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A Comparison of Two Strategies for Affinity Maturation of a BH3 Peptide toward Pro-Survival Bcl-2 Proteins

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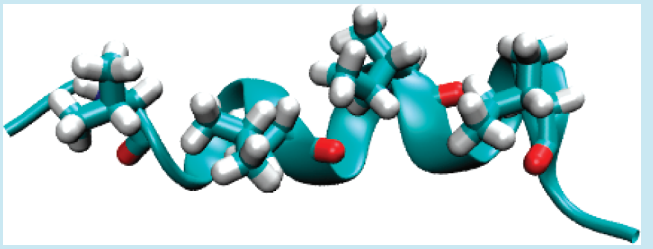
Supporting Information

ABSTRACT: The Bcl-2 family of proteins regulates apoptosis at the level of mitochondrial permeabilization. Pro-death members of the family, including Bak and Bax, initiate apoptosis, whereas pro-survival members such as Bcl-x_L and Mcl-1 antagonize the function of Bak and Bax via heterodimeric interactions. These heterodimeric interactions are primarily mediated by the binding of the helical amphipathic BH3 domain from a pro-death protein to a hydrophobic cleft on the surface of the pro-survival protein. Since high levels of pro-survival Bcl-2 proteins are present in many cancers, peptides corresponding to pro-death BH3 domains hold promise as therapeutics. Here we apply a high-throughput flow cytometry assay to engineer the Bak BH3 domain for improved affinity toward the pro-survival proteins Bcl-x_L and Mcl-1. Two strategies, engineering the hydrophobic face of the Bak BH3 peptide and increasing its overall helicity, are successful in identifying Bak BH3 variants with improved affinity to Bcl-x_L and Mcl-1. Hydrophobic face engineering of the Bak BH3 peptide led to variants with up to a 15-fold increase in affinity for Bcl-x_L and increased specificity toward Bcl-x_L. Engineering of the helicity of Bak BH3 led to modest (3- to 4-fold) improvements in affinity with retention of promiscuous binding to all pro-survival proteins. HeLa cell killing studies demonstrate that the affinity matured Bak BH3 variants retain their expected biological function.



Protein−protein interactions are central to most biological processes, including signaling, structure formation, and catalysis.[1] Two important descriptors for any biomolecular interaction are the affinity and the specificity of the interaction. The affinity, often expressed as an equilibrium association or dissociation constant (K_a or K_d, respectively), provides information about how strongly the two binding partners interact, whereas specificity describes whether a protein has only a single binding partner or many partners. One class of interactions that is particularly ripe for asking questions about affinity and specificity is the set of interactions that occur between members of the Bcl-2 family of proteins.[2−5] The Bcl-2 family consists of important regulators of apoptosis and includes members that have pro-death (pro-apoptotic) and pro-survival (anti-apoptotic) functions. The multidomain pro-death members of the family, exemplified by the proteins Bak and Bax, induce apoptosis via permeabilization of the mitochondrial outer membrane.[6,7] The pro-survival members, including Bcl-x_L, Bcl-2, and Mcl-1, attenuate the function of Bak, Bax, and other pro-death proteins via heterodimeric interactions.[8] The binding between a pro-death and a pro-survival protein is mediated by the interaction between the highly conserved helical amphipathic BH3 (Bcl-2 homology 3) domain of the pro-death protein and a surface cleft on the pro-survival protein.[9,10] Because of difficulties in observing interactions between full-length pro-death and pro-survival proteins *in vitro*, these interactions are most commonly studied by binding a peptide corresponding to the BH3 domain of the pro-death protein to a full-length pro-survival protein.[9,11−14]

Previously, we demonstrated that the binding between a BH3 domain peptide and its pro-survival protein binding partners can be recapitulated on the *E. coli* cell surface via display of the BH3 peptide on the engineered outer membrane protein eCPX.[15] In these experiments, cells displaying the BH3 peptide are treated with biotin-labeled pro-survival protein. Following staining with fluorescent streptavidin, the interactions between the BH3 domain and the pro-survival protein can be quantitatively assessed using flow cytometry (Figure 1a). More recently, we have carried out a survey of the binding of nearly all known BH3 domains in the human genome to each of the five pro-survival proteins (Bcl-x_L, Bcl-2, Bcl-w, Mcl-1, and A1 (also known as Bfl-1)) using the same techniques.[16] This study confirmed and expanded upon prior results[12] showing that although some BH3 peptides bind promiscuously to all five pro-survival proteins, other BH3 peptides exhibit selectivity and bind only to a subset of the pro-survival proteins. Here we have selected a single, promiscuous BH3 peptide from the pro-apoptotic protein Bak and subjected it to affinity maturation toward both Bcl-x_L and Mcl-1 using eCPX mediated surface display and fluorescence activated cell sorting (FACS). Two methods for affinity maturation were evaluated: engineering of the hydrophobic face of the amphipathic Bak peptide and engineering of the overall helical propensity of the peptide. The rationale for carrying out this work was 2-fold. First, we wanted to determine the extent of affinity improvement that is possible in the Bak BH3 peptide. BH3 peptides

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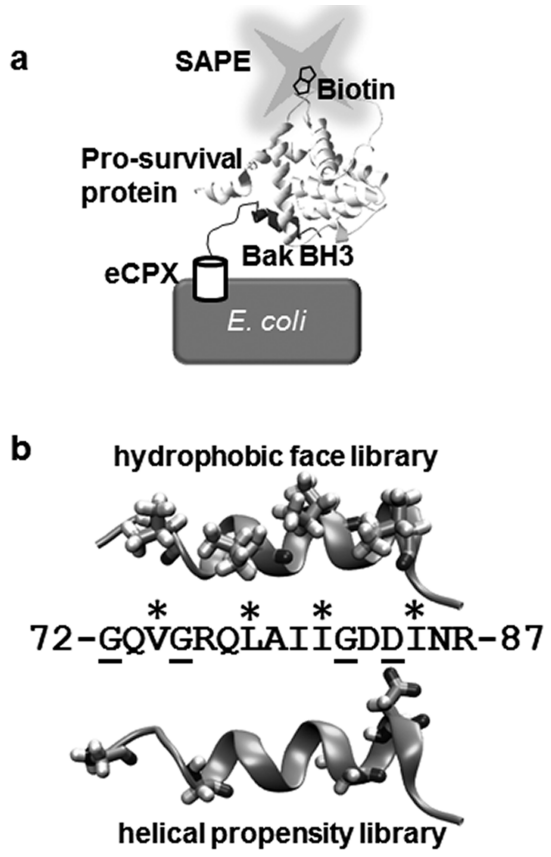

Figure 1. Cell surface display of Bak BH3 peptide libraries. (a) Schematic of the Bak BH3 peptide: pro-survival protein interaction on the *E. coli* cell surface, not to scale. The Bak BH3 peptide (dark gray) is tethered to the outer membrane protein eCPX and binds in a surface cleft of the pro-survival protein (white). A biotin tag on the N-terminus of the pro-survival protein interacts with streptavidin-phycoerythrin (SAPE) allowing for detection of the interaction by flow cytometry. (b) Sequence and structure of the Bak BH3 peptide. Residues mutagenized in the hydrophobic face library are indicated by asterisks, and residues mutagenized in the helical propensity library are underlined. The positions of these residues are highlighted on the model of the Bak BH3 peptide. Coordinates taken from PDB file 1BXL.

from pro-death proteins hold great promise as cancer therapeutics,[3,17,18] and improvements in the affinity of a BH3 peptide for its targets may result in increased efficacy of such therapeutics. Second, we were interested in whether improvements in the affinity of the Bak BH3 peptide resulted in changes to its specificity in binding pro-survival proteins. We find that both hydrophobic face engineering and helical propensity engineering are useful strategies for improving the affinity of BH3 peptides for their pro-survival targets. Furthermore, we demonstrate that one of the highest affinity Bak BH3 variants isolated from our screens is more cytotoxic to HeLa cells than the wild-type Bak peptide.

