Published in final edited form as:

ACS Chem Biol. 2014 March 21; 9(3): 831–837. doi:10.1021/cb4003305.

Distinct BimBH3 (BimSAHB) Stapled Peptides for Structural and Cellular Studies

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Abstract

Hydrocarbon stapling is a chemical approach to restoring and fortifying the natural α -helical structure of peptides that otherwise unfold when taken out of context from the host protein. By iterating the peptide sequence, staple type, and sites of insertion, discrete compositions can be generated to suit a diversity of biochemical, structural, proteomic, cellular, and drug development applications. Here, we reinforce key design considerations to avoid pitfalls and maximize progress when applying stapled peptides in chemistry and biology research.

Chemists and biologists have long sought to harness the affinity and specificity of natural peptides to dissect and target protein interactions. Despite the remarkable diversity afforded by such amino acid polymers, the amide bond backbone renders peptides vulnerable to degradation *in vivo*, limiting their applications as therapeutics. What's more, structured peptides taken out of context from the host protein can unfold, resulting in loss of conformation-based biological activity. We and others have applied a technique called hydrocarbon stapling(1), which installs an all-hydrocarbon crosslink within unfolded peptides to restore α -helical shape. The all-hydrocarbon crosslink itself has the important attribute of being chemically and metabolically stable *in vivo*, an advantage over alternative approaches that rely on amide or disulfide crosslinks, which can undergo hydrolysis or reduction, respectively. We previously reported that depending on a combination of biophysical parameters, including degree of α -helicity, hydrophobicity/amphipathicity, and charge, appropriately designed stapled peptides can also achieve cellular uptake(2, 3). Thus, the capacity of hydrocarbon stapling to recapitulate native α -helical shape, which can in turn promote structural stability, protease resistance, and cellular penetrance, has fueled the development and successful application of stapled peptides in a host of biological systems (Table 1).

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In order to maximize the potential for success in designing stapled peptides for basic research and therapeutic development, a series of important considerations must be kept in mind to avoid potential pitfalls. For example, Okamoto et al. recently reported in *ACS Chemical Biology* that a hydrocarbon-stapled BIM BH3 peptide (BIM SAHB) manifests neither improved binding activity nor cellular penetrance compared to an unmodified BIM BH3 peptide, and thereby caution that peptide stapling does not necessarily enhance affinity or biological activity(4). These negative results underscore an important point about peptide stapling: insertion of any one staple at any one position into any one peptide to address any one target provides no guarantee of stapling success. In this particular case, it is also noteworthy that the authors based their conclusions on a construct that we previously reported was weakened-by-design to accomplish a specialized NMR study of a transient ligand-protein interaction(5) and was not used in cellular studies(5, 6) because of its relatively low α -helicity, weak binding activity, overall negative charge, and diminished cellular penetrance (Fig. 1). Thus, the Okamoto et al. report provides an opportunity to reinforce key learnings regarding the design and application of stapled peptides, and the biochemical and biological activities of discrete BIM SAHB peptides.

The first step in determining the success of peptide stapling is to evaluate the degree of induced α -helical stabilization. For example, Okamoto et al. chose as their major focus of study a BIM BH3 peptide composed of amino acids 145-164 that, in the unmodified form, manifests approximately 21% alpha-helical content in solution(4). Upon substitution of a hydrocarbon staple at the R154/E158 *i, i+4* position, the α -helicity is only modestly increased to 39%(4). Thus, stapling in this context is only partially effective, rendering the construct suboptimal for certain applications. It is important to remember that when substituting into the peptide sequence the requisite non-natural amino acids for stapling, natural amino acids will be eliminated, which carries the risk of negating important intrapeptide or peptide-target molecular interactions. If the conformational benefit conferred by the staple does not overcome the penalty incurred by removing select natural amino acids, the goals of peptide stapling may not be achieved. In agreement with these design principles, the authors find that insertion of the staple at the R154/E158 (“A”) position of BIM BH3 (aa 145-164) impairs binding to three BCL-2 family anti-apoptotic targets compared to the unmodified peptide. Notably, however, there is little to no negative impact of stapling on binding to two other anti-apoptotic targets(4). We previously found that this same BIM SAHB_A (aa 145-164) construct binds to pro-apoptotic BAX (enabling the discovery of the BAX trigger site(5)), whereas the unmodified BIM BH3 peptide (aa 145-164) shows negligible binding to full-length BAX. Taken together, these data demonstrate that the same peptide template with identical staple composition and positioning, applied to different protein targets, can yield distinct *target-dependent* results. Thus, the authors' conclusion that “unexpectedly, we found that such modified peptides have *reduced* affinity for their targets”(4) is partially, but not entirely, correct with regard to the binding activity of BIM SAHB_A (aa 145-164).

