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Letters

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Stabilizing the Pro-Apoptotic BimBH3 Helix (BimSAHB) Does Not Necessarily Enhance Affinity or Biological Activity

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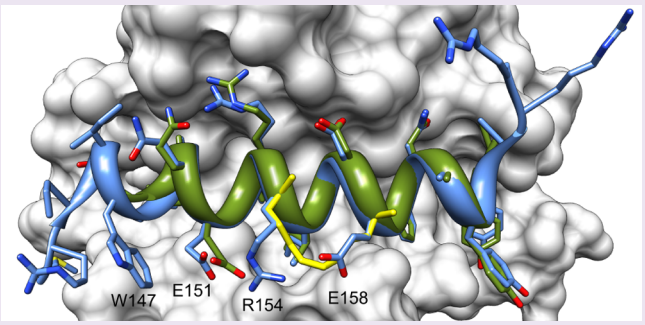
Supporting Information

ABSTRACT: An attractive approach for developing therapeutic peptides is to enhance binding to their targets by stabilizing their α-helical conformation, for example, stabilized BimBH3 peptides (BimSAHB) designed to induce apoptosis. Unexpectedly, we found that such modified peptides have *reduced* affinity for their targets, the pro-survival Bcl-2 proteins. We attribute this loss in affinity to disruption of a network of stabilizing intramolecular interactions present in the bound state of the native peptide. Altering this network may compromise binding affinity, as in the case of the BimBH3 stapled peptide studied here. Moreover, cells exposed to these peptides do not readily undergo apoptosis, strongly indicating that BimSAHB is not inherently cell permeable.



Stabilized helical peptides are designed to mimic an α-helical structure through a constraint imposed by covalently linking two residues on the same helical face (e.g., residue *i* with *i* + 4). "Stapling" the peptide into a preformed helix might be expected to lower the energy barrier for binding by reducing entropic costs, with a concomitant increase in binding affinity. Additionally, stabilizing the peptide may reduce degradation by proteases[1,2] and, in the case of hydrocarbon linkages, reportedly enhance transport into cells,[3] thereby improving bioavailability and their potential as therapeutic agents.[3−5] The findings we present here for the stapled BH3 peptide (BimSAHB), however, do not support these claims, particularly in regards to affinity and cell permeability. We observe a reduction in binding upon BimBH3 stapling, which we attribute to the loss of a network of stabilizing intramolecular interactions on the peptide. Thus, in addition to the primary consideration for staple placement in peptide design, that of avoiding key binding interfaces, our observations reveal a new consideration, that staples should also avoid disruption of favorable interactions within the peptide itself.

It has previously been reported that a stapled version of the BimBH3 peptide (dubbed: "stabilized α-helices of Bcl-2 domains", BimSAHB), where two native residues were replaced with (*S*)-pentenyl alanine derivatives and covalently joined through a metathesis reaction,[2,3] kills cells by directly activating Bax through an interface involving residue Lys21.[6] This stabilized peptide has also been reported to have enhanced binding for the pro-survival proteins.[7] When mouse embryonic fibroblasts (MEFs) (Supplementary Figure 1) or Jurkat cells (data not shown) were treated with BimSAHB, we observed no cell death, in contrast to the potent killing induced by the well-characterized activator of apoptosis, etoposide. This was true for both the 20-mer BimSAHB peptide used below and the 21-mer BimSAHB peptide previously used in cellular assays.[8]

Given that no cell killing was observed when BimSAHB was added to cells in culture, we decided to test the ability of these peptides to induce mitochondrial cytochrome *c* release *in vitro* from cells permeabilized with a low concentration of the detergent digitonin. This would determine if the absence of killing activity could be attributed to a lack of cellular uptake. In such assays, we found that BimSAHB was indeed capable of liberating mitochondrial cytochrome *c* (Figure 1a,b), consistent with the conclusion that BimSAHB, under the conditions tested, does not readily enter cells in sufficient amounts to induce apoptosis (although proteolytic degradation or non-specific binding effects could also account for this observation).