RESULTS AND DISCUSSION

Construction of Bak BH3 Peptide Libraries for Affinity Maturation. The Bak BH3 domain corresponds to an amphipathic α-helix, the hydrophobic face of which is buried in a cleft when complexed with its binding partners including Bcl-x_L and Mcl-1.[9,19] Thus perhaps the simplest strategy for affinity improvement of the Bak BH3 peptide toward its pro-survival partners is to mutagenize the hydrophobic face residues. An alternative strategy is to attempt to increase the overall helicity of the BH3 peptide via mutagenesis. Single amino acid variants of the Bad BH3 peptide aimed at increasing the helicity of the peptide resulted in improvements of peptide affinity toward Bcl-x_L.[11] Walensky and colleagues have also demonstrated that locking a BH3 peptide into its helical conformation via hydrocarbon stapling results in increased BH3 peptide affinity toward its pro-survival binding partners.[17] Thus, the overall helicity of a BH3 peptide seems to correlate in a positive fashion with its affinity. To test these two trajectories for affinity maturation independently, we created two separate libraries of the 16-mer Bak BH3 peptide that we[15] and others[9] have previously studied. We have previously described a hydrophobic face library in which the four hydrophobic positions (Val-74, Leu-78, Ile-81, and Ile-85; see Figure 1b) were allowed to vary to other large, hydrophobic amino acids.[15] Here we have constructed a much larger library that allows these four hydrophobic positions to vary to any of the 20 amino acids. The library was encoded at the gene level using the degenerate codon NNK where N is any of the four bases and K is either G or T. For the helical propensity library, we chose four residues with low helical propensity (Gly-72, Gly-75, Gly-82, and Asp-84; Figure 1b) and mutagenized these positions with the NNG degenerate codon. The NNG codon encodes for a set of 13 amino acids that is biased toward amino acids with high helical propensity (Supplementary Table S1).[20] Arora and colleagues have found previously that substitution of the Asp-84 position with acetyllysine led to an increase in helicity in the Bak BH3 peptide with a concomitant increase in its binding affinity to Bcl-x_L.[21] The other positions changed in this library are all glycines, an amino acid with very low helical propensity.[20] The hydrophobic face NNK library contained 4×10^6 clones, whereas the helical propensity NNG library contained 9×10^5 clones, allowing for a thorough sampling of the sequence space encoded by the libraries. For the sake of simplicity in nomenclature, we will refer to these libraries as the NNK and NNG libraries for the remainder of the paper.

Affinity Maturation of the Bak BH3 Peptide by Hydrophobic Face Engineering. With the Bak BH3 NNK library in hand, we sought to screen it for variants with improved affinity toward either Bcl-x_L or Mcl-1. We have previously determined that the apparent K_d for the Bak BH3 16-mer bound to Bcl-x_L is 275 nM,[16] a value that agrees well with K_d values determined by solution binding methods.[9,11] Thus we treated cells with a sub-K_d concentration of Bcl-x_L (40 nM) to identify library members with improved affinity. The cells that exhibited the highest fluorescence within the library were sorted and regrown. Two additional rounds of sorting were carried out with the Bcl-x_L concentration at 40 nM for round 2 and 4 nM for round 3. A total of 27 individual clones isolated from the round 2 and round 3 sorts exhibited improved affinity toward Bcl-x_L. These clones were given the names XNNK1 to XNNK27 where the "X" refers to the fact that the peptide was matured against Bcl-x_L and the NNK reflects the library from which the clone originated. DNA sequencing of clones XNNK1 to XNNK27 revealed 9 distinct sequences (Table 1), two of which (XNNK4 and XNNK7) had been previously identified in our earlier screen for Bak BH3 peptides with improved affinity to Bcl-x_L.[15] Several trends are apparent in these sequences; the leucine in position 78 that is highly conserved across different BH3 peptides (Supplementary Figure S1) is unchanged in all clones. In all 9 sequences the isoleucine in position 85 is changed to a larger aromatic amino acid with phenylalanine predominating. There is more variability in the amino acid substitutions at positions 74 and 81; the valine

Table 1. Sequences of Variants Isolated from the NNK Library

Bak BH3	74	78	81	85	K_d vs Bcl-x_L (nM)	K_d vs Mcl-1 (nM)
WT	V	L	I	I	275	103
clones selected against Bcl-x_L						
XNNK4	L	L	L	F		
XNNK5	L	L	Y	F		
XNNK6	I	L	W	F	37.6	
XNNK7	L	L	F	F		
XNNK12	I	L	M	F	35.0	
XNNK14	V	L	L	F	18.7	
XNNK17	L	L	W	F	37.8	
XNNK19	A	L	L	F		
XNNK20	L	L	Y	Y		
clones selected against Mcl-1						
MNNK1	G	M	S	N	70.9	26.3

residue in position 74 is either retained or replaced by another aliphatic amino acid, while Ile-81 is replaced by an isosteric aliphatic amino acid or a larger aromatic amino acid. Collectively, these results indicate that increasing the size of the Ile-81 and Ile-85 residues is an effective strategy for increasing the affinity of the Bak BH3 for Bcl-x_L. A previous study demonstrated that substitution of Ile-85 in the Bak 16-mer with several large aromatic unnatural amino acids led to improvements in affinity of the peptide with Bcl-x_L,[22] consistent with our findings here. To quantify the affinity improvements, we determined the apparent K_d values for several of the affinity matured peptides using flow cytometry (Table 1 and Supplementary Figure S4a−e). The gains in apparent affinity were substantial with the best variant, XNNK14, exhibiting an apparent K_d of 18.7 nM, a nearly 15-fold improvement over the wild-type peptide.

Encouraged by our success in finding Bak BH3 variants with improved affinity toward Bcl-x_L in the NNK library, we next screened the library against Mcl-1. The wild-type Bak BH3 16-mer peptide has an apparent affinity of 103 nM toward Mcl-1,[16] so we screened the library by incubating it with 50 nM Mcl-1 for one round and 25 nM Mcl-1 in a second round. In analysis of sorted clones from the second round, only a single clone with improved affinity to Mcl-1 emerged. This clone was named MNNK1 since it originated from the NNK library screened with Mcl-1. Sequencing of this clone revealed four unexpected amino acid substitutions in the hydrophobic face residues: V74G, L78M, I81S, and I85N (Table 1). These substitutions abolish much of the hydrophobic character of the BH3 peptide, though it has been demonstrated previously that Mcl-1 can retain binding to BH3 ligands with nonconservative replacements in the four hydrophobic positions.[23] Binding isotherm analysis of the MNNK1 clone confirmed that this peptide has an apparent affinity of 26.3 nM toward Mcl-1 (Table 1 and Supplementary Figure S4p), a 4-fold improvement relative to the wild-type peptide. Overall the experiments on the NNK library indicate that while hydrophobic face engineering of the Bak BH3 peptide is an excellent method for affinity maturation toward Bcl-x_L, this method is not necessarily generalizable to all of the pro-survival proteins including Mcl-1.