The authors then applied this BIM SAHB_A (aa 145-164) construct in cellular studies and observed no biological activity, leading to the conclusion that “BimSAHB is not inherently cell permeable”(4). However, before applying stapled peptides in cellular studies, it is very

important to directly measure cellular uptake of fluorophore-labeled SAHBs by a series of approaches, including FACS analysis, confocal microscopy, and fluorescence scan of electrophoresed lysates from treated cells, as we previously reported(3, 6–8). Indeed, we did not use the BIM SAHB_A (aa 145-164) peptide in cellular studies, specifically because it has relatively low α -helicity, weakened binding activity, and overall negative charge (–2), all of which combine to make this particular BIM SAHB construct a poor candidate for probing cellular activity (Fig. 1). As indicated in our 2008 *Methods in Enzymology* review, “anionic species may require sequence modification (e.g., point mutagenesis, sequence shift) to dispense with negative charge”(2), a strategy that emerged from our earliest studies in 2004 and 2007 to optimize the cellular penetrance of stapled BID BH3 and p53 peptides for cellular and *in vivo* analyses(7, 9), and also applied in our 2010 study involving stapled peptides modeled after the MCL-1 BH3 domain(8). In our 2011 *Current Protocols in Chemical Biology* article, we emphasized that “based on our evaluation of many series of stapled peptides, we have observed that their propensity to be taken up by cells derives from a combination of factors, including charge, hydrophobicity, and α -helical structure, with negatively charged and less structured constructs typically requiring modification to achieve cell penetrance. Successful interventions include (1) substituting Gln for Glu and/or Asn for Asp, or appending native charged residues at the N or C terminus to adjust the overall charge to 0 to +2, and (2) producing constructs with greater α -helical content through differential staple placement”(3). Our review of the literature for this *Point of View* article found that the majority of stapled peptides successfully developed for cellular and *in vivo* work by us and independently by others typically exhibited high α -helical content and overall neutral or positive charge (Table 1). As BIM SAHB_A (aa 145-164) bears a charge of –2, a reported α -helicity of 39%, and relatively weak affinity for select BCL-2 family targets, we agree with Okamoto et al. that this BIM SAHB_A (aa 145-164) construct shows little to no cellular activity.

Over the past seven years, we have developed a variety of BIM SAHB constructs for biochemical, structural, and biological studies. In 2006, we published the synthesis and binding activity of our first stapled BIM BH3 peptide, named BIM SAHB_A, which was comprised of amino acids 146-166 and contained an *i, i+4* staple at position R154/E158(10). BIM SAHB_A (aa 146-166) exhibited 81% α -helical content in solution, a significant improvement upon the 20% α -helicity of the corresponding unmodified BIM BH3 peptide (aa 146-166)(10). In fluorescence polarization binding assays, BIM SAHB_A (aa 146-166) bound better to anti-apoptotic BCL-X_L C than the unmodified BIM BH3 peptide, and exhibited low nanomolar binding to pro-apoptotic BAX, whereas the corresponding unmodified peptide showed no detectable BAX interaction(10). The comparative binding activity of BIM SAHB_A (aa 145-164) and BIM SAHB_A (aa 146-166) represents an example of two different peptide templates bearing the identical staple composition and positioning, applied to the same set of targets, yielding distinct *sequence-dependent* results.

