM, Gellman SH, Fairlie WD. *ChemBioChem*. 2011; 12:2025–2032. [PubMed: 21744457] (b) Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. *Science*. 1997; 275:983–986. [PubMed: 9020082] (c) Smits C, Czabotar PE, Hinds MG, Day CL. *Structure*. 2008; 16:818–829. [PubMed: 18462686]
- (28). Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, Huang DCS, Fairlie WD, Hinds MG, Colman PM. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:6217–6222. [PubMed: 17389404]
- (29). Ku B, Liang C, Jung JU, Oh BH. *Cell Res.* 2011; 21:627–641. [PubMed: 21060336]
- (30). Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DCS. *Genes Dev.* 2005; 19:1294–1305. [PubMed: 15901672]
- (31). Okamoto T, Segal D, Zobel K, Fedorova A, Yang H, Fairbrother WJ, Huang DCS, Smith BJ, Deshayes K, Czabotar PE. *ACS Chem. Biol.* 2014; 9:838–839. [PubMed: 24547872]
- (32). Sinclair JKL, Schepartz A. *Org. Lett.* 2014; 16:4916–4919. [PubMed: 25207804]
- (33). Bird GH, Madani N, Perry AF, Princiotto AM, Supko JG, He X, Gavathiotis E, Sodroski JG, Walensky LD. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:14093–14098. [PubMed: 20660316]
- (34)(a). Bernal F, Tyler AF, Korsmeyer SJ, Walensky LD, Verdine GL. *J. Am. Chem. Soc.* 2007; 129:2456–2457. [PubMed: 17284038] (b) Bird GH, Gavathiotis E, LaBelle JL, Katz SG, Walensky LD. *ACS Chem. Biol.* 2014; 9:831–837. [PubMed: 24358963] (c) Bautista AD, Appelbaum JS, Craig CJ, Michel J, Schepartz A. *J. Am. Chem. Soc.* 2010; 132:2904–2906. [PubMed: 20158215]
- (35). Glaser SP, Lee EF, Trounson E, Bouillet P, Wei A, Fairlie WD, Izon DJ, Zuber J, Rappaport AR, Herold MJ, Alexander WS, Lowe SW, Robb L, Strasser A. *Genes Dev.* 2012; 26:120–125. [PubMed: 22279045]
- (36). Li YC, Rodewald LW, Hoppmann C, Wong ET, Lebreton S, Safar P, Patek M, Wang L, Wertman KF, Wahl GM. *Cell Rep.* 2014; 9:1946–1958. [PubMed: 25464845]
- (37). Bhattacharya S, Zhang H, Cowburn D, Debnath AK. *Biopolymers*. 2011; 97:253–264. [PubMed: 22170623]
- (38). Horne WS, Price JL, Gellman SH. *Proc. Natl. Acad. Sci. U.S.A.* 2008; 105:9151–9156. [PubMed: 18587049]
- (39). Lee EF, Dewson G, Smith BJ, Evangelista M, Pettikiriachchi A, Dogovski C, Perugini MA, Colman PM, Fairlie WD. *Structure*. 2011; 19:1467–1476. [PubMed: 22000515]
- (40). Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K, Matayoshi ED, Oltersdorf T, Fesik SW. *Proc. Natl. Acad. Sci. U.S.A.* 2001; 98:3012–3017. [PubMed: 11248023]
- (41). Smith BJ, Lee EF, Checco JW, Evangelista M, Gellman SH, Fairlie WD. *ChemBioChem*. 2013; 14:1564–1572. [PubMed: 23929624]
- (42). Kabsch W. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2010; 66:125–132. [PubMed: 20124692]
- (43)(a). McCoy AJ, Grosse-Kunstleve RW, Storoni LC, Read RJ. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2005; 61:458–464. [PubMed: 15805601] (b) Read RJ. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2001; 57:1373–1382. [PubMed: 11567148] (c) Storoni LC, McCoy AJ, Read RJ. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2004; 60:432–438. [PubMed: 14993666]
- (44). Emsley P, Cowtan K. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2004; 60:2126–2132. [PubMed: 15572765]

- (45). Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2002; 58:1948–1954. [PubMed: 12393927]
- (46). Gill SC, Vonhippel PH. Anal. Biochem. 1989; 182:319–326. [PubMed: 2610349]

Figure 1.

(A) Primary sequences of α - and α/β -peptides used in this study. The crosslinked α -peptide α -1 has been referred to as **BimSAHB** in prior reports.^{15,16} Non-natural amino acid residues are indicated by colored circles: green for S5 residue used for crosslinking, and orange for cyclic β -residues. A horizontal line connecting two S5 residues indicates that these residues have been crosslinked using olefin metathesis. (B) Structures of a generic α -residue, the S5 residue (*), two crosslinked S5 residues, and the cyclic β -residues ACPC (X) and sAPC (U).

Figure 2.
Far-UV circular dichroism spectra of α - and α/β -peptides at 50 μ M in water at 20 $^{\circ}$ C.

Figure 3.

Crystal structures of α/β -peptides α/β -1 and α/β -1-LIN bound to Bcl-2. (A) Structure of α/β -1 bound to Bcl-2 (PDB: 5AGW). (B) Structure of α/β -1-LIN bound to Bcl-2 (PDB: 5AGX). In A and B, the α/β -peptide is colored by residue type (yellow for natural α -residues, green for S5 residues, and orange for cyclic β -residues) and the Bcl-2 surface is shown in gray. (C) Overlay of α/β -1 and α/β -1-LIN with previously reported structures of an analogue of α -Bim (bound to Bcl-x_L, PDB: 3FDL)²⁶ and an analogue of α -1 (bound to Bcl-x_L, PDB: 2YQ6)¹⁹. Positions of key protein-contacting hydrophobic residues h1-h4 and the position of the crosslink (for α -1 and α/β -1) are labeled.

