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Perspective

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Hydrocarbon-Stapled Peptides: Principles, Practice, and Progress

Miniperspective

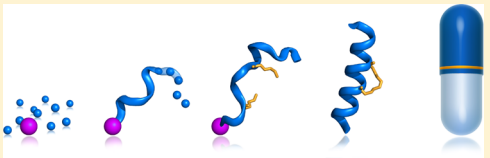
Loren D. Walensky*[,†,‡,§] and Gregory H. Bird*[,†,‡]

[†]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02215, United States

[‡]Linde Program in Cancer Chemical Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02215, United States

[§]Department of Pediatrics, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts 02115, United States

ABSTRACT: Protein structure underlies essential biological processes and provides a blueprint for molecular mimicry that drives drug discovery. Although small molecules represent the lion's share of agents that target proteins for therapeutic benefit, there remains no substitute for the natural properties of proteins and their peptide subunits in the majority of biological contexts. The peptide α-helix represents a common structural motif that mediates communication between signaling proteins. Because peptides can lose their shape when taken out of context, developing chemical interventions to stabilize their bioactive structure remains an active area of research. The all-hydrocarbon staple has emerged as one such solution, conferring α-helical structure, protease resistance, cellular penetrance, and biological activity upon successful incorporation of a series of design and application principles. Here, we describe our more than decade-long experience in developing stapled peptides as biomedical research tools and prototype therapeutics, highlighting lessons learned, pitfalls to avoid, and keys to success.



Chemists and biologists have long sought to recapitulate the shape and bioactivity of the peptide α-helix for basic science and therapeutic applications. A diversity of clever approaches to reinforcing α-helical structure, spanning non-covalent and covalent strategies, have been advanced over the past several decades.[1,2] For example, designs that include helical caps between terminal side chains and the peptide backbone,[3] hydrogen bonding or electrostatic interactions between side chains at select positions,[4] and introduction of α,α-disubstituted amino acids,[5,6] such as aminoisobutyric acid, have yielded peptides with improved α-helical structure in solution. Covalent approaches based on installing disulfide[7] and lactam[8−10] bridges into the peptide architecture have provided even further enhancements. With proof-of-concept for chemical stabilization of peptide helices in hand, a critical next step was to transform structured peptides into reagents that could withstand the in vivo proteolytic environment, target and penetrate intact cells, and ultimately achieve clinically relevant biological activity. The purpose of this review is to describe our practical experience to date with inserting all-hydrocarbon cross-links into bioactive peptide motifs and how this chemical intervention created a new class of structured peptides for biological discovery and clinical translation.

The all-hydrocarbon cross-link for peptide α-helix stabilization was first published in 2000 by Verdine and colleagues, who sampled a large series of α,α-disubstituted non-natural amino acids bearing olefin tethers to determine optimal length and stereochemistry for ruthenium-catalyzed ring-closing metathesis (RCM) across one or two α-helical turns.[11] This work was an extension of the pioneering studies of Blackwell and Grubbs, who created a cross-link between *O*-allylserine residues on a peptide template to form a covalent bond using the Grubbs catalyst.[12] Importantly, Verdine and colleagues combined the principles of RCM with α,α-disubstitution of the amino acid chiral carbon and on-resin peptide synthesis to ultimately achieve the goal of structural stabilization[11] (Figure 1). The resultant constructs were later dubbed stapled peptides,[13] based

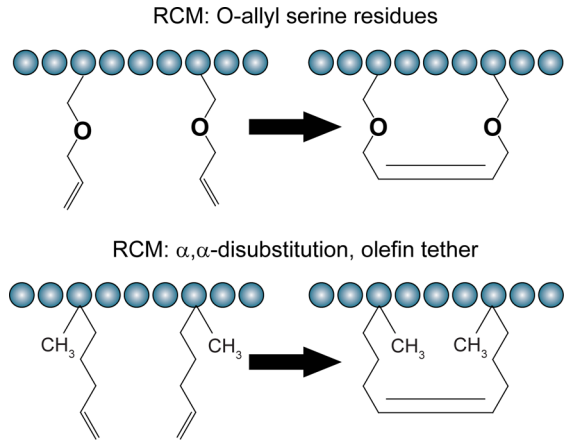


Figure 1. Application of ruthenium-catalyzed olefin metathesis to install macrocyclic cross-links into synthetic peptides. Blackwell and Grubbs performed the metathesis reaction on a pair of *O*-allylserine residues (top), whereas Schafmeister and Verdine employed α,α-disubstituted non-natural amino acids bearing all-hydrocarbon tethers (bottom). The latter approach yielded peptide constructs with marked α-helical stabilization.

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