Because BIM SAHB_A (aa 146-166) was among the first stapled BH3 peptides to manifest quantifiable binding interactions with BAX(10), resulting in direct BAX activation *in vitro*, we were eager to deploy it to localize the long-elusive BH3-binding site on BAX using structural methods. Collaborating with the laboratory of Dr. Nico Tjandra, who first solved

the solution structure of full-length, inactive BAX(11), we found that BIM SAHB_A (aa 146-166) triggered such rapid oligomerization of BAX that NMR analysis of the interaction was precluded. Thus, we adjusted the composition to weaken its binding activity in an effort to slow down the BAX activation process, while also optimizing its solubility for the high concentrations required for structural analysis. Ultimately, removal of the two C-terminal arginine residues (aa 165-166) and addition of one native N-terminal glutamate residue (aa 145) accomplished this goal(5). Consistent with its weakened BAX-binding and BAX-activating capacity, BIM SAHB_A (aa 145-164) also demonstrated weaker binding affinity for anti-apoptotic BCL-X_L C, as compared to the original BIM SAHB_A (aa 146-166)(6, 12).

Applying the adjusted BIM SAHB_A (aa 145-164) construct in a series of NMR analyses led to the identification of a novel BH3 “trigger site” at the confluence of alpha-helices 1 and 6 at the N-terminal face of BAX(5). In this study, reported in *Nature* in 2008, we also functionally validated the calculated model structure of the BIM BH3/BAX complex by stapled peptide and protein mutagenesis, linking interaction at the novel site to direct BAX activation *in vitro* and in cells. Of note, in the body of the *Nature* article we referred to our stapled peptide constructs using the generic term “BIM SAHB,” as employed by Okamoto et al., but we defined in the first paragraph of our Methods section entitled “SAHB Synthesis and Characterization” the explicit compositions of BIM SAHBs applied in each study: “BIM SAHB_A used in the NMR and *in vitro* studies is an N-acetylated, C-amidated 20-amino-acid peptide Ac-¹⁴⁵EIWIAQELRXIGDXFNAYYA¹⁶⁴-CONH₂, in which X represents the non-natural amino acid inserted for olefin metathesis...For MEF studies, the cell-permeable N-acetylated, C-amidated 21-amino-acid peptide Ac-¹⁴⁶IWIAQELRXIGDXFNAYYARR¹⁶⁶-CONH₂ (ref. 10) and its R153D mutant were used”(5).

In a 2009 commentary on our *Nature* article, Czabotar et al. wrote, “we note that fast exchange reported here [ref 5] is indicative of considerably weaker affinity than the value previously reported by these same authors for the stapled BimBH3/Bax interaction, 24 nM [ref 10],” suggesting the possibility of a BIM SAHB/BAX binding discrepancy between our two studies(5, 10). However, the weak affinity was a property of our weakened-by-design BIM SAHB_A (aa 145-164)(5) and the potent nanomolar binder was a feature of the original highly α -helical BIM SAHB_A (aa 146-166)(10), as reflected by the different sequence compositions reported in the main figures of these two studies. Since that time, we continued to reiterate the sequence-specific details regarding the two distinct BIM SAHB constructs. In the Introduction of our 2010 *Molecular Cell* study that delineated the series of allosteric conformational changes induced upon BIM SAHB_A (aa 145-164) engagement of the novel BAX trigger site, we noted, “Among the SAHBs tested for BAX-binding activity, BIM SAHB displayed the strongest binding affinity (EC₅₀, 24 nM) (ref 10) and was subsequently employed in nuclear magnetic resonance (NMR) spectroscopy studies designed to localize the BH3-binding site on BAX. However, chemical shift perturbation mapping of ¹⁵N-BAX upon BIM SAHB titration was precluded by the potency of BIM SAHB in binding and triggering BAX oligomerization in solution. To achieve a complex stable enough for short-term NMR acquisitions, we screened a series of BIM SAHBs of differential length and sequence composition, ultimately identifying a BIM SAHB_A

construct that, while still triggering BAX, exhibited sufficiently weak binding activity to monitor the complex (ref 5)"(13). In our subsequent 2011 review of the BAX activation pathway, we again wrote, "Initial NMR analyses of ^{15}N -BAX upon BIM SAHB titration highlighted the catch-22 of trying to study the structure of, literally, a moving target. Because ligand binding triggered rapid BAX oligomerization, precluding structural analysis, the composition of BIM SAHB was adjusted to weaken its binding activity in an effort to slow down the activation process. Ultimately, removal of two C-terminal residues and addition of one native N-terminal residue accomplished this goal"(14).