Affinity Maturation of the Bak BH3 Peptide by Helical Propensity Engineering. The NNG library, which targets residues with low helical propensity, was screened against Bcl-x_L and Mcl-1 in a similar fashion. After two rounds of sorting against 80 nM Bcl-x_L,

Table 2. Sequences of the Variants Isolated from the NNG Library

Bak BH3	72	75	82	84	K_d vs Bcl-x_L (nM)	K_d vs Mcl-1 (nM)	specificity index[c]
WT	G	G	G	D	275	103	2.67
clones selected against Bcl-x_L							
XNNG2	L	G	G	A	68.0	88.3	0.770
XNNG4	G	G	A	W			
XNNG8[a]	E	G	G	V	88.0	39.1	2.25
XNNG10	G	G	G	A			
XNNG12[b]	E	G	G	E	99.5	30.0	3.32
XNNG16	E	G	A	W	73.2	398	0.184
clones selected against Mcl-1							
MNNG1[a]	E	G	G	V	88.0	39.1	2.25
MNNG2	E	G	G	S			
MNNG3	A	G	G	W	59.3	25.3	2.34
MNNG4	A	G	G	A			
MNNG5[b]	E	G	G	E	99.5	30.0	3.32
MNNG7	Q	G	G	L	41.9	39.6	1.06
MNNG8	E	G	G	A			
MNNG9	E	G	G	T			
MNNG10	E	G	G	R	78.3	14.8	5.29
MNNG11	E	G	G	W			
MNNG12	M	G	G	K	113	54.1	2.09

[a] XNNG8 and MNNG1 clones are identical and were isolated independently from both libraries. [b] XNNG12 and MNNG5 clones are identical and were isolated independently from both libraries. [c] Specificity index is defined as $K_{d,Bcl\text{-}xL}/K_{d,Mcl\text{-}1}$

six distinct clones emerged with improved affinity (Table 2). Subsequently, we performed two sorts of the NNG library against Mcl-1, first at 50 nM and then at 25 nM protein. Twelve distinct clones were isolated from the screen against Mcl-1 (Table 2). Interestingly, two of the clones that appeared from the screen against Bcl-x_L (XNNG8 and XNNG12) also appeared in the Mcl-1 screen (MNNG1 and MNNG5). This indicates that helical propensity engineering of BH3 peptides may be a more general solution for affinity maturation toward their pro-survival binding partners. Consistent with this notion, the same amino acid substitutions that were beneficial for Bcl-x_L binding appeared to also be beneficial for Mcl-1 binding. In general, the glycines in positions 75 and 82 were largely unchanged in affinity matured variants. Since Gly-82 is highly conserved across BH3 domains (Supplementary Figure S1), its recalcitrance to substitution was expected. The Gly-75 residue, however, is not strongly conserved, so it was somewhat surprising that it was retained in all of the affinity matured variants. This result is not completely unprecedented, however, since Gly-75 was retained in the majority of variants obtained from an affinity selection of miniature protein/Bak BH3 chimeras that bind to Bcl-2.[24] The Gly-72 position was frequently changed to glutamic acid in clones from both screens while Asp-84 was replaced with a wide size range of high-helical propensity amino acids, from alanine to tryptophan (Table 2). There appears to be no clear consensus among the substitutions at the Asp-84 position other than that the helical propensity increases upon substitution. To confirm improvements in affinity among these clones, we generated binding isotherms for XNNG8 and XNNG12, the two clones that were isolated from both screens. These variants exhibit a ~3-fold decrease in apparent K_d

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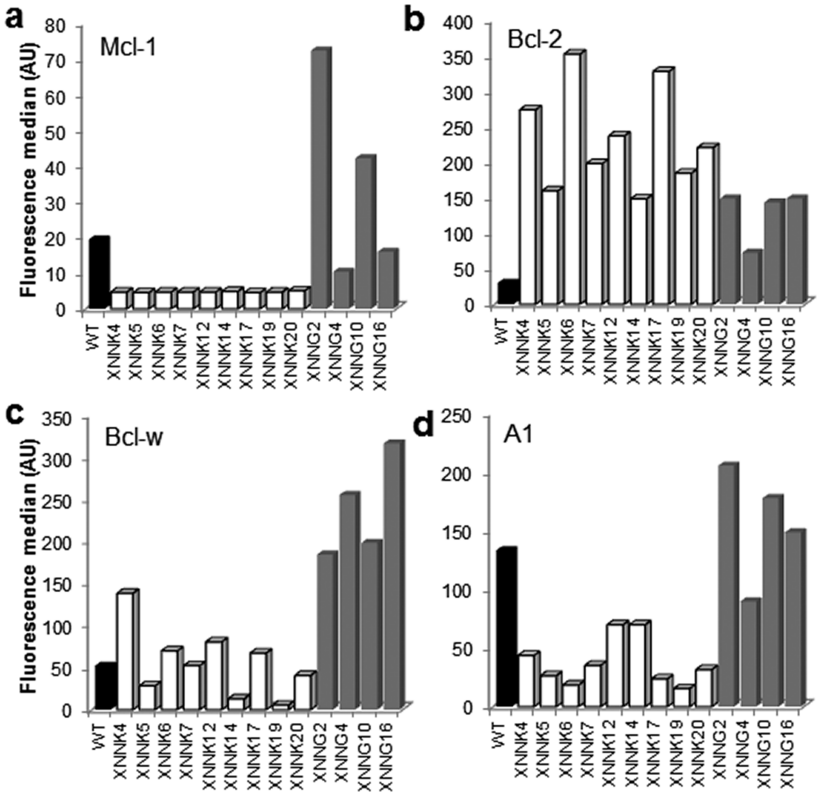

Figure 2. Binding profile of Bak BH3 variants affinity matured toward Bcl-x_L. Variants matured toward Bcl-x_L were tested for binding to each of the four other pro-survival proteins. Binding was carried out at a single subsaturation concentration of pro-survival protein, and the median fluorescence is reported. (a) Mcl-1 (20 nM), (b) Bcl-2 (100 nM), (c) Bcl-w (400 nM), (d) A1 (50 nM). Black bars represent the wild-type Bak BH3 peptide, white bars are variants isolated from the NNK library, and gray bars are variants isolated from the NNG library. Since the XNNG8 and XNNG12 variants were also isolated in screens against Mcl-1, data for these variants appear in Figure 3.

toward both Bcl-x_L and Mcl-1 relative to the wild-type Bak BH3 peptide (Supplementary Figure S4h,i,r,s) and thus have more modest increases in apparent affinity as compared to the XNNK variants.