We next tested the role of the Bax interface encompassing Lys21 proposed to be essential for its activation.[6] To undertake

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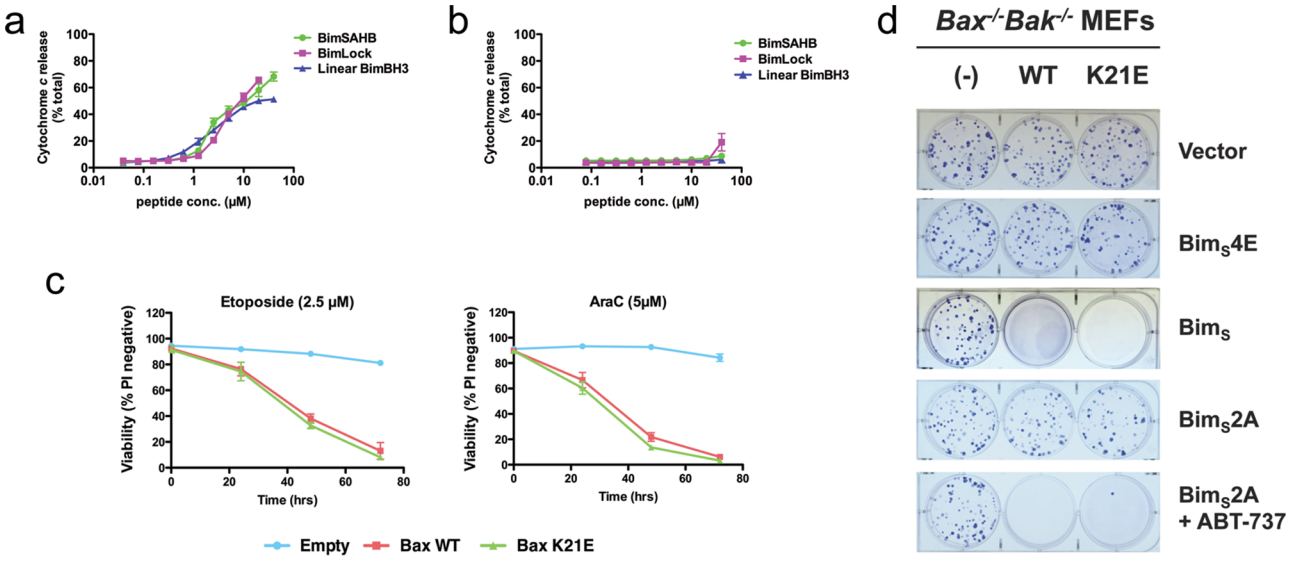

Figure 1. Bioactivity of constrained BimBH3 peptides. Cytochrome *c* release experiments were performed on permeabilized cells (MEFs) that were derived from either (a) wild-type or (b) from $Bax^{-/-}Bak^{-/-}$ mice. The cell pellets, containing intact mitochondria, were treated with increasing concentrations of the indicated peptides. The quantity of cytochrome *c* release was assessed by ELISA (R&D Systems) analyses of supernatants postpeptide treatment as compared to mitochondria treated with the permeabilizing agent 1% Triton X-100 (100% activity). (c) Killing assay of $Bax^{-/-}Bak^{-/-}$ MEF cells reconstituted with either wild-type Bax (WT) or Bax(K21E) in response to DNA damaging agents etoposide or Ara-C. Cell viability was monitored by propidium iodide (PI) exclusion determined by flow cytometry. Expression of wild-type Bax and BaxK21E were confirmed by Western blot analysis (Supplementary Figure 2). (d) Colony formation assays of $Bax^{-/-}Bak^{-/-}$ MEF cells reconstituted with either wild-type Bax (WT) or Bax (K21E) and transfected with vector encoding an inactive Bim variant (Bim_S4E),[20] wild-type Bim_S (targeting all pro-survival proteins), a Bim_S variant targeting only Mcl-1 (Bim_S2A),[21] or Bim_S2A in combination with ABT-737 treatment. Error bars in panels a–c represent SEM of 2 independent experiments.

this, we reconstituted MEFs lacking the essential cell death mediators Bax and Bak with wild-type (WT) Bax or the K21E mutant that was reported to be inert.[6] Unexpectedly, cells reconstituted with either WT or K21E mutant Bax behaved identically in short- or long-term survival assays (Figure 1c,d), suggesting that this interface was not required for Bax activation. Equivalent cell death to that observed for BimBH3 overexpression was observed when ABT-737 (to target Bcl-2, Bcl-x_L, and Bcl-w[7]) was combined with Bim_S2A (to target Mcl-1[9]), suggesting that apoptosis in these cells is primarily the result of inhibition of pro-survival proteins (Figure 1d).[10] By implication, the mechanism by which BimBH3 peptides initiate cytochrome *c* release in our experiments using isolated mitochondria could be accounted for by relieving the pro-survival proteins restraining Bax and Bak[10] and possibly by direct activation of Bax via an alternative interface such as that shown for Bak.[11]