In our latest BIM SAHB paper, published in the *Journal of Clinical Investigation* in 2012, we demonstrated that the original potent and cell-permeable BIM SAHB_A (aa 146-166) analog reactivated the death pathway in a series of resistant hematologic cancer cells by broadly engaging anti-apoptotic targets and pro-apoptotic BAX, and demonstrated sequence-specific *in vivo* efficacy in suppressing both leukemic growth and *Bim*^{-/-} autoimmune disease in two mouse models(6). We again defined the sequence of this potent, cell-permeable BIM SAHB_A (aa 146-166) analog, indicating "To explore the breadth of BIM BH3 activity, we generated a hydrocarbon-stapled BIM BH3 helix (amino acids 146-166)(ref 10) and a negative control peptide based on an R153D reverse polarity mutation within the core BH3 sequence (ref 5, Figure 1A, and Supplemental Table 1)"(6). By assembling our published documentation of the explicit sequence compositions of BIM SAHBs and their distinct properties and scientific applications, as also summarized in Fig. 1, we hope to resolve any confusion generated by the Okamoto et al. study.

Finally, in one supplementary experiment, Okamoto et al. did test the cellular activity of our potent and cell-permeable BIM SAHB_A (aa 146-166) analog, which indicates that the authors were aware of the two distinct BIM SAHB_A compositions. The authors found that BIM SAHB_A (aa 146-166) does not impair the viability of wild-type mouse embryonic fibroblasts (MEFs), in agreement with our previously reported experiment showing that this same construct had little to no effect on wild-type MEF viability at the same dose and time point(6). Although based on these results the authors concluded that "BimSAHB is not inherently cell permeable"(4), we had performed cellular uptake analyses on the treated MEFs, and found that BIM SAHB_A (aa 146-166) was indeed taken up by the cells(6), but the fibroblasts manifested relative resistance compared to the observed effects on a series of hematologic cancer cell lines – highlighting a potential therapeutic window for BIM SAHB_A (aa 146-166) treatment(6). In agreement with our conclusions, senior Walter and Eliza Hall Institute (WEHI) investigator Dr. Jerry Adams wrote in his complimentary preview of our 2012 *JCI* article, "Notably, at low μM levels the BIM SAHB killed the cultured tumor cells but not nontransformed fibroblasts, and the cell death had all the hallmarks of apoptosis" (15).

To probe the sequence dependence of BIM SAHB_A (aa 146-166) on direct activation of BAX in genetically-defined cells, we previously reconstituted *Bax*^{-/-} *Bak*^{-/-} MEFs with wild-type BAX and then treated with BIM SAHB_A (aa 146-166) in an initial serum-free, detached-cell setting, which enabled us to detect a sequence-specific difference in apoptosis induction that linked the cellular activity of BIM SAHB_A (aa 146-166) to direct BAX interaction(5). These cellular findings correlated with our structural, biochemical, and *in*

vitro functional results(5, 13). In relation to these studies, Okamoto et al. state that our K21E mutant form of BAX “was reported to be inert” and that Lys21 was “essential for activation” (4). However, in our two studies of the direct BAX activation mechanism, we demonstrated that this single point mutation at the trigger site slows the kinetics of direct BAX activation rather than render the protein inactive(5, 13). Thus, cellular studies that explore 20 hour and longer time points, including long-term clonogenic assays, may not be suitable for detecting the kinetic difference between wild-type and K21E BAX activation. In addition, BAX can be activated by a variety of other mechanisms that would remain intact in this context.

