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Cellular Uptake and Ultrastructural Localization Underlie the Proapoptotic Activity of a Hydrocarbon-stapled BIM BH3 Peptide

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Abstract

Hydrocarbon stapling has been applied to restore and stabilize the α -helical structure of bioactive peptides for biochemical, structural, cellular, and *in vivo* studies. The peptide sequence, in addition to the composition and location of the installed staple, can dramatically influence the properties of stapled peptides. As a result, constructs that appear similar can have distinct functions and utilities. Here, we perform a side-by-side comparison of stapled peptides modeled after the pro-apoptotic BIM BH3 helix to highlight these principles. We confirm that replacing a salt-bridge with an *i, i* + 4 hydrocarbon staple does not impair target binding affinity and instead can yield a biologically and pharmacologically enhanced α -helical peptide ligand. Importantly, we demonstrate by electron microscopy that the pro-apoptotic activity of a stapled BIM BH3 helix correlates with its capacity to achieve cellular uptake without membrane disruption and accumulate at the organellar site of mechanistic activity.

Graphical Abstract

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Supporting Information

Supporting Information Figures 1–4 and Supporting Information Table 1. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.5b00214.

Therapeutic targeting of intracellular protein–protein interactions (PPIs) remains a formidable challenge. The key interaction surfaces are often large and flat, requiring agents that are more sizable, the very feature that can limit cellular penetrance. Indeed, of the estimated 20 000 proteins in the human proteome, only ~3000 have been deemed reasonable drug targets for conventional therapeutics, leaving nearly three-quarters of established disease-driving protein surfaces “undruggable.”¹ Peptide α -helices commonly mediate PPIs and participate in approximately 40% of homodimeric interfaces and 26% of heterodimeric interfaces.² The prevalence of such interactions make α -helices an attractive motif for molecular mimicry. If α -helical peptides themselves, already evolutionarily honed to provide a “best fit” for the target interface, could be adapted for cellular and *in vivo* application, a large diversity of biological targets could become “druggable.” Thus, a variety of chemical techniques have been developed to harness the transformative potential of structured α -helical peptides.³

We and others have applied all-hydrocarbon peptide stapling to dissect and target a broad range of protein interactions.⁴ For example, we have generated stapled peptides for high affinity protein binding,⁵ intracellular targeting,^{6,7} binding site discovery,^{8–12} and for potential therapeutic application in cancer,^{13–17} diabetes,¹⁸ viral infection,^{19,20} and vaccine development.²¹ That said, much remains to be learned about what factors dictate the range of biochemical and biological activities observed and how to best optimize stapled peptides for clinical development. As new studies continue to explore the relationships between stapled peptide design, biophysical properties, and cell penetration,^{4,22,23} some of the misconceptions about how stapled peptides operate will be resolved.^{4,24–27}

To that end, here we compare and contrast the biophysical, biochemical, and cellular activities of two “stabilized α -helices of BCL-2 domains,” or SAHBs, modeled after the BH3 death domain of the pro-apoptotic BCL-2 family protein BIM. The first BIM SAHB we generated, BIM SAHB_A, encompassed amino acid residues 146–166 of the BH3 domain and was found to have notably high α -helicity in aqueous solution and low nanomolar binding affinity for its BCL-2 family protein targets.⁵ We subsequently found that this same construct manifested robust cellular uptake and induced sequence-dependent cell death of cancer cells in culture and *in vivo*, with cell morphology and second messenger signaling characteristics of mitochondrial apoptosis.¹⁵ To explore the mechanism of how BIM SAHB_A directly bound to and activated pro-apoptotic BAX, a key executioner protein of the apoptotic pathway, we conducted a series of NMR analyses, which were initially hampered by the rapid BAX activation/oligomerization properties of BIM SAHB_A (aa 146–166). To facilitate our correlative biochemical and structural studies, we increased the solubility and weakened the BAX-activating properties of our prototype BIM SAHB_A, slowing down ligand-induced BAX activation/oligomerization sufficiently to perform the structural work, ultimately leading to our identification of a novel “trigger site” on BAX.^{10,28} In contrast to the prototype BIM SAHB_A (aa 146–166), the revised construct (aa 145–164) had relatively lower α -helical content in solution and was negatively charged, properties we previously reported to be suboptimal for cellular work in general.^{13,22,29} Indeed, when BIM SAHB_A (aa 145–164) was applied by an independent group in cellular studies, no biological activity was observed, leading to the conclusion that stabilizing the BIM BH3 helix with our Arg154/E158-positioned hydrocarbon staple does not confer cell penetrance or induce

apoptosis.²⁷ In follow-up work based on our published study¹⁵ and response,²⁴ application of the appropriate BIM SAHB_A (aa 146–166) yielded the expected cytotoxic activity.²⁶