In sharp contrast to the reported studies,[3] our studies suggest that BimSAHB is not inherently cell permeable. Moreover, the linear control BimBH3 of precisely the same length as the stapled peptide was just as active at inducing cytochrome *c* release *in vitro* (Figure 1a). As the proposed interface for Bax activation by BimSAHB did not appear to play a major role (Figure 1c,d), we undertook a detailed biochemical and structural analysis of the constrained BimSAHB and its interactions with pro-survival proteins. To undertake these studies, we also employed a second approach, which we termed BimLOCK, to link the side-chains of a glutamate with a lysine through a lactam bridge.[12,13] These modified residues were identical to the (*S*)-pentenyl alanines used to construct BimSAHB.

Circular dichroism (CD) was employed to confirm that covalent linkages enhanced helical content (Figure 2a). Both BimSAHB (39% helix) and BimLOCK (49%) displayed enhanced helical content in an aqueous solution compared to an equivalent linear peptide (21%). Additionally, we determined crystal structures for both constrained peptides in complex with the pro-survival Bcl-2 family protein Bcl-x_L (BCL2L1) (Figure 2b,c). These structures revealed that both constrained peptides bind to Bcl-x_L analogously to the linear BimBH3 peptide and that neither the hydrocarbon staple nor the lactam bridge interacts with residues on Bcl-x_L. Because of the increased helicity, we anticipated an approximate 4-fold increase in binding affinity (see Supporting Information). Instead, these peptides have reduced affinities for pro-survival proteins (Table 1a). Direct binding assays confirmed this observation for two pro-survival proteins, Bcl-x_L and Mcl-1. The penalty imposed by the staple for Bcl-x_L is due to slower on- and faster off-rates (1.8- and 13-fold, respectively). Similarly, the loss in affinity for Mcl-1 resulted predominantly from a faster off-rate (2.8-fold increase, compared to a 1.3-fold reduction in on-rate) (Table 1b).

Inspection of the structure of BimBH3-peptide in complex with Bcl-x_L (PDB code, 3FDL[14]) reveals how the side-chain groups of residues on the exposed face of the peptide form a series of interactions with one another; Glu151 and Glu158 (numbering based on human Bim_{EL}) form a pair of salt bridges with Arg154, and the aliphatic methylene moiety of Glu151 packs tightly with the indole side-chain of Trp147. Additionally, Arg154 of Bim forms a water-mediated interaction with Arg103 of Bcl-x_L (Supplementary Figure 3a). Critically, none of these interactions are observed in either structure of the stapled peptides in complex with Bcl-x_L (Supplementary Figure 3b,c).

We have calculated the pairwise interaction energy between the side-chain groups of each of these residues employing the AUTODOCK empirical function: the AUTODOCK potential

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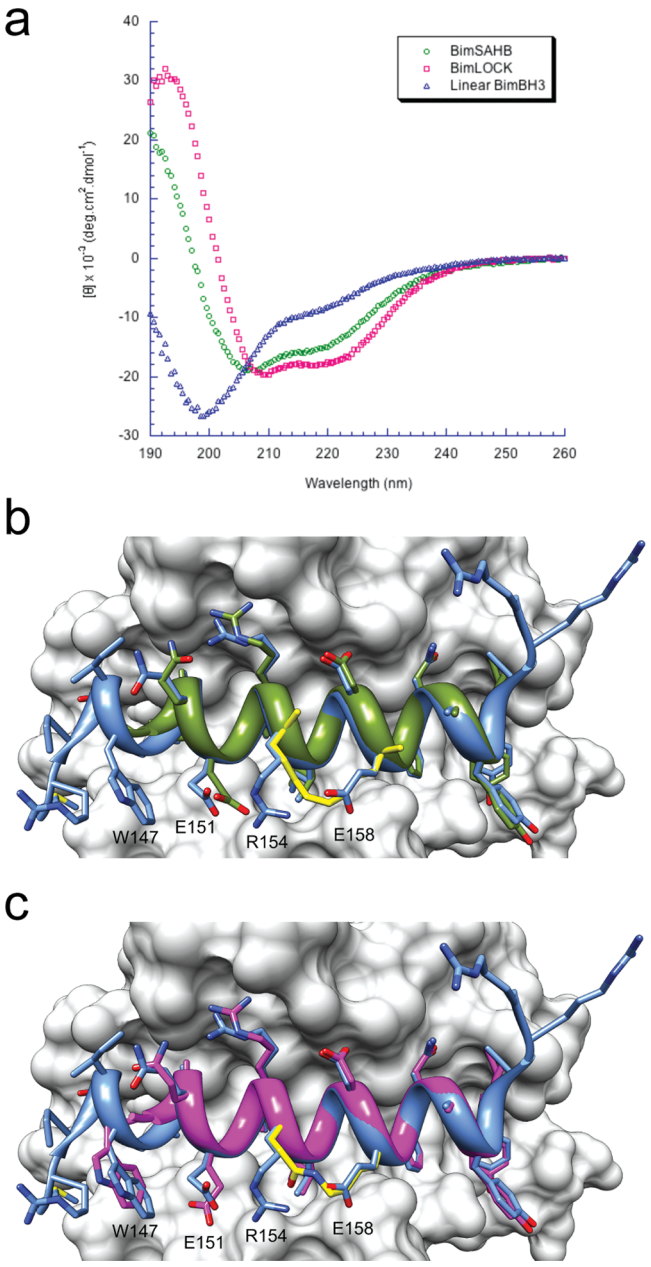