As the biological focus of the Okamoto et al. study relates to the relevance of direct BAX activation by BIM BH3 helices, it is also noteworthy that our laboratories are now closer than ever to consensus regarding the importance of this apoptotic mechanism. Dating back to the discovery of the BH3-only protein BID in 1996, Dr. Stanley Korsmeyer and colleagues proposed that BID's BH3 domain could directly trigger BAX/BAK by a “hit and run” mechanism. Since that time, a variety of laboratories obtained biochemical, structural, cellular, and *in vivo* evidence in support of such a mechanism(14). Advocating for a different model that relies exclusively on an indirect mechanism for BAX/BAK activation, negating a role for direct BH3 interactions with BAX or BAK, Dr. P. Czabotar and WEHI colleagues reported in *Science* in 2007, “Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak”(16). Now, as presented in their 2013 *Cell* study, Czabotar et al. change course and agree that discrete BH3 domains, such as that of BID and BIM, can directly activate BAX, as evidenced by structural studies employing a C-terminally truncated form of BAX(17). The authors do not capture a triggering BH3 interaction at the $\alpha 1/\alpha 6$ site of BAX by X-ray crystallography, likely due to its transient “hit and run” nature and bypass of this regulatory step by their use of a C-terminally deleted form of BAX. We previously found using full-length BAX protein that BIM SAHB_A (aa 145-164) triggering at the $\alpha 1/\alpha 6$ site induces allosteric release of the C-terminal $\alpha 9$ helix, resulting in the translocation of cytosolic BAX to the mitochondria, a key initiating step of the direct BAX activation pathway(13). Once $\alpha 9$ is released, we observe binding compatibility between direct activator BH3 domains and the C-terminal canonical BH3-binding pocket on BAX, as detected by our combined photoreactive SAHB affinity labeling/mass spectrometry approach(18–20). These latter interactions have been structurally validated by Czabotar et al.(17) and likely play a role in propelling the activation and oligomerization of mitochondria-localized BAX. In agreement with publications from our group and others supporting a multistep model for BH3-mediated direct BAX activation(5, 13, 20, 21), Czabotar et al. acknowledge in their recent *Cell* paper, “Our studies, however, do not address whether some such alternative site contributes to earlier activation steps, such as release of $\alpha 9$ from the groove. Hence BH3 interactions with two different sites on Bax may play sequential roles in activation, as some biochemical evidence suggests”(17).

As for any new technology or mechanism of action, the path to success is paved with passion, persistence, rigorous and logical experimentation, and careful handling and conveyance of the scientific facts. To study a target of interest, we and others have generated panels of stapled peptides, iterating the designs as needed for the desired application (Table 1). For those readers interested in developing stapled peptides for a variety of research

applications, including a troubleshooting section on how to optimize peptides for each indication, please see our 2011 review that details a decade of learnings about the peptide stapling technology(3). Anticipate that generating the optimal stapled peptide for a particular scientific application is likely to require exploration of differential sequence templates, staple scanning, and several rounds of synthetic iteration to achieve optimal solubility, structural stability, cell permeability, and biological activity (Fig. 2). Like small molecules, and depending upon the sequence composition, stapled peptides can be differentially affected by serum, with binding to albumin previously documented(18). Because serum can reduce cellular exposure(19, 22), it is important to perform direct measures of cellular uptake so that serum-free, serum-free window followed by serum replacement, or full-serum conditions can be applied accordingly in cellular assays, as demonstrated successfully across the broad spectrum of published reports (Table 1). Increasing the dosing level for cellular and *in vivo* studies and/or limited amino acid sequence adjustments can often overcome the effect of serum, which is also offset by the striking proteolytic stability of stapled peptides(9, 18). As with any new technology, much remains to be learned as we and others continue to decipher the rules, make improvements, and discover new applications. However, a commitment to taking a rigorous, stepwise, and detail-oriented approach to the production, optimization, and application of stapled peptides is the best formula for success in developing these reagents for protein interaction research and therapeutic targeting. We are heartened by the successes of many independent investigators and drug companies who have designed and deployed stapled peptides to advance their basic research and therapeutic programs (Table 1), and we look forward to continued progress. Indeed, the very first clinical trial to evaluate a stapled peptide drug in man was successfully completed this year, with additional clinical trials already being planned for the year ahead.