Given the nuances of stapled peptide design for diverse applications, we felt it would be instructive to conduct a thorough side-by-side comparison of BIM SAHB_{As} to demonstrate their similarities and differences. In doing so, we employed electron microscopy to address one of the key issues in the structured peptide field, namely whether the biological activity of a given stapled peptide results from intracellular access mediated by membrane disruption or physiologic cellular uptake.

RESULTS AND DISCUSSION

Comparative Biophysical and Biochemical Properties of BIM BH3 Peptides.

The unmodified and *i, i + 4* stapled analogs of the BIM BH3 domain corresponding to amino acid sequences 146–166 (named BIM BH3₁, BIM SAHB_{A1}) and 145–164 (named BIM BH3₂, BIM SAHB_{A2}) were resynthesized side-by-side and the N-termini derivatized with either acetyl, FITC, or Biotin-PEG caps, according to our established methods^{22,30} (Figure 1A, Supporting Information Figure 1). The compounds were isolated at >95% purity by HPLC (Supporting Information Figure 2) and identities confirmed by mass spectrometry (Supporting Information Figure 3) and then rigorously quantitated by amino acid analysis. BIM SAHB_{As} emerge from the HPLC column at later elution times compared to their unmodified counterparts, consistent with the increased hydrophobicity of hydrocarbon-stapled peptides. We recorded circular dichroism spectra of the four BIM BH3 constructs to compare their α -helical content in solution. To achieve solubility of all constructs in the same solvent for optimal comparison, we employed a 20% acetonitrile in water solution (Figure 1B). Although the addition of organic solvent influences the estimated α -helical content, resulting for example in enhanced α -helicity of unmodified peptides compared to previously reported values in aqueous solution,^{5,27} the relative enhancements observed upon hydrocarbon stapling remain evident. In addition, BIM SAHB_{A1} demonstrates greater α -helical content in solution than BIM SAHB_{A2}, as designed.¹⁰

We next evaluated the comparative binding activities of the BIM BH3 constructs for exemplary BCL-2 family targets, including antiapoptotic BCL-X_L and pro-apoptotic BAX. Whereas BCL-2 family protein binding studies have historically been performed using constructs lacking their C-terminus (C),³¹ which both facilitates protein expression and directly exposes the BH3-binding site at the C-terminal face, we favor the analysis of full-length proteins as well, particularly in the case of BAX, where two BH3-binding sites exist.^{10,11,32} In the case of BCL-X_L C, where the BH3-binding site is relatively deep and exposed, BIM BH3₁, BIM SAHB_{A1}, and BIM SAHB_{A2} all bind within a similar 15–22 nM range as measured by a fluorescence polarization (FP) direct binding assay, with the BIM BH3₂ peptide showing somewhat weaker binding activity at 68 nM (Figure 1C). This pattern is essentially recapitulated when full-length BCL-X_L is applied, except that overall affinities are decreased, likely due to competition with the endogenous BCL-X_L α 9-helix for pocket engagement (Figure 1D). The BIM BH3₂ template sequence again binds more weakly to BCL-X_L than BIM BH3₁ (327 nM vs 91 nM), with the benefits of peptide stapling more

apparent in the context of full-length BCL-X_L (α 9-competition), as best evidenced by the comparison between BIM BH3₂ and BIM SAHB_{A2} (327 nM vs 68 nM).