Specificity of Affinity Matured Bak BH3 Peptides. Having isolated affinity matured Bak BH3 variants from both the NNK and NNG libraries, we next determined whether these variants retained promiscuous binding to all five of the pro-survival proteins. As mentioned above, some BH3 domain peptides, such as the peptides from Bak and Bim, bind avidly to all five of the pro-survival proteins.[12,16] In contrast, some BH3 peptides exhibit specificity toward a subset of the pro-survival proteins. The Bad BH3 peptide binds well only to Bcl-x_L, Bcl-2, and Bcl-w, while the Noxa BH3 binds only to Mcl-1 and A1.[12] We tested the affinity matured XNNK clones for binding against Bcl-2, Bcl-w, Mcl-1, and A1. The protein concentrations were chosen to be below the apparent K_d value for the wild-type peptide such that we were examining the linear portion of the binding isotherm. Each of the XNNK clones exhibited improved binding toward Bcl-2 relative to the wild-type, but each of the clones also exhibited diminished binding toward Mcl-1 and A1 (Figure 2). Thus affinity maturation via hydrophobic face engineering converted the promiscuous Bak BH3 peptide into a peptide with "Bad-like" specificity to a subset of the pro-survival proteins. Some variants, most notably XNNK19, also lose affinity for Bcl-w (Figure 2), resulting in a peptide that has specificity for only two of the five pro-survival proteins. It is noteworthy that this specificity arose solely as a result of affinity maturation; no counterselection against Mcl-1 or A1 was required.

In stark contrast to the XNNK clones, the single MNNK clone exhibited improved binding to all five of the pro-survival proteins (Figure 3, Supplementary Figure S4f,p). Since the amino acid changes in the MNNK1 variant were so nonconservative, it was important to establish that it still bound to the canonical BH3 binding cleft on Bcl-x_L. We measured the equilibrium binding of cell-surface-displayed MNNK1 in the presence of soluble wild-type Bak BH3 peptide and soluble XNNK12 peptide (Supplementary Figure S5). In these competition experiments, increasing the concentration of soluble peptide led to a decrease in signal in the flow cytometry assay, indicating that the MNNK1 clone does bind to the canonical BH3 binding cleft.

The XNNG and MNNG clones displayed a similar pattern to the MNNK1 variant with these variants nearly universally exhibiting improved binding to all of the pro-survival proteins (Figure 2, Figure 3, Supplementary igure S4). From these studies we can conclude that both affinity and specificity can be engineered into the Bak BH3 peptide via hydrophobic face engineering. In contrast, helical propensity engineering toward a single pro-survival protein results in affinity improvements toward all five pro-survival proteins.

To more quantitatively analyze the specificity of the NNG clones for Bcl-x_L vs Mcl-1, we performed binding isotherm analysis on more than half of the isolated clones (Table 2, Supplementary Figure S4). The apparent K_d values we measured follow the trends observed in the single point measurements presented in Figure 3. Dividing the apparent K_d values of the variants for Bcl-x_L and Mcl-1 ($K_{d,Bcl\text{-}xL}/K_{d,Mcl\text{-}1}$) provides a simple specificity

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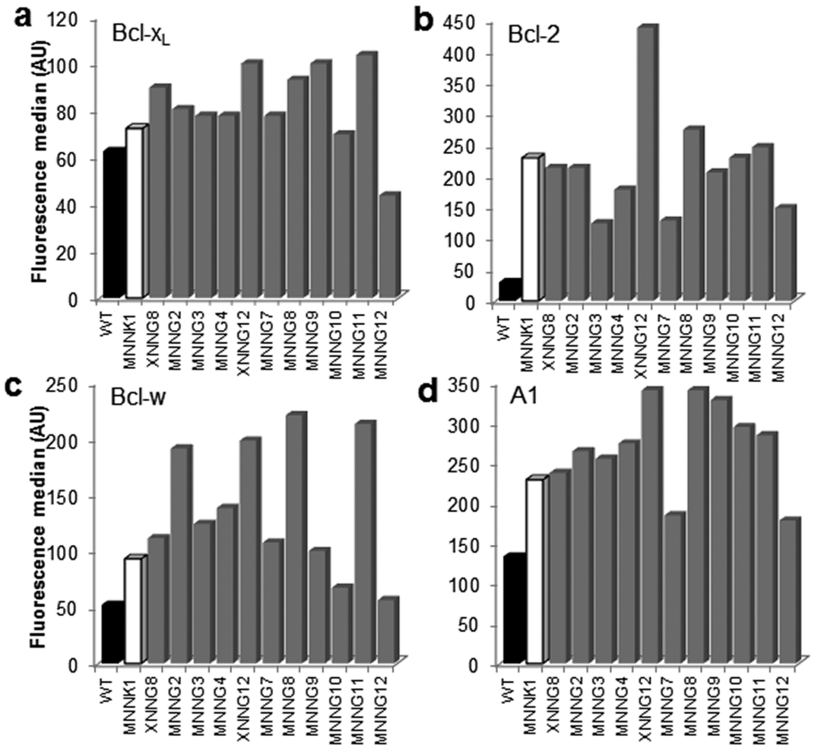

Figure 3. Binding profile of variants affinity matured toward Mcl-1. Binding to subsaturation concentrations of (a) Bcl-x_L (50 nM), (b) Bcl-2 (100 nM), (c) Bcl-w (400 nM), (d) A1 (50 nM) was carried out using the flow cytometric binding assay. The median fluorescence from these assays is reported. As in Figure 2, black bars represent the wild-type Bak BH3 peptide, white bars are variants isolated from the NNK library, and gray bars are variants isolated from the NNG library.

index with which the variants can be compared; larger values of this index indicate specificity toward Mcl-1, while smaller values correspond to Bcl-x_L specificity. The specificity index for the wild-type Bak peptide is 2.7, while this value ranges from 0.2 to 3.3 for the XNNG clones and from 1.1 to 5.3 for the MNNG clones (Table 2). These data indicate that the XNNG and MNNG clones have not made any radical shifts in specificity, though in general there is some bias in affinity toward the pro-survival protein used in screening. In other words, the MNNG variants generally develop weak specificity toward Mcl-1 and the XNNG variants develop weak specificity toward Bcl-x_L.

Solution Binding Assays. A common concern when using cell surface display techniques to determine apparent affinities is whether these measurements agree with affinity measurements from solution binding techniques. To confirm that the apparent K_d values we measured using surface display correlate with solution K_d values, we observed the binding of soluble BH3 peptide and soluble biotinylated pro-survival protein using flow cytometry. In these assays, adapted from a protocol by Shusta and co-workers,[25] the pro-survival protein concentration is kept constant, and soluble, label-free BH3 peptide is added at differing concentrations and allowed to bind the protein. The amount of unbound protein remaining is titrated by binding to *E. coli* cells that display a ligand to which the protein binds tightly. Following treatment with fluorescent streptavidin, the amount of protein bound to the cells can be quantified using flow cytometry. We tested the binding of the wild-type Bak BH3 and XNNK12 to Bcl-x_L using this method and determined K_d values of 545 and 18 nM, respectively (Supplementary Figure S2). These solution K_d values are within a factor of 2 of the apparent K_d values we determined using surface display.