Acknowledgments

We thank E. Smith for editorial assistance and figure preparation. This work was supported by NIH grants 5R01CA050239 and 5R01GM090299 to L.D.W.

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Figure 1. A tale of two BIM SAHBs

In order to accomplish a challenging NMR analysis of the hit-and-run interaction between BIM BH3 and BAX (left), we adjusted the sequence of our prototype high α -helicity, high affinity, and cell permeable BIM SAHB_A (aa 146–166) peptide (right) to enhance its solubility and weaken (i.e. slow down) its BAX-activating capability (left). Whereas Okamoto et al. successfully applied this refashioned BIM SAHB_A (145–164) peptide for structural determination (left), their application of this weakened-by-design BIM SAHB_A (145–164) in binding and cellular studies (red arrow) led to the conclusion that stapling BIM BH3 does not enhance binding affinity or biological activity. In agreement with these results, we never used BIM SAHB_A (145–164) in cellular studies because of its relatively low α -helicity and weak binding activity, and instead applied our original highly α -helical, potent, and cell permeable BIM SAHB_A (aa 146–166) construct in cellular and *in vivo* analyses(5, 6, 10). Indeed, the versatility of peptide chemistry in general and hydrocarbon stapling in particular allows for the production of a diverse spectrum of stapled peptides that can be tailored to suit a host of research and therapeutic goals.

Figure 2. Design and synthesis of stapled peptides for protein interaction research and therapeutic targeting

Single peptide α -helices embedded within proteins can serve as key binding determinants for protein interaction, reflecting Nature's solution to high fidelity protein targeting. Although potentially well-suited as prototype therapeutics for disrupting pathologic protein interactions, when taken out of context from the full-length protein, such structured peptides can unfold, limiting their biological activity and rendering them susceptible to rapid proteolysis *in vivo*. Hydrocarbon stapling can restore bioactive secondary structure, and combined with facile iteration of peptide sequence composition (varying template length and charge), staple type, staple placement, and a host of derivatizations, a library of stapled peptides can be generated to suit a diversity of research and therapeutic applications.

Table 1

research.

Analyses	Proteolytic stability testing	Structure	Cellular uptake analysis	Cellular assays	In vivo PK, efficacy	%Helicity linear	%Helicity stapled	Charge atpH7	Reference	Institution
X	X			X	X	13	40	-7	Bird et al. <i>Proc Natl Acad Sci</i> , 2010	Dana-Farber Cancer Institute
X	X			X		17	97	0	Sviridov et al. <i>Biochem Biophys Res Comm</i> , 2011	NIH
X	X	X						+2	Phillips et al. <i>JACS</i> , 2011	Pfizer, U Portsmouth (UK), CPC Scientific
X	X			X	X	15	83	-2	Platt et al. <i>J Biol Chem</i> 2012	University of Utah, University of Gudansk
X	X			X		15	44	+5	Chapuis et al. <i>Amino Acids</i> , 2012	Academy of Sciences of the Czech Republic
X	X			X		12	56	+4	Chapuis et al. <i>Amino Acids</i> , 2012	Academy of Sciences of the Czech Republic
X	X			X	X	1	23	+4	Green et al. <i>Bioorg Med Chem</i> , 2013	University of Utah
X	X			X	X	4	20	+3	Green et al. <i>Bioorg Med Chem</i> , 2013	University of Utah
X	X			X		6	57	-1	Cui et al. <i>Bioorg Med Chem</i> , 2013	Tsinghua-Peking Center for Life Sciences
X	X			X		4	47	+3	Pham et al. <i>Bioorg Med Chem Lett</i> , 2013	Dongguk University, South Korea
X	X					40	84	-1	Schafmeister et al. <i>J Am Chem Soc</i> , 2000	Harvard University
X	X		X	X [□]		34	70	0	Kim et al. <i>Org Lett</i> , 2010	Harvard University
						25	69,74	0	Shim et al. <i>Chem Biol Drug Des</i> 2013	Harvard University
X	X	X	X	X [†]	X	14, 16	88,86	-1,0	Walensky et al. <i>Science</i> 2004; <i>Mol Cell</i> 2006	Dana-Farber Cancer Institute
		X ^{**}						-1	Leshchiner et al. <i>PNAS</i> , 2013	Dana-Farber Cancer Institute
		X						-1	Moldoveanu et al. <i>Nat Struct Mol Biol</i> , 2013	St. Jude Children's Research Hospital