To distinguish between the two discrete BH3-binding sites on BAX, we employed BAX_C and BAX A112C/V177C constructs. We previously demonstrated that BAX_C favors BH3-binding to the exposed site at the C-terminal face, whereas BAX A112C/V177C, a disulfide-tethered construct that occludes the C-terminal BH3-binding surface, favors interaction at the N-terminal trigger site.³³ Comparing the direct binding of the four FITC-BIM-BH3 peptides to BAX_C, we find that each of the stapled analogs outperforms the unmodified counterparts, and the binding activity of the aa 145–164 template, whether unmodified (BIM BH3₂) or stapled (BIM SAHB_{A2}), is weaker than the peptides designed based on the aa 146–166 sequence (BIM BH3₁, BIM SAHB_{A1}) (Figure 1E). Consistent with our prior analyses of BID and PUMA BH3 peptides,^{5,33} the unmodified BIM BH3 peptides are notably less effective binders of the N-terminal trigger site on BAX, as compared to the corresponding stapled analogs (Figure 1F). These data are consistent with the shallower topography of the N-terminal trigger site compared to the C-terminal BH3-binding site,¹⁰ such that prefolding of the BIM BH3 peptide, rather than relying on induced folding, results in a binding advantage for the BIM SAHB_{As}.

In contrast to a prior report,²⁷ we do not observe reductions in binding affinity for either of our FITC-BIM SAHB_A constructs as a result of replacing the Arg154 and Glu158 salt-bridge with the all-hydrocarbon staple, as measured in our solution-phase direct binding FP assay using recombinant and tagless BCL-2 family proteins. To rule out any discrepancy based on the use of FITC-vs acetylated BIM BH3 peptides, we performed a competitive FP assay comparing the binding activities of Ac-BIM BH3₁ and Ac-BIM SAHB_{A1} for BCL-X_L

C. In this experimental context, Ac-BIM SAHB_{A1} outperformed Ac-BIM BH3₁ by more than 6-fold when competing with a FITC-BID BH3 peptide for BCL-X_L C engagement, consistent with a binding advantage for the prefolded stapled peptide (Figure 2A,B). To rule out a discrepancy based on conducting binding studies using free (e.g., FP) vs immobilized (e.g., surface plasmon resonance) components, we generated Biotin-PEG derivatized BIM BH3₁ and BIM SAHB_{A1} peptides for biolayer interferometry³⁴ (BLI) analyses, in which streptavidin-coated biosensor tips are decorated with biotinylated peptide for “dip and bind” kinetic assays employing serial dilutions of recombinant BCL-X_L C. In this alternative direct binding assay that employs an immobilized ligand, we again see no impairment in BCL-X_L C binding as a result of replacing the Arg154 and Glu158 salt-bridge with an all-hydrocarbon *i, i + 4* staple (Figure 2C–E). Thus, across three distinct binding analysis platforms that employ three different N-terminal derivatizations of BIM BH3 peptides, the impact of stapling is either equivocal or beneficial (Figures 1C–F, 2A–E, Supporting Information Table 1).

Taken together, the utility of stapling is most evident in the context of shallow binding pockets (e.g., N-terminal trigger site of BAX) or noncovalently occluded pockets (e.g., endogenous BCL-X_L α 9 or exogenous BH3 peptide competition), where ligand prefolding confers a clear benefit. Finally, it is important to point out that—irrespective of any observed binding differences between unmodified and stapled BH3 constructs—only the stapled peptides, in contrast to unmodified or substituted-but-unstapled peptides, are protease

resistant (Figure 2F), which translates into a key pharmacologic advantage of the all-hydrocarbon stapling approach.^{7,19}

Comparative Pro-Apoptotic Activity and Cellular Uptake of BIM BH3 Peptides.

We next tested the effect of our acetylated BIM BH3 peptides on cells, including the OCI-AML3 leukemia cell line and wild-type and *Bax*^{-/-}*Bak*^{-/-} (DKO) mouse embryonic fibroblasts (MEFs). As expected, BIM BH3₂ and BIM SAHB_{A2} had no effect on any of the cells, with BIM BH3₁ beginning to impair viability at the highest dose (20 μ M) in OCI-AML3 cells only (Figure 3A). In contrast, BIM SAHB_{A1} demonstrated dose-responsive cytotoxicity in OCI-AML3 cells (IC₅₀, ~5.5 μ M), notably less potent but detectable activity in wild-type MEFs (IC₅₀, ~20 μ M), and negligible activity in DKO MEFs (Figure 3A). Importantly, the dose-responsive killing activity of BIM SAHB_{A1} in OCI-AML3 cells and wild-type MEFs coincided with caspase 3/7 activation in the identical dose range. No caspase 3/7 activation was detected in DKO MEFs, even at the highest dose (20 μ M), where low-level cytotoxicity was observed (Figure 3B). Thus, of the BIM BH3 peptides, only BIM SAHB_{A1} exhibits cellular activity, and its adverse effect on viability, when accompanied by caspase 3/7 activation, is consistent with a pro-apoptotic mechanism of action.¹⁵