QSAR Analysis of Affinity Matured Bak BH3 Peptides. To determine whether there were quantifiable trends in the affinity matured Bak BH3 variants, we carried out a quantitative structure−activity relationship (QSAR) analysis using four chemical descriptors (Figure 4). In addition to the widely used z_1, z_2, and z_3 descriptors suggested by Wold and colleagues,[26] we calculated an overall helical propensity energy using the helical propensity scale of Pace and Scholtz (Supplementary Table S1).[20] In this helical propensity scale alanine, the amino acid with the highest helical propensity, is assigned an energy of 0 kcal/mol and all other amino acids have energies greater than 0 kcal/mol. Thus a small value of the overall peptide energy corresponds to a peptide with high helical propensity. Several trends are immediately apparent in analyzing the QSAR data. Each of the NNG clones has helical propensity higher than that of the wild-type Bak peptide, demonstrating that the NNG library design worked as planned. Two of the NNK clones (XNNK4 and XNNK19) also have increased helicity relative to the wild-type peptide. The helical propensity energy of the wild-type Bak peptide is 8.3 kcal/mol, while the average helical propensity energy of the NNK and NNG clones is 8.1 and 7.2 kcal/mol, respectively. The z_1 descriptor, which measures the lipophilicity or hydrophobicity of the peptide, is fairly constant across the XNNK clones. In contrast, clone MNNK1 is much less hydrophobic than the wild-type peptide and any of the other variants isolated from either library. About half of the NNG clones exhibit increased hydrophobicity (lower values of z_1). This change can be explained by the fact that the NNG library involves the replacement of glycine and aspartate, two amino acids with low hydrophobicity. The z_2 descriptor accounts for the size of the amino acids with higher values of z_2 corresponding to larger amino acids.

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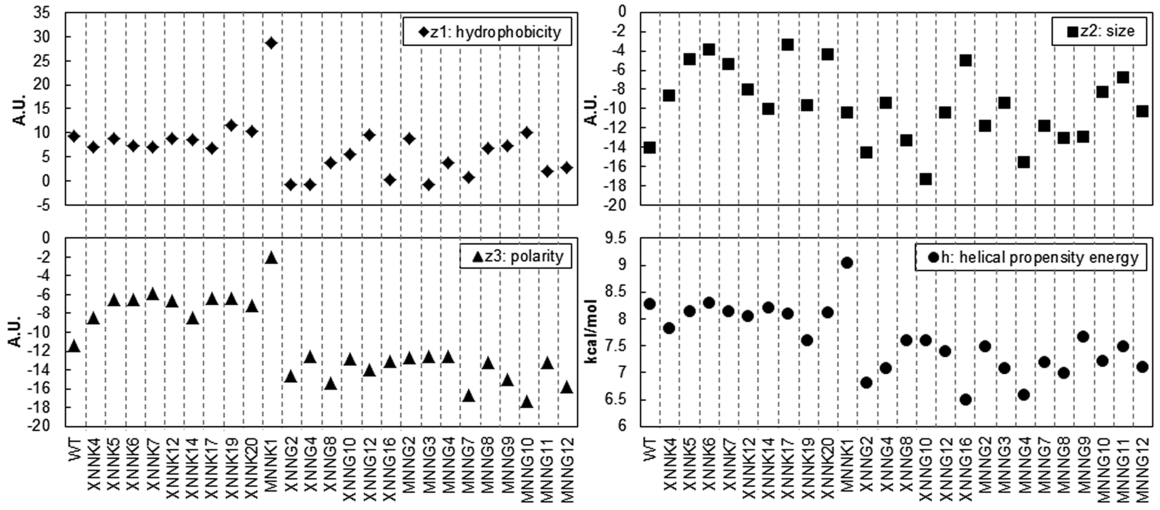

Figure 4. Quantitative structure−activity relationship analysis of affinity matured Bak BH3 variants. The z_1, z_2, and z_3 descriptors correlate to lipophilicity, size, and polarity, respectively, and are from ref 26. The helical propensity scale[20] is such that lower values of the helical propensity energy correspond to high helical propensities.

Each of the NNK clones has a z_2 value larger than that of the wild-type Bak peptide, while there is no discernible trend among the z_2 values for the NNG clones. The z_3 descriptor captures the polarity of the amino acids; amino acids with high electronegativity have values of z_3 higher than those of amino acids that are electrophilic.[26] The clones isolated from the two libraries are readily distinguished by their overall z_3 scores: the NNK clones all exhibit z_3 values larger than that of the wild-type peptide, while the NNG clones all have z_3 values lower than that of the wild-type peptide. In summary, the trends we observe from the QSAR analysis are that the affinity matured NNK variants exhibit increased size and electronegativity relative to the wild-type peptide. In contrast, the NNG clones are less hydrophobic, more electrophilic, and much more helical.

Biological Activity of Affinity Matured Bak BH3 Peptides. To confirm that the affinity matured Bak BH3 variants retained biological activity, we tested the ability of these peptides to kill HeLa cells. Though the 16-mer Bak BH3 peptide itself is unable to enter HeLa cells, it has been previously demonstrated that a Bak BH3 peptide fused to the C-terminus of the 16-aa cell-penetrating peptide penetratin is able to induce apoptosis in HeLa cells.[27] We obtained fusions of three of our affinity matured Bak BH3 peptides (XNNK12, MNNK1, and XNNG8) to penetratin along with a wild-type penetratin-Bak BH3 peptide as a control. The ability of these peptides to kill HeLa cells was assessed by two independent methods: propidium iodide staining of cells followed by flow cytometry and the MTT assay. Both of these assays demonstrate that each of the affinity matured Bak BH3 variants is able to kill cells essentially as well as the wild-type Bak peptide (Figure 5a and b). Though it is difficult to decouple the transport of the penetratin-Bak peptides into the cell from their activities within the cell, we tested the ability of the wild-type penetratin-Bak and the penetratin-Bak XNNK12 variants to kill HeLa cells at a range of concentrations from 0.35 to 45 μM. The XNNK12 peptide was more efficacious at killing HeLa cells at each of the concentrations tested (Supplementary Figure S6), suggesting that affinity increases in BH3 peptides may lead to more potent cell killing activity.

Discussion. Here we have demonstrated two successful strategies for the affinity maturation of the 16-mer Bak BH3 peptide for its pro-survival binding partners: hydrophobic face engineering and helical propensity engineering. Engineering of the hydrophobic face of the Bak BH3 peptide led to large (up to 15-fold) increases in the apparent affinity of this peptide for Bcl-x_L (Table 1). However, isolation of Bak BH3 variants that bound Mcl-1 strongly from the hydrophobic face library was considerably more difficult. Thus hydrophobic face engineering appears to be a high risk/high reward strategy for the engineering of affinity matured BH3 peptides; this strategy may not succeed for all pro-survival targets, but when it does succeed, it can lead to large increases in apparent affinity. On the other hand, Bak BH3 variants with increased apparent affinity were readily isolated from the helical propensity library regardless of whether the library was screened for binding against Bcl-x_L or Mcl-1. Moreover, two of the same variants were isolated independently from Bcl-x_L and Mcl-1 screens. This implies that helical propensity engineering is a more general route for increasing the affinity of a BH3 peptide for its targets. However, this generality comes at a cost; the variants isolated from the helical propensity library have modest increases in affinity (3- to 5-fold) relative to the variants from the hydrophobic face library.