Figure 4.

Cytochrome *c* release assay on permeabilized wild-type (wt) or *bax*^{-/-}/*bak*^{-/-} mouse embryonic fibroblasts (MEFs) treated with 10 μ M of the indicated α - or α/β -peptide for 1 hour. α/β -1, α/β -2, and α -Bim were able to induce cytochrome *c* release from the mitochondria-containing pellet fraction (P) to the soluble cytosolic fraction (S) in wt MEFs, but not in *bax*^{-/-}/*bak*^{-/-} MEFs, as indicated by western blot for cytochrome *c*. No cytochrome *c* release was seen for negative control **Bim4E** (DMRPEIWEAQEERREGDEENAYYARR-OH). The lower panel shows western blot for Bak as a control for membrane permeabilization. Bak is a mitochondrial protein and therefore should not appear in the cytosolic fraction. *bax*^{-/-}/*bak*^{-/-} MEFs do not have Bak, and therefore no band is apparent. Irrelevant intervening lanes between α/β -1 and α/β -2 have been cropped from the image.

Figure 5.

Viability of various cell lines (relative to DMSO control) when exposed to 10 μ M α -1 (black bars) or α/β -1 (white bars) for 24 hours, as measured using CellTiter-Glo assay. Each bar represents the mean $\pm$ SD for two independent experiments. [Although the data seem clear, I wonder if we should give some indication of statistical significance. Specifically, can we state or indicate with symbols that for all cells OTHER THAN U937, growth in the presence of peptides is statistically indistinguishable from a "no treatment" control? In terms of layout, can we should move U937 next to wtMEF, or somewhere in the middle, to make it clearer that there is one cell line that is susceptible? It is easy to miss this information with U937 at the end, since there are no "bars".]

Figure 6.

Viability of U937 cells (relative to DMSO control) when treated with various concentrations of α - or α/β -peptides in the absence (red) or presence (blue) of 50 μ M caspase inhibitor Q-VD-OPh (QVD), as determined by CellTiter-Glo assay. Cells were treated with the indicated α - or α/β -peptide for 2 hours under serum-free conditions, followed by the addition of serum-containing media and additional incubation until the indicated time point. Each data point represents the mean $\pm$ SEM for 3-5 replicate measurements.

Figure 7.

(A) Analysis of U937 cell killing over time induced by 5 μ M α/β -1 or α -1 in the absence or presence of 50 μ M QVD-OPh (QVD). Green cells indicate those stained for CellTracker Green dye (living cells). Red cells indicate cells stained by propidium iodide (dead cells). (B) Close up of the indicated conditions from panel A, highlighting morphological changes resulting from treatment with α/β -1 relative to DMSO control.

Figure 8.

Cytochrome *c* release assay on unpermeabilized U937 cells treated with 10 μ M of the indicated α - or α/β -peptide for 2 hours. **α/β -1**, but not **α/β -2** or **α -Bim**, was able to induce cytochrome *c* release from the mitochondria-containing pellet fraction (P) to the soluble cytosolic fraction (S). The lower panel indicates western blot for Bak as a control for membrane permeabilization.

Figure 9.

Analysis of U937 cellular uptake of fluorescein (Flu)-labeled analogues of α - and α/β -peptides by live-cell confocal microscopy. Cells were treated with 2 μ M of the indicated α - or α/β -peptide in serum-free media for 3 hours prior to imaging. Images are obtained under 90 $\times$ magnification, showing the channels for green (fluorescein, peptide), red (WGA488, outer membrane stain), and blue (Hoescht 33342, nuclear stain). Scale bars represent 25 μ m.

Figure 10.

Analysis of MEF cellular uptake of fluorescein (Flu)-labeled analogues of α - and α/β -peptides by live-cell confocal microscopy. Cells were treated with 2 μ M of the indicated α - or α/β -peptide in serum-free media for 3 hours prior to imaging. Images are obtained under 60 $\times$ magnification, showing the channels for green (fluorescein, peptide), red (WGA488, outer membrane stain), and blue (Hoescht 33342, nuclear stain). Scale bars represent 25 μ m.

Table 1Binding to anti-apoptotic proteins by SPR and proteolysis data for α - and α/β -peptides

Oligomer	IC ₅₀ (nM) [SPR] ^a				t _{1/2} (min) ^b
	Bcl-x _L	Mcl-1	Bcl-2		
α-Bim	4.1 (0.5)	4.4 (0.4)	3.6 (0.6)		0.27
α-1	22.2 (4.8)	3.3 (0.4)	66.6 (13.6)		1.6
α/β-1	4.5 (3.4)	4.1 (2.9)	10.2 (1.4)		150
α-1-LIN	80.0 (7.5)	4.0 (0.2)	189 (33)		-
α/β-1-LIN	5.5 (0.8)	4.0 (0.5)	50.5 (10.1)		-
α/β-2	579 (202)	15.7 (5.6)	1323 (234)		-

^a Relative binding of oligomers to anti-apoptotic proteins determined by competition SPR assays, based on 2-5 independent experiments. Numbers in parentheses indicate standard deviation.

^b Half-life of 40 μ M α - or α/β -peptide in the presence of 10 μ g/mL proteinase K in TBS, pH 7.5 with 8% DMSO. Dashes indicate that the oligomer was not tested in the assay.