To explore what factor(s) could underlie the dramatic differences in cytotoxicity observed for the BIM BH3 peptides, we first examined their cellular uptake potential. The three cell lines were treated with FITC-derivatized peptide constructs, followed by repeated cell washing, detergent lysis, isolation of the supernatant, electrophoresis, and FITC scan. Only the lysates from cells treated with BIM SAHB_{A1} were found to contain FITC-peptide above the baseline (Figure 4A). These data suggest that the biological activity of BIM SAHB_{A1} derives from its capacity to access the intracellular environment. Importantly, the relative absence of cytotoxic activity in DKO MEFs does not result from any difference in cellular uptake, because all three lines manifest FITC-BIM SAHB_{A1} accumulation in lysates from treated cells. To rule out the possibility that BIM SAHB_{A1} achieves cellular penetrance by nonspecifically disrupting the plasma membrane, we performed lactate dehydrogenase release assays (LDH), an important screening tool for identifying membrane-disruptive peptides.^{4,22} We observed no LDH release above the background for any of the three cell lines tested at the 0–20 μ M dosing range, indicating that BIM SAHB_{A1} does not achieve cellular penetrance or induce cell death simply by puncturing the plasma membrane (Figure 4B).

Electron Microscopy of Cells Treated with BIM BH3 Peptides.

To obtain further evidence that the biological activity of BIM SAHB_{A1} derives from its capacity to reach the intracellular environment, we performed electron microscopy on FITC-BIM SAHB_{A1}-treated cells, using vehicle and FITC-BIM-BH3₁ exposure as negative controls. We first examined the comparative morphology of wild-type MEFs upon treatment with vehicle or 5 μ M peptides. Blinded evaluation of the electron micrographs from treated specimens revealed no morphologic differences among the cells, with the plasma membrane clearly intact in BIM SAHB_{A1}-treated cells (Figure 5A). We next performed immunoelectron microscopy to determine the intracellular location of FITC-BIM SAHB_{A1}. Whereas little to no immunogold labeling was observed for FITC-BIM-BH3₁-treated cells,

robust labeling of mitochondria was observed in FITC-BIM SAHB_{A1}-treated cells (Figure 5B). These data are consistent with BIM SAHB_{A1} targeting the very organelle that is enriched in BCL-2 family protein targets. We also observed labeling of microvesicular bodies (Supporting Information Figure 4), consistent with an endosomal mechanism of cellular import, as suggested by our earliest study of cell-penetrating stapled peptides.⁷

Conclusions.

The results of our comparative study of distinct stapled BIM BH3 peptides highlight several key principles of stapled peptide design, evaluation, and application. All-hydrocarbon stapling increases the hydrophobicity of stapled peptides compared to their unmodified counterparts, as evidenced by a rightward shift of the HPLC profile. Circular dichroism can be used to assess the relative benefits of hydrocarbon stapling in enhancing peptide α -helicity. Inserting an all-hydrocarbon staple of identical composition and position into peptides of slightly different length and sequence can, among other parameters (e.g., net charge), result in different degrees of α -helical induction, which can impact stability, and biochemical and biological activities. Comparative binding analyses are especially helpful for determining whether the inserted staple increases, decreases, or has no effect on target engagement. We have previously observed that placement of the hydrocarbon staple at the peptide/target binding interface, or replacing a key interacting residue with a stapling amino acid, can negatively impact binding activity, providing useful negative controls for biochemical and biological studies.^{6,10} In designing stapled peptides, we typically locate the staples at the noninteracting surface of the peptide α helix to avoid disrupting the native binding interface.^{7,10} Indeed, for stapling the BIM BH3₂ template, we previously observed that a series of constructs bearing differentially placed staples at the noninteracting surface all behaved similarly, with enhanced potency in BAX-related biochemical assays compared to the unmodified peptide template.¹⁰ Although a recent report indicated that replacement of the Arg154–Glu158 amino acid pair with the *i, i + 4* hydrocarbon staple in BIM SAHB_A peptides is detrimental to BCL-2 family protein binding activity,^{26,27} our data across three distinct binding assay platforms, including direct FP with FITC-peptides, competitive FP with Ac-peptides, and BLI with Biotin-PEG-peptides, do not support that conclusion. Staples placed at the amphipathic boundary of peptide binding interfaces can occasionally engage the target surface through supplementary hydrophobic interactions, strengthening the binding interactions of stapled peptides even further.⁶