Affinity matured variants isolated from the NNG library retain promiscuous binding to all five of the pro-survival proteins. In fact, nearly all of the variants isolated from the NNG library display improved affinity to each of the five pro-survival proteins relative to the wild-type Bak BH3 peptide. In contrast, Bak BH3 variants isolated against Bcl-x_L from the NNK library (XNNK clones) develop specificity toward a subset of pro-survival proteins (Figure 2). This specificity arises solely due to the affinity maturation since no counterselection against pro-survival targets was carried out. The XNNK clones exhibit improved binding to Bcl-x_L, Bcl-2, and in most cases Bcl-w but exhibit diminished binding to Mcl-1 and A1, mimicking the binding spectrum of the Bad BH3 peptide[12] and the small molecule BH3 mimetic ABT-737.[28] Thus we can hypothesize that the specificity observed in modern Bcl-2 proteins such as Bad and Noxa may have evolved solely because of a selective pressure to bind more tightly to a single pro-survival target. The major changes to the XNNK variants involve increasing the side chain size at the third and fourth positions of the hydrophobic face (Ile-81 and Ile-85,

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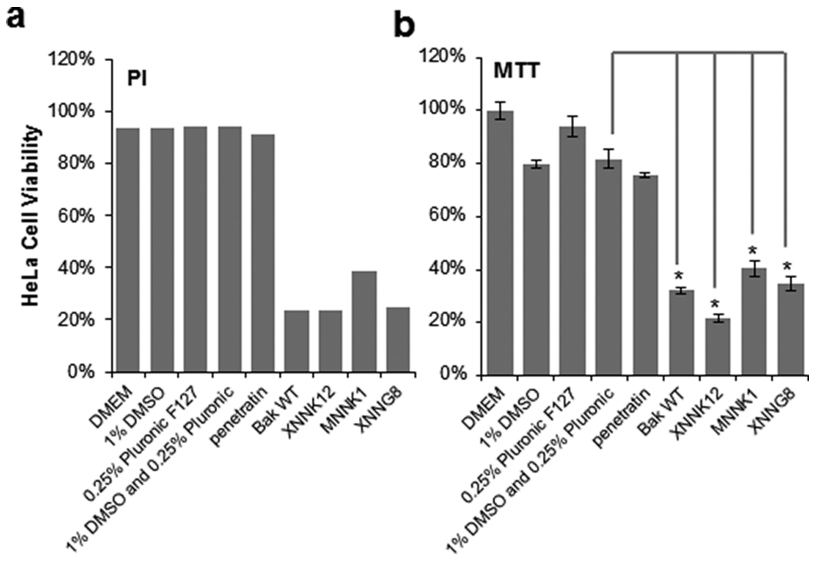

Figure 5. HeLa killing by Bak BH3 variants. (a) Penetratin-Bak BH3 (50 μM) peptides were incubated with HeLa cells, and viability was assessed using propidium iodide (PI) staining. The percent viability was calculated as the ratio of live cells to total cells. (b) As described in panel a but with viability assessment by the MTT assay; 100% viability corresponds to the MTT signal from untreated cells grown in DMEM. Assays were repeated in triplicate, $*$ indicates p-values <0.001. The penetratin control in panels a and b is 50 μM penetratin with 1% DMSO and 0.25% Pluronic F127, indicating that the cell-penetrating peptide alone has minimal cytotoxic effect on the cells.

respectively). In comparing the structures of a Bak BH3:Bcl-x_L complex[9] to the Bim BH3:Mcl-1 complex,[29] it is difficult to rationalize why a size increase at these positions would lead to disruption of BH3 peptide binding to Mcl-1. The clefts in which the BH3 peptides reside in Bcl-x_L and Mcl-1 are similar in size, and Mcl-1 can accommodate BH3 peptides with aromatic residues in the fourth hydrophobic position, including the Bim BH3 peptide. No structure currently exists for the complex between the Bak BH3 peptide and Mcl-1, so it is possible that the Bak BH3 peptide binds in a slightly different fashion than does the Bim BH3.

The biological activity of the affinity matured peptide variants was tested by fusing them to a cell penetrating peptide. We found that the amino acid substitutions in the peptide did not cause any appreciable loss of biological activity. Since the uptake efficiency governed by the cell-penetrating peptide cannot be decoupled from the killing activity of the Bak peptide, this method is unable to directly probe whether any of the affinity matured variants exhibit improved killing efficacy. Other cell-permeable peptide constructs, such as stapled peptides,[17,21,30,31] can be used in future studies to quantify the improvement on biological activity.

Although our work here focuses on native BH3 peptides, other scaffolds have been used to investigate the affinity and specificity of BH3 peptide interactions with their pro-survival binding partners. Gellman and colleagues have carried out extensive studies on BH3-mimetic foldamers consisting of a segment of alternating α- and β-amino acids followed by an all-α-amino acid segment.[32−34] One foldamer with particularly strong binding to Bcl-x_L included the I85F amino acid substitution we frequently observed in our affinity matured NNK variants (Table 1).[32] Analysis of the crystal structure of this foldamer in complex with Bcl-x_L revealed that this Phe residue was well buried within the Bcl-x_L surface cleft.[34] Gellman and colleagues also generated α/β foldamers based on the Puma BH3 peptide and found that the location of the β-amino acid within the foldamer can have large effects on its affinity and specificity for pro-survival proteins; whereas the native Puma BH3 peptide binds with single nanomolar affinity to Bcl-x_L and Mcl-1, two of the α/β foldamers show a pronounced preference for binding Bcl-x_L.[35] As mentioned briefly above, Schepartz and co-workers have grafted residues from the Bak BH3 peptide onto the avian pancreatic polypeptide (aPP) miniature protein scaffold and used phage display to isolate variants with strong binding to Bcl-2.[24] The high affinity variants of the aPP/Bak BH3 chimera in this study all contained an I81F amino acid substitution that we observed in one of our affinity matured NNK variants (Table 1). The same aPP/Bak chimera was further evolved using phage display to bind specifically to Bcl-2 rather than Bcl-x_L.[36] This experiment required a selection for Bcl-2 binding alternated with a counterselection against Bcl-x_L binding. In contrast, the specificity we observe here in the XNNK variants arises solely from affinity maturation rather than via cycles of selection and counterselection.

Our work herein uses a combinatorial library approach to engineer affinity and specificity into the Bak BH3 peptide. The advantage of a combinatorial approach is underscored by the fact that it would be difficult to predict that many of the variants isolated in our screens would exhibit improved binding or altered specificity using rational protein engineering techniques. While we have applied a combinatorial method here to improve the affinity of a BH3 peptide, other combinatorial, high-throughput tools have also been used to probe Bcl-2 family interactions. Phage display has been used to isolate peptides with specificity toward Mcl-1 from a random peptide library,[37] and a high-throughput yeast two-hybrid approach was recently used to isolate peptides that bind specifically to A1.[38] Keating and co-workers displayed the Bim BH3 peptide on the yeast cell surface and used semirandom mutagenesis of the peptide and FACS to uncover elements governing the specificity of Bim BH3 binding to Bcl-x_L and Mcl-1.[39] There remain many unexplained complexities associated with Bcl-2 family recognition and the biological consequences of these interactions, but future applications of

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combinatorial tools to the study of Bcl-2 family interactions promises to shed light on some of these complexities.