Helical Ligand	Protein Target	Biophysical/ biochemical analyses	Proteolytic stability testing	Structure	Cellular uptake analysis	Cellular assays	<i>In vivo</i> PK, efficacy	%Helicity linear
BAD BH3	BCL-2 family, glucokinase	X			X	X [†]		21
Phospho-BAD BH3	Glucokinase	X		X ^{**}		X [†]		21
BIM BH3	BCL-2 family	X			X	X [†]	X	20
BIM BH3	BAX	X		X				
BIM BH3	BCL-2 anti-apoptotics	X		X		X [†]		21
MCL-1 BH3	MCL-1	X		X	X	X [□]		18
MCL-1 BH3	MCL-1			X [*]				
PUMA BH3	BAX, BCL-2 family	X		X ^{**}	X	X ^{□†‡}		35
BH3, photoreactive	BCL-2 family, glucokinase	X		X ^{**}				
p53	MDM2	X	X		X	X [†]		11
p53	MDM2/MDMX	X			X	X [□]	X	11
p53	MDM2/MDMX	X		X				11
p53	MDM2/MDMX					X [†]	X	11
p53 [^]	MDM2	X			X	X [□]		
p53	MDM2	X		X [*]				
p53	MDM2/MDMX			X [*]				

Helical Ligand	Protein Target	Biophysical/ biochemical analyses	Proteolytic stability testing	Structure	Cellular uptake analysis	Cellular assays	<i>In vivo</i> PK, efficacy	%Helicity linear
P53	MDM2/MDMX	X				X [†]		Bird et al.
p53	MDM2/MDMX	X		X	X	X [†]	X	11
P53	MDM2	X				X [†]		
HIV-1 capsid	Gag	X		X	X	X [†]		
HIV-1 capsid	Gag	X		X	X	X [†]		
HIV-1 integrase	HIV-1 Integrase	X			X	X [†]		21
Mastermind	Notch	X			X	X [†]	X	23
BCL9	β-catenin	X			X	X [†]	X	33
Axin	β-catenin	X		X	X	X [†]		15
Axin	β-catenin	X		X	X	X [†]		28
p110α	IRS1	X				X [†]	X	
Borealin	Survivin	X	X					52
eIF4G	eIF4E	X		X*				0
EZH2	EED	X	X		X	X [†]		

Methodologic Reviews

Synthesis and biophysical characterization of stabilized alpha-helices of BCL-2 domains

Dissection of the BCL-2 family signaling network with stabilized alpha-helices of BCL-2 domains

Synthesis of all-hydrocarbon stapled alpha-helical peptides by ring-closing olefin metathesis

Stapled Peptides for Intracellular Targets

Helical Ligand	Protein Target	Biophysical/ biochemical analyses	Proteolytic stability testing	Structure	Cellular uptake analysis	Cellular assays	<i>In vivo</i> PK, efficacy	%Helicity linear
Chemical synthesis of hydrocarbon- stapled peptides for protein interaction research and therapeutic targeting								

Walensky laboratory or collaborative studies, Independent laboratory studies

* Atomistic simulations,

** Photocrosslinking/mass spectrometry-based binding site identification,

^ Includes β -peptide analogs,

$\square$ Serum-free conditions,

$\dagger$ Serum-free window followed by serum replacement,

$\ddagger$ Serum-containing media throughout