The cellular penetrance of appropriately designed stapled peptides affords potentially transformative clinical translation opportunities. In developing stapled peptides for cellular and *in vivo* studies, it is critically important to measure cellular uptake and membrane disruption potential to determine whether any given construct, and at what dosing range, is appropriate for such applications. Here, we demonstrate that the biological inactivity of BIM BH3₁, BIM BH3₂, and BIM SAHB_{A2} peptides correlates with their inability to gain access to the intracellular environment, whereas the cytotoxic and caspase 3/7 activation properties of BIM SAHB_{A1} is consistent with its cellular uptake and nanomolar target-binding activity. Importantly, the observed cellular penetrance and biological activity of BIM SAHB_{A1} does not derive from plasma membrane lysis, as monitored by LDH release assay and electron microscopy at bioactive doses. Instead, we find that BIM SAHB_{A1} localizes to the

mitochondria and multivesicular bodies of intact cells, consistent with an endosomal import mechanism and tropism for the very organelle enriched in its BCL-2 family protein targets. Taken together, we find that a stepwise approach to stapled peptide development and optimization, which incorporates comparative assessments of biophysical and biochemical properties, cellular uptake, and mechanism-based biological activities, provides the best chance for advancing a prototype stapled peptide therapeutic with the desired mechanism of action toward clinical translation.

METHODS

Stapled Peptide Synthesis and Characterization.

All-hydrocarbon stapled peptides were synthesized; derivatized at the N-terminus with FITC, Ac, or Biotin-PEG; and purified to >95% homogeneity by LC/MS as previously described.²⁵ Acetylated peptides were dissolved in 20% (v/v) acetonitrile in water for circular dichroism analyses, performed on an Aviv Biomedical spectrophotometer, as reported.²⁵

Recombinant Protein Production.

BCL-X_L C was expressed as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli* BL21 (DE3) from the pGEX2T vector (Pharmacia Biotech) and purified by affinity chromatography using glutathione sepharose beads (GE Healthcare), followed by thrombin cleavage of the GST tag. Full-length BCL-X_L was expressed as a histidine-tagged protein in *Escherichia coli* BL21 (DE3) from the pET-19b vector (EMD Millipore), purified by affinity chromatography using Ni-NTA agarose (Qiagen), eluted with 300 mM imidazole, and dialyzed into a 300 mM NaCl solution containing 20 mM Hepes, pH 7.2. BAX C and BAX A112C/V177C were expressed in *Escherichia coli* BL21 (DE3) from the pTYB1 vector and purified by affinity chromatography using chitin beads (New England BioLabs) and the chitin tag cleaved by overnight incubation in 50 mM dithiothreitol. In each case, pure, monomeric protein was isolated by gel filtration FPLC.

Fluorescence Polarization Binding Assay.

For direct FP binding assays, FITC-derivatized BIM BH3 peptides (50 nM) were added to serial dilutions of recombinant protein in binding buffer (BCL-X_L C: 100 mM NaCl, 50 mM Tris, pH 8.0; full-length BAX and BCL-X_L: 140 mM NaCl, 50 mM Tris, pH 7.4) in 96-well black opaque plates. For binding assays employing BAX A112C/V177C, the protein stock and binding buffer was supplemented with GSSG (0.5 mM). The plates were incubated in the dark at RT and then fluorescence polarization measured at 20 min on a microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices). Dissociation constants (K_D) were calculated by nonlinear regression analysis of dose-response curves with Prism software 5.0 (GraphPad) using the ligand depletion formula for FP binding assays, as described.^{13,35} For competitive FP binding assays, FITC-derivatized BID BH3 peptide (25 nM) composed of the sequence DIIRNIARHLAQVGDSBDRSI (where B is norleucine) was added to BCL-X_L C protein (250 nM) and serial dilutions (starting from 1 μ M) of Ac-BIM BH3₁ or Ac-BIM SAHB_{A7} in 96-well black opaque plates. The plates were incubated in the dark at RT and then FP measured at 20 min as above. IC50s were calculated

by nonlinear regression analysis of dose-response curves using Prism software 5.0 (GraphPad) and K_i s calculated according to the formula, $K_i = IC_{50}/(1 + [L/K_D])$.