METHODS

Bacterial Strains, Plasmid and Library Construction. The MC1061 strain of *E. coli* was used in library construction and in all surface display experiments. The BL21 *E. coli* strain was used for expression of the pro-survival proteins. Bak BH3 libraries were constructed as N-terminal fusions to the enhanced circularly permuted OmpX (eCPX) outer membrane protein[40,41] in an arabinose-inducible pBAD33 vector.[42] The restriction enzymes used to clone the libraries are identical to those used in our previous work.[15] The plasmid library was transformed into electrocompetent MC1061 and rescued in liquid LB medium containing chloramphenicol to select for transformants. Clones were sequenced from the naive libraries, and no biases were observed at any of the mutagenized positions. Aliquots of this cell library were mixed with an equal volume of 65% glycerol and frozen at −80 °C until needed. Plasmids for the expression of pro-survival proteins have been described in detail previously.[16] Briefly, these constructs contain an N-terminal biotin acceptor peptide tag[43] and a C-terminal histidine tag for biotinylation and purification, respectively. These plasmids are derivatives of the tetracycline-inducible pASK-75 plasmid.[44]

Pro-survival Bcl-2 Protein Expression and Purification. BL21 cells were co-transformed with a plasmid encoding a pro-survival protein and the pMON-BirA plasmid, which encodes a constitutively expressed copy of the *E. coli* biotin ligase. In a typical experiment, a 1-L culture was grown to an OD_{600} of 0.2, and biotin was added to a final concentration of 2 mg/L. After the culture reached an OD_{600} of 1, a final concentration of 0.2 mg/L of anhydrotetracycline was added to induce protein expression. After 2−4 h of induction at room temperature, cells were spun down and lysed via sonication. The protein was purified using Ni-NTA resin (Qiagen), and its purity was confirmed by SDS-PAGE. The purified protein was buffer exchanged using a PD-10 column (Biorad) into 2x phosphate-buffered saline (PBS) and stored at 4 °C until needed. Typical protein yields were 10 mg/L of culture for Bcl-x_L and 0.5−1 mg/L culture for other proteins.

Binding Assays, Library Screening, and Determination of Apparent K_d Values. In a typical binding experiment, 50 μL of an overnight culture of MC1061 harboring a plasmid with the desired Bak BH3-eCPX fusion was subcultured into 5 mL of LB medium and grown to OD_{600} of 0.6. After inducing with arabinose (0.02 wt %) for 2.5 h at RT, 1 mL of cells was pelleted and resuspended in PBS. A 50-μL aliquot of these cells was diluted 10-fold in PBS, and pro-survival protein was added to the desired concentration. This 500-μL mixture was rotated for 1 h at RT, and the cells were pelleted again. The cells were resuspended in 100 μL of PBS, and streptavidin-phycoerythrin (SAPE) was added to a final concentration of 50 nM. The cell suspension was incubated on ice for 30 min, pelleted, and resuspended in 2 mL PBS for analysis using Partec CyFlow ML flow cytometer. In library screening experiments, an aliquot of the library was thawed and added directly to LB on the day of the experiment. Pro-survival protein was added to the library at a sub-K_d concentration. Additional rounds of screening were carried out with lower concentrations of pro-survival protein until no further improvements were observed; details for each screen are provided in the Results and Discussion section. The clones with the highest fluorescence were isolated using a FACSVantage SE cell sorter. To determine apparent K_d values, the binding experiment was carried out at a range of different concentrations. The median fluorescence from the flow cytometry measurements was plotted as a function of protein concentration and fit to a single-site saturation model to determine an apparent K_d.

HeLa Culture and Killing Assays. Penetratin-Bak BH3 fusion peptides (wild-type peptide, XNNK12, MNNK1, and XNNG8) were custom synthesized by Genscript. These peptides consist of the 16-aa penetratin sequence (also referred to as the Antennapedia homeoprotein internalization domain)[27,45] followed by the 16-mer Bak BH3 peptide. HeLa cells were maintained in DMEM (Gibco), with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin at 37 °C with 5% CO_2. Cells from passages 9−12 were used for all killing assays and were plated at a density of 200,000 per well in 6-well plates or 10,000 cells/well in a 96-well plate. After an overnight incubation, the cells were washed with PBS and treated with penetratin-Bak peptides (concentrations from 0.35 to 50 μM) in serum-free (SF)-DMEM with 1% DMSO and 0.25% Pluronic F-127 to assist with solubilization of the peptide. After 4 h of incubation with peptides in SF-DMEM, FBS was added to a final volume of 10%, and the cells were incubated for another 20 h. To collect cells for flow cytometry analysis, cells on 6-well plates were trypsinized and combined with the supernatant portion (to include detached cells), pelleted, and resuspended in 1 mL of 0.5 μg/mL propidium iodide (PI)/PBS for a 30-min incubation on ice. The cells were analyzed for forward scatter and fluorescence (FL3, 630 nm bandpass on a Partec CyFlow ML cytometer). Dead cells were gated on the basis of their fluorescence in the FL3 channel, and percent viability was calculated as the number of live cells divided by the total number of cells. A minimum of 10,000 events were collected for each sample. To assess viability via MTT assays, 10 μL of 5 mg/mL MTT in PBS was added to each well in a 96-well plate (100 μL culture) and incubated at 37 °C with 5% CO_2 for 4 h. The medium was then aspirated, and 200 μL of DMSO was added to each well to solubilize the purple formazan crystals. The absorbance was read at 570 nm with a reading at 690 nm as an internal reference. All MTT assays were carried out in triplicate.

ASSOCIATED CONTENT

Supporting Information. Supplementary figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Schreiber, G., and Keating, A. E. (2011) Protein binding specificity versus promiscuity. *Curr. Opin. Struct. Biol. 21*, 50–61.

(2) Cory, S., and Adams, J. M. (2002) The BCL2 family: Regulators of the cellular life-or-death switch. *Nat. Rev. Cancer 2*, 647–656.

(3) Walensky, L. D. (2006) BCL-2 in the crosshairs: tipping the balance of life and death. *Cell Death Differ. 13*, 1339–1350.

(4) Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J., and Green, D. R. (2010) The BCL-2 family reunion. *Mol. Cell 37*, 299–310.

(5) Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol. 9*, 47–59.

(6) Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev. 14*, 2060–2071.

(7) Wei, M. C., Zong, W. X., Cheng, E. H. Y., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacCregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science 292*, 727–730.

(8) Minn, A. J., Kettlun, C. S., Liang, H., Kelekar, A., Vander Heiden, M. G., Chang, B. S., Fesik, S. W., Fill, M., and Thompson, C. B. (1999) Bcl-x(L) regulates apoptosis by heterodimerization-dependent and -independent mechanisms. *EMBO J. 18*, 632–643.

(9) Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) Structure of Bcl-x(L)-Bak peptide complex: Recognition between regulators of apoptosis. *Science 275*, 983–986.

(10) Petros, A. M., Olejniczak, E. T., and Fesik, S. W. (2004) Structural biology of the Bcl-2 family of proteins. *Biochim. Biophys. Acta, Mol. Cell Res. 1644*, 83–94.

(11) Petros, A. M., Nettesheim, D. G., Wang, Y., Olejniczak, E. T., Meadows, R. P., Mack, J., Swift, K., Matayoshi, E. D., Zhang, H. C., Thompson, C. B., and Fesik, S. W. (2000) Rationale for Bcl-x(L)/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Sci. 9*, 2528–2534.

(12) Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., and Huang, D. C. S. (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol. Cell 17*, 393–403.

(13) Hinds, M. G., Smits, C., Fredericks-Short, R., Risk, J. M., Bailey, M., Huang, D. C. S., and Day, C. L. (2007) Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death Differ. 14*, 128–136.

(14) Day, C. L., Smits, C., Fan, F. C., Lee, E. F., Fairlie, W. D., and Hinds, M. G. (2008) Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. *J. Mol. Biol. 380*, 958–971.