Figure 2. Characterization of constrained BimBH3 peptides. (a) Circular dichroism was used to measure the percentage of helical content for BimSAHB, BimLOCK, and an equivalent linear BimBH3 peptide. Both constrained BimBH3 peptides were found to have significant helical content (BimSAHB 39% helix; BimLOCK 49% helix), while the linear peptide was found to have a modest secondary structure content (21% helix). (b) Structure of the Bcl-x_L:BimSAHB complex overlaid with linear BimBH3 peptide in complex with Bcl-x_L (PDB code, 3FDL;[14] note that the peptide used for the 3FDL structure is longer than those used in this study). Peptide regions of BimSAHB are colored green and the hydrocarbon staple yellow. (c) Structure of the Bcl-x_L:BimLOCK complex overlaid with BimBH3. Peptide regions of BimLOCK are colored magenta and the lactam bridge yellow. BimBH3 is colored blue, and Bcl-x_L is represented in surface format in both complexes. Peptide sequences are as detailed in Table 1 unless otherwise stated.

includes five major contributions to the total free energy of interaction: the van der Waals, hydrogen-bond, electrostatic, conformational entropy, and desolvation energies.[15] The individual energies of interaction calculated were, between Trp147−Glu151, −0.3 kJ mol^{-1}, Glu151−Arg154, −7.0 kJ mol^{-1}, and Arg154−Glu158, −2.7 kJ mol^{-1} (using the Bcl-x_L/Bim coordinates), yielding a total interaction energy between all four residues of −10.0 kJ mol^{-1}. Assuming these side-chains do not interact with one another in the free peptide, the cumulative effect of increased helicity of the peptide and estimated loss in binding energy due to missing side-chain interactions, the anticipated reduction in binding affinity for BimSAHB and BimLOCK is 24-fold and 16-fold, respectively. These estimates compare favorably with the observed 26-fold reduction from the competition assay and 8-fold reduction from the direct binding assay for BimSAHB (Table 1, Supplementary Table 2) and, likewise, 3-fold reduction from the competition assay for BimLOCK.

For Mcl-1 and A1, where the reduction in binding affinity observed by the introduction of the constraint is not as large as that observed for Bcl-2 and Bcl-x_L, either the side-chain groups may maintain some interaction with one another or the side-chain groups may interact with the pro-survival receptor to improve binding affinity. To further assess the contribution of the intramolecular bonding network on the exposed face of the peptide and to ensure loss of affinity could not be attributed to steric hindrance imposed by the staple, we tested the binding affinity of an unstapled Bim peptide (linear Bim with Glu151 and Arg154 substituted with pentenylalanine residues but not subjected to ring closing metathesis) (Table 1b). This peptide bound Bcl-x_L with a further reduced affinity as compared to the stapled Bim, as would be anticipated with the combined effects of a reduction in helicity (due to loss of the staple) and the loss of the stabilizing intramolecular network.

Upon binding their pro-survival receptor, the BH3 peptides adopt an α-helical geometry, stabilized by the interactions between receptor and peptide *and* by the intramolecular interactions within the peptide, specifically the classical hydrogen bond between the carbonyl oxygen and the NH groups (i with $i + 4$) and also the interactions between the side-chain groups. Disrupting the latter of these interactions results in a decrease in binding affinity; in our estimate, this is ∼10 kJ mol^{-1} in the case of the BimBH3 peptide. The introduction of the staple into the peptides has two consequences: (i) while the staple is able to preorganize the helix and reduce the entropic penalty for the association, the introduction of a covalent bond between side-chains removes the contribution to the binding energy that comes about when the two residues interact (in the salt bridge formed between Arg154 and Glu158, we estimate this to be 2.7 kJ mol^{-1}), and (ii) the chemical nature of the staple may not be conducive to forming stable interactions with the side-chain group of the residue one turn away. Loss of a stable interaction with the staple can be perpetuated down the chain; in both stapled peptides here, the result is the loss of all stabilizing interactions between side-chains, most critically the loss of the salt-bridge between Glu151 and Arg154. The binding free energy reflects the change in free energy between unbound and bound states, and since the covalent linkage is present in both states, there is no change in free energy of interaction across the staple upon binding. In contrast, in the native peptide, we calculate a significant favorable change in intramolecular energy of the ligand upon binding.

The structures of the peptides in complex with pro-survival Bcl-x_L reveal that they bind analogously to unrestrained BimBH3, neither modification interacting directly with the target protein unlike that observed with another stabilized peptide in complex with Mcl-1[16] where the staple itself makes

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Table 1. Analyses of Binding of BimBH3 Peptides to Pro-Survival Proteins Using Biacore-Based Assays

a					
K_i (nM)	Bcl-2	Bcl-x_L	Bcl-w	Mcl-1	A1
BimSAHB	460 ± 80	300 ± 37	370 ± 61	3.4 ± 0.1	4.1 ± 0.3
BimLOCK	800 ± 27	35 ± 3.7	37 ± 3.0	3.7 ± 0.3	4.7 ± 0.1
Linear Bim	6.8 ± 1.6	11 ± 0.6	25 ± 2.3	<1.4	<2.5
b	on-rate K_a (1/Ms)		off-rate K_d (1/s)		affinity K_D
Bcl-x_L					
BimSAHB	3.2×10^6		1.5×10^{-1}		45 nM
unstapled BimSAHB	7.9×10^5		7.5×10^{-2}		95 nM
linear BimBH3	1.7×10^6		1.1×10^{-2}		5.7 nM
Mcl-1					
BimSAHB	3.7×10^6		2.7×10^{-3}		0.73 nM
linear BimBH3	4.9×10^6		9.7×10^{-4}		0.20 nM
c	sequence		note		
BimSAHB	Ac-^{145}EIWIAQELRXIGDXFNAYYA^{164}-NH_2		X represents linked (*S*)-pentenylalanine residues		
BimLOCK	Ac-^{145}EIWIAQELRRIGDEFNAYYA^{164}-NH_2		R154 and E158 linked by lactam bridge		
linear BimBH3	Ac-^{145}EIWIAQELRRIGDEFNAYYA^{164}-NH_2				

(a) Competition experiments performed using a Biacore 3000 as previously described.[20] Values are presented as a K_i in nM, with SD (n = 3 independent experiments). (b) Direct affinity measurements for binding of linear BimBH3 and BimSAHB to Bcl-x_L and Mcl-1. Measurements were performed using a BIAcore S51. Peptides were passed over a sensor chip to which either recombinant Bcl-x_L-GST or Mcl-1-GST fusion proteins had been coupled via an anti-GST antibody. Direct association and dissociation rates were measured at a range of concentrations from which K_D values were calculated. Unstapled BimSAHB refers to a BimSAHB peptide in which pentylalanine residues have not been linked. (c) Sequences for peptides.

intimate hydrophobic contact with the side-chain of Phe318, and one of the flanking α-disubstitution methyl groups contacts Gly262. Unexpectedly, enhanced helicity did not improve activity with regards to affinity for the pro-survival proteins or bioactivity, as measured by induction of cytochrome *c* release from mitochondria and cellular uptake. Our findings recapitulate earlier observations[17,18] that stapling of peptides to enforce helicity does not necessarily impart enhanced binding affinity for target proteins and support the notion that interactions between the staple and target protein may be required for high affinity interactions in some circumstances.[19] Thus, the design of stapled peptides should consider how the staple might interact with both the target *and* the rest of the peptide, and particularly in the latter case whether its introduction might disrupt otherwise stabilizing interactions.