Biolayer Interferometry.

BLI binding measurements were performed using an Octet Red384 System (ForteBio Inc.). Super Streptavidin (SSA) sensors were prewetted in PBS containing 2% (v/v) DMSO for 10 min prior to use, loaded with $10 \mu\text{g mL}^{-1}$ Biotin-PEG-BIM BH3₁ or Biotin-PEG-BIM SAHB_{AI} peptides, quenched with 0.1 mg mL^{-1} biocytin for 2 min, and washed with kinetics buffer for 2 min (PBS containing 1% [w/v] BSA, 0.1% [v/v] Tween-20, 0.05% [w/v] NaN₃). The sensors were then transferred into 2-fold serial dilutions (starting from 375 nM) of BCL-X_L C in kinetics buffer for 5 min (association step), followed by kinetics buffer alone for 10 min (dissociation step). Negative control runs were performed as above except that no Biotin-PEG peptide was loaded onto the sensors. Binding parameters were calculated using the accompanying Octet Software version 9 (ForteBio, Inc.).

Peptide Protease Resistance Analysis.

In vitro proteolytic degradation was measured by LC/MS (Agilent 1200) using the following parameters: 20 μL injection, 0.6 mL per minute flow rate, 15 min run time consisting of a gradient of water (0.1% [v/v] formic acid) and 20% [v/v] to 80% acetonitrile (0.75% formic acid) over 10 min, 4 min wash to revert to starting gradient conditions, and 0.5 min post-time. The mass spectrometer was set to scan mode at $(M + 3H)/3, \pm 1$ mass units. Integration of the signal yielded areas under the curve of $>10^8$ counts. Reaction samples were composed of 5 μL of peptide in DMSO (1 mM stock) and 195 μL of buffer consisting of 150 mM sodium chloride and 50 mM sodium phosphate buffer, pH 7.4. Upon injection of the sample (time 0), 2 μL of 40 ng μL^{-1} Asp-N (Roche) was added, and the amount of intact peptide was quantitated by serial injection over time. A plot of area versus time yielded an exponential decay curve, and half-lives were determined by nonlinear regression analysis using Prism software 5.0 (GraphPad).

Cell Culture.

Wild-type and *Bax*^{-/-}*Bak*^{-/-} mouse embryonic fibroblasts were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FBS, 100 U mL⁻¹ penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, and 50 μM β -mercaptoethanol. OCI-AML3 cells were maintained in RPMI 1640 GlutaMAX (Invitrogen) supplemented with 10% (v/v) FBS and 100 U mL⁻¹ penicillin/streptomycin.

Cell Viability and Caspase-3/7 Activation Assays.

Wild-type and *Bax*^{-/-}*Bak*^{-/-} mouse embryonic fibroblasts were plated in 96-well plates (2.5×10^3 cells per well), allowed to adhere overnight, and then washed and incubated in OptiMEM containing 100 U mL⁻¹ penicillin/streptomycin. OCI-AML3 cells were plated in 96-well plates (1×10^4 cells per well) in OptiMEM containing 100 U mL⁻¹ penicillin/streptomycin. Serial dilutions of the BIM BH3 peptides from a 10 mM DMSO stock, or vehicle, were added to the cells in a final volume of 100 μL and incubated at 37 °C for 2 h, followed by the addition of 10 μL of FBS (serum replacement to 10% [v/v]). For viability

analysis, cells were analyzed at 24 h post-treatment using the CellTiter-Glo chemiluminescence reagent (Promega). To measure caspase-3/7 activation, cells were analyzed after 6 h of treatment using the Caspase-Glo 3/7 chemiluminescence reagent (Promega). Luminescence was detected by a microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices).

Cellular Uptake Analysis.