(15) Sun, J. J., Abdeljabbar, D. M., Clarke, N., Bellows, M. L., Floudas, C. A., and Link, A. J. (2009) Reconstitution and engineering of apoptotic protein interactions on the bacterial cell surface. *J. Mol. Biol. 394*, 297–305.

(16) Zhang, S. Y., and Link, A. J. (2011) Bcl-2 family interactome analysis using bacterial surface display. *Integr. Biol. 3*, 823–831.

(17) Walensky, L. D., Kung, A. L., Escher, I., Malia, T. J., Barbuto, S., Wright, R. D., Wagner, G., Verdine, G. L., and Korsmeyer, S. J. (2004) Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science 305*, 1466–1470.

(18) Verdine, G. L., and Walensky, L. D. (2007) The challenge of drugging undruggable targets in cancer: Lessons learned from targeting BCL-2 family members. *Clin. Cancer Res. 13*, 7264–7270.

(19) Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. S. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-x(L), but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev. 19*, 1294–1305.

(20) Pace, C. N., and Scholtz, J. M. (1998) A helix propensity scale based on experimental studies of peptides and proteins. *Biophys. J. 75*, 422–427.

(21) Wang, D. Y., Liao, W., and Arora, P. S. (2005) Binding properties of artificial a helices derived from a hydrogen-bond surrogate: Application to Bcl-xL. *Angew. Chem., Int. Ed. 44*, 6525–6529.

(22) Frey, V., Viaud, J., Subra, G., Cauquil, N., Guichou, J. F., Casara, P., Grassy, G., and Chavanieu, A. (2008) Structure-activity relationships of Bak derived peptides: Affinity and specificity modulations by amino acid replacement. *Eur. J. Med. Chem. 43*, 966–972.

(23) Fire, E., Gulla, S. V., Grant, R. A., and Keating, A. E. (2010) Mcl-1-Bim complexes accommodate surprising point mutations via minor structural changes. *Protein Sci. 19*, 507–519.

(24) Chin, J. W., and Schepartz, A. (2001) Design and evolution of a miniature bcl-2 binding protein. *Angew. Chem., Int. Ed. 40*, 3806–3809.

(25) Pavoor, T. V., Cho, Y. K., and Shusta, E. V. (2009) Development of GFP-based biosensors possessing the binding properties of antibodies. *Proc. Natl. Acad. Sci. U.S.A. 106*, 11895–11900.

(26) Sandberg, M., Eriksson, L., Jonsson, J., Sjostrom, M., and Wold, S. (1998) New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *J. Med. Chem. 41*, 2481–2491.

(27) Holinger, E. P., Chittenden, T., and Lutz, R. J. (1999) Bak BH3 peptides antagonize Bcl-x(L) function and induce apoptosis through cytochrome c-independent activation of caspases. *J. Biol. Chem. 274*, 13298–13304.

(28) Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B. L., Wendt, M. D., Zhang, H. C., Fesik, S. W., and Rosenberg, S. H. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature 435*, 677–681.

(29) Czabotar, P. E., Lee, E. F., van Delft, M. F., Day, C. L., Smith, B. J., Huang, D. C. S., Fairlie, W. D., Hinds, M. G., and Colman, P. M. (2007) Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc. Natl. Acad. Sci. U.S.A. 104*, 6217–6222.

(30) Schafmeister, C. E., Po, J., and Verdine, G. L. (2000) An all-hydrocarbon cross-linking system for enhancing the helicity and metabolic stability of peptides. *J. Am. Chem. Soc. 122*, 5891–5892.

(31) Kim, Y. W., Grossmann, T. N., and Verdine, G. L. (2011) Synthesis of all-hydrocarbon stapled alpha-helical peptides by ring-closing olefin metathesis. *Nat. Protoc. 6*, 761–771.

(32) Sadowsky, J. D., Fairlie, W. D., Hadley, E. B., Lee, H. S., Umezawa, N., Nikolovska-Coleska, Z., Wang, S. M., Huang, D. C. S., Tomita, Y., and Gellman, S. H. (2007) alpha/beta+alpha)-Peptide antagonists of BH3 Domain/Bcl-x(L) recognition: Toward general strategies for foldamer-based inhibition of protein-protein interactions. *J. Am. Chem. Soc. 129*, 139–154.

(33) Sadowsky, J. D., Murray, J. K., Tomita, Y., and Gellman, S. H. (2007) Exploration of backbone space in foldamers containing alpha- and beta-amino acid residues: Developing protease-resistant oligomers that bind tightly to the BH3-recognition cleft of Bcl-x(L). *ChemBioChem 8*, 903–916.

(34) Lee, E. F., Sadowsky, J. D., Smith, B. J., Czabotar, P. E., Peterson-Kaufman, K. J., Colman, P. M., Gellman, S. H., and Fairlie, W. D. (2009) High-resolution structural characterization of a helical alpha/beta-peptide foldamer bound to the anti-apoptotic protein Bcl-x(L). *Angew. Chem., Int. Ed. 48*, 4318–4322.

(35) Horne, W. S., Boersma, M. D., Windsor, M. A., and Gellman, S. H. (2008) Sequence-based design of alpha/beta-peptide foldamers that mimic BH3 domains. *Angew. Chem., Int. Ed. 47*, 2853–2856.

(36) Gemperli, A. C., Rutledge, S. E., Maranda, A., and Schepartz, A. (2005) Paralog-selective ligands for Bcl-2 proteins. *J. Am. Chem. Soc. 127*, 1596–1597.

(37) Lee, E. F., Fedorova, A., Zobel, K., Boyle, M. J., Yang, H., Perugini, M. A., Colman, P. M., Huang, D. C. S., Deshayes, K., and

Fairlie, W. D. (2009) Novel Bcl-2 homology-3 domain-like sequences identified from screening randomized peptide libraries for inhibitors of the pro-survival Bcl-2 proteins. *J. Biol. Chem. 284*, 31315–31326.

(38) Brien, G., Debaud, A.-L., Bickle, M., Trescol-Biemont, M.-C., Moncorge, O., Colas, P., and Bonnefoy-Berard, N. (2011) Characterization of peptide aptamers targeting Bfl-1 anti-apoptotic protein. *Biochemistry 50*, 5120–5129.

(39) Dutta, S., Gulla, S., Chen, T. S., Fire, E., Grant, R. A., and Keating, A. E. (2010) Determinants of BH3 binding specificity for Mcl-1 versus BcI-x(L). *J. Mol. Biol. 398*, 747–762.

(40) Rice, J. J., and Daugherty, P. S. (2008) Directed evolution of a biterminal bacterial display scaffold enhances the display of diverse peptides. *Protein Eng., Des. Sel. 21*, 435–442.

(41) Rice, J. J., Schohn, A., Bessette, P. H., Boulware, K. T., and Daugherty, P. S. (2006) Bacterial display using circularly permuted outer membrane protein OmpX yields high affinity peptide ligands. *Protein Sci. 15*, 825–836.

(42) Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P-Bad promoter. *J. Bacteriol. 177*, 4121–4130.

(43) Beckett, D., Kovaleva, E., and Schatz, P. J. (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci. 8*, 921–929.

(44) Skerra, A. (1994) Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in Escherichia coli. *Gene 151*, 131–135.

(45) Deshayes, S., Morris, M. C., Divita, G., and Heitz, F. (2005) Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cell. Mol. Life Sci. 62*, 1839–1849.