In conclusion, we undertook an investigation of stapled peptides as part of our on going studies into the mechanism of the Bcl-2 family of proteins. We found that the addition of a hydrocarbon staple to the BimBH3 peptide, either via Grubbs metathesis or formation of an external amide bond, does not change the peptide-like properties of the molecules in a manner that makes them significantly more drug-like. While modification of peptides may yet prove beneficial for drug development, in this case, we describe that the addition of external constraints does not enhance either binding affinity or cell permeability.

METHODS

Circular Dichroism Measurements. Circular dichroism was used to measure the percentage of helical content for BimSAHB, BimLOCK, and an equivalent linear BimBH3 peptide. Spectra were performed on 50 μM peptide solutions in 0.1 M potassium phosphate (pH 7) at 25 °C. Percent helicity was calculated as previously described.[22,23]

Structure Determination. Bcl-x_L protein preparation, crystallization, and structure determination for both the Bcl-x_L:BimSAHB complex and the Bcl-x_L:BimLOCK complex were as previously described for the Bcl-x_L:BimBH3 complex.[14] Crystallographic statistics for the two complexes are reported in Supplementary Table 1.

Cytochrome *c* Release. Cells were pelleted and lysed in 0.025% (w/v) digitonin containing lysis buffer (20 mM Hepes, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 250 mM sucrose, supplemented with Complete Protease Inhibitor Cocktail from Roche). The crude lysates, containing mitochondria, were incubated with or without 1% (w/v) Triton-X100 or increasing concentrations of Bim peptides for 1 h at 30 °C, pelleted at 4000 rpm at 4 °C for 15 min, and the supernatant collected. The amount of released cytochrome *c* was determined by ELISA (R&D systems), according to the manufacturer's protocol.

BIAcore Competition Assays. BIAcore competition assays were performed as previously described.[20,21] Pro-survival proteins (5 nM) (Bcl-2, Bcl-x_L, and Bcl-w prepared as described in ref , Mcl-1 prepared as described in ref 24, A1 prepared as described in ref 25) were incubated with increasing concentrations of BimBH3 peptides for 2 h in running buffer prior to injection onto a CM5 chip onto which either a wild-type 26-mer BimBH3 peptide or an inert BimBH3 mutant peptide (Bim4E) was immobilized.

Direct Affinity Measurements. Direct binding assays were performed with a Biacore S51 as previously described.[9] Briefly, anti-GST antibody was immobilized to a CM5 chip using amine coupling. Recombinant GST-tagged Bcl-x_L or GST-tagged Mcl-1 (100 μg mL^{-1}) was captured to the chip followed by injection of peptide at a variety of concentrations. Sensograms were generated by subtracting the binding response from that of a reference spot to which GST alone had been captured.

Killing Assays. MEFs were trypsinized, collected, and then washed twice in serum-free media followed by plating of cells (5×10^4/well) in 50 μL, exposed to BimSAHB (20 μM) or vehicle in serum-free media for 2 h, and serum replacement (20% (v/v) serum in 50 μL media) for an overall treatment duration as indicated.

Colony Survival Assays. Retroviruses encoding BH3-only proteins were transduced as described.[20] Infected MEFs (GFP(+) cells) were sorted by FACS and long-term assays of colony formation performed as described.[20]

Molecular Modeling. The free energy of interaction between the side-chain groups on the BimBH3-peptide was calculated using the AUTODOCK function.[15] In its original formulation, the AUTODOCK potential (model C) includes terms that represent the entropic

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penalty for restriction of conformational freedom and desolvation of the ligand only. Here, we have included these two components for both molecular constituents and consequently reduced the contribution to the total free energy of interaction of each by half. The AMBER all-atom partial atomic charges[26] were used to calculate the electrostatic interaction energy. Hydrogen atoms were added to fill valencies using the UCSF Chimera package.[27]

Detailed methods for peptide synthesis, structure determination, K_i calculations, and binding analysis are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

BimSAHB peptide does not kill MEFs; reconstituted $Bax^{-/-}Bak^{-/-}$ MEF cells express comparable levels of wild-type Bax and Bax K21E. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID codes, 2YQ6 for Bcl-xL:BimSAHB and 2YQ7 for Bcl-xL:BimLOCK).

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Notes

The authors declare the following competing financial interest(s): Genentech is a member of the Roche Group. Roche Pharma and Aileron Therapeutics have an independent collaboration developing stapled peptide technology. Between 2007 and 2010, the Walter and Eliza Hall Institute of Medical Research had a research collaboration agreement with Genentech and Abbott in the field of apoptosis, specifically the Bcl-2 protein family.

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