Wild-type and *Bax*^{-/-} *Bak*^{-/-} mouse embryonic fibroblasts were plated in six-well plates (5×10^5 cells per well), allowed to adhere overnight, and then washed and incubated in OptiMEM (Invitrogen) containing 100 U mL⁻¹ penicillin/streptomycin. OCI-AML3 cells were plated in six-well plates (1×10^6 cells per well) in OptiMEM containing 100 U mL⁻¹ penicillin/streptomycin. FITC-BIM-BH3 peptides (1 mM stock in DMSO) or vehicle was diluted into 1 mL cultures for a final treatment concentration of 1 μ M and incubated at 37 °C for 2 h, followed by washing twice in PBS, trypsinizing for 10 min to remove any surface-bound peptide, and washing in PBS twice more. Cells were then lysed in 1% CHAPS buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 1% [w/v] CHAPS, 1 mM EDTA, 1.5 mM MgCl₂, complete protease inhibitor tablet [Roche]) on ice and incubated for 20 min. Supernatants were collected after table top centrifugation, electrophoresed, and intracellular FITC peptide detected by fluorescence imaging using a Typhoon 9400 (GE Healthcare Life Sciences).

Lactate Dehydrogenase Release Assay.

MEFs were plated in 96-well format (1.5×10^4 cells per well), and after overnight incubation, full media was replaced with serum-free DMEM. OCI-AML3 cells were plated in 96-well format (2.5×10^4 cells per well) in OptiMEM containing 100 U mL⁻¹ penicillin/streptomycin. Serial dilutions of BIM BH3 peptides from a 10 mM DMSO stock, or vehicle, was added to the cells in a final volume of 100 μ L and incubated at 37 °C for 2 h. The plate was spun down at 1500 rpm for 5 min at 4 °C, and 80 μ L of cell culture media was transferred to a clear plate (Corning), incubated with 80 μ L of LDH reagent (Roche) for 15 min while shaking, and absorbance measured at 490 nm on a microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices).

Electron Microscopy.

For electron microscopy studies, wild-type MEFs were maintained in DMEM (Invitrogen) supplemented with 10% (v/v) FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, and 50 μ M β -mercaptoethanol. Cells were plated in six-well format (1×10^5 cells per well) and, after overnight incubation, were washed in Opti-MEM and then incubated with 5 μ M FITC-BIM-BH3₁, FITC-BIM SAHB₄₁, or vehicle (0.05% [v/v] DMSO) for 2 h in Opti-MEM lacking phenol red (Invitrogen). The cells were washed twice with PBS and fixed in the dish using a solution of 2.5% (w/v) glutaraldehyde, 1.25% (w/v) paraformaldehyde, and 0.03% (w/v) picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at RT. The cells were then washed in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed for 30 min in 1% (w/v) osmium tetroxide/1.5% (w/v) potassium ferrocyanide, washed in water three times, and then incubated in 1% (w/v) aqueous uranyl acetate for 30 min, followed by two washes in water and subsequent dehydration in graded ethanol (5 min each of 50%, 70%, 95%, and then twice in 100%). The

cells were removed from the dish in propylene oxide, pelleted at 3000 rpm for 3 min, and incubated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc.). The samples were subsequently embedded in TAAB Epon and polymerized at 60 °C for 48 h. Ultrathin sections (~80 nm) were cut on a Reichert Ultracut-S microtome, placed onto copper grids, stained with 0.2% (w/v) lead citrate, and then examined on a JEOL 1200EX transmission electron microscope. Images were recorded using a 2k CCD camera (Advanced Microscopy Techniques).

For immunoelectron microscopy, wild-type MEFs were treated with peptides as above, washed twice with PBS, detached using 0.05% (w/v) trypsin (Invitrogen) for 5 min, and then washed twice with PBS. The cells were fixed at RT for 2 h in a solution of 4% paraformaldehyde (Electron Microscopy Sciences) containing 0.1% (w/v) glutaraldehyde (Electron Microscopy Sciences). The cells were pelleted and cryo-protected using 2.3 M sucrose in PBS containing 0.2 M glycine (to quench free aldehyde groups) for 15 min. The cells were frozen in liquid nitrogen, and ultrathin sections (~80 nm) were cut at -120 °C. The sections were transferred to Formvar-carbon coated copper grids, floated on PBS, and then immunogold labeling was performed at RT on parafilm. The grids were blocked on drops of 1% (w/v) bovine serum albumin (BSA) for 10 min, transferred to 5 μ L drops of primary antibody (rabbit polyclonal anti-FITC, Invitrogen 71–1900) diluted 1:50 in 1% (w/v) BSA/PBS, and incubated for 30 min. The grids were then washed in four drops of PBS for a total of 15 min, transferred to 5 μ L drops of protein-A gold 10 nm (University Medical Center Utrecht) diluted 1:50 in 1% (w/v) BSA/PBS for 20 min, and washed in four drops of PBS and then six drops of double-distilled water. Contrasting of the labeled grids was carried out on ice in 0.3% (w/v) uranyl acetate containing 2% (w/v) methyl cellulose for 10 min. Grids were removed and excess liquid blotted with filter paper, leaving a thin coat of methyl cellulose. The grids were examined on a JEOL 1200EX transmission electron microscope, and images were recorded using a 2k CCD camera (Advanced Microscopy Techniques).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparative amino acid sequence, α -helical content, and BCL-2 family protein direct binding activities of BIM BH3 peptides. (A) Amino acid sequence and staple position of unmodified and stapled BIM BH3 peptides. (B) Circular dichroism analysis of unmodified and stapled Ac-BIM BH3 peptides, dissolved in 20% acetonitrile/water. (C–F) Comparative direct binding activities of FITC-BIM-BH3 peptides for (C) BCL-X_L C, (D) full-length (FL) BCL-X_L, (E) BAX C, and (F) full-length BAX A112C/V177C, as measured by FP assay. Plotted data are mean $\pm$ SD for experiments performed in triplicate; K_D 's are mean $\pm$ SEM of the calculated dissociation constants from biological replicates.

Figure 2.

Comparative competitive and ligand-immobilized binding activities, and proteolytic stability, of BIM BH3₁ peptides. (A) Binding affinity of an unmodified FITC-BID BH3 peptide for BCL- X_L C. (B) Competitive binding of Ac-BIM BH3₁ and Ac-BIM SAHB_{A1} peptides to BCL- X_L C in the presence of FITC-BID BH3, as measured by FP. (C–E) Comparative binding affinities of (C) vehicle, (D) Biotin-PEG-BIM BH3₁, and (E) Biotin-PEG-BIM SAHB_{A1} for BCL- X_L C, as measured by biolayer interferometry (OCTET, BioForté) using streptavidin biosensor tips loaded without (C) or with (D,E) Biotin-PEG-peptides. (F) Half-lives of unmodified, substituted but unstapled, and stapled BIM BH3₁ peptides exposed to Asp-N, as analyzed by LC/MS. Plotted data for FP and proteolysis are mean $\pm$ SD for experiments performed in triplicate; an exemplary BLI trace is shown for each experimental condition. Binding parameters for competitive FP and BLI are mean $\pm$ SEM of the calculated constants from biological replicates.

Figure 3.

Comparative cellular effects of BIM BH3 peptides on viability and caspase 3/7 activation. (A) Impact of BIM BH3 peptide treatments on the viability of OCI-AML3 cells, and wild-type and *Bax*^{-/-}*Bak*^{-/-} MEFs at 24 h. (B) Caspase 3/7 activation was measured at 6 h for the OCI-AML3 and MEF cells treated with the corresponding dose range of BIM BH3 peptides. Data are normalized to vehicle control, and plotted data are mean ± SD for experiments performed in triplicate.

Figure 4.

Cellular uptake and membrane permeabilization analyses of BIM BH3 peptides. (A) OCI-AML3 cells, and wild-type and *Bax*^{-/-}*Bak*^{-/-} MEFs, were exposed to FITC-BIM-BH3 peptides for 2 h at 37 °C, followed by washing, trypsinization, cell lysis, electrophoresis of the isolated supernatant, and fluorescence detection. (B) OCI-AML3 cells and wild-type and *Bax*^{-/-}*Bak*^{-/-} MEFs were treated with Ac-BIM SAHB_{A1} at the same dose range used for cell viability and caspase 3/7 experiments, and LDH release into the supernatant was quantified 2 h after peptide treatment. T, Triton X-100. Plotted data are mean ± SD for experiments performed in triplicate.

Figure 5. Ultrastructural localization of BIM SAHB_{A1} in peptide-treated cells. (A) Morphology of wild-type MEFs treated with vehicle, FITC-BIM-BH3₁, or FITC-BIM SAHB_{A1}, as examined by electron microscopy. (B) Ultrastructural localization of FITC-BIM SAHB_{A1}, as detected by immunoelectron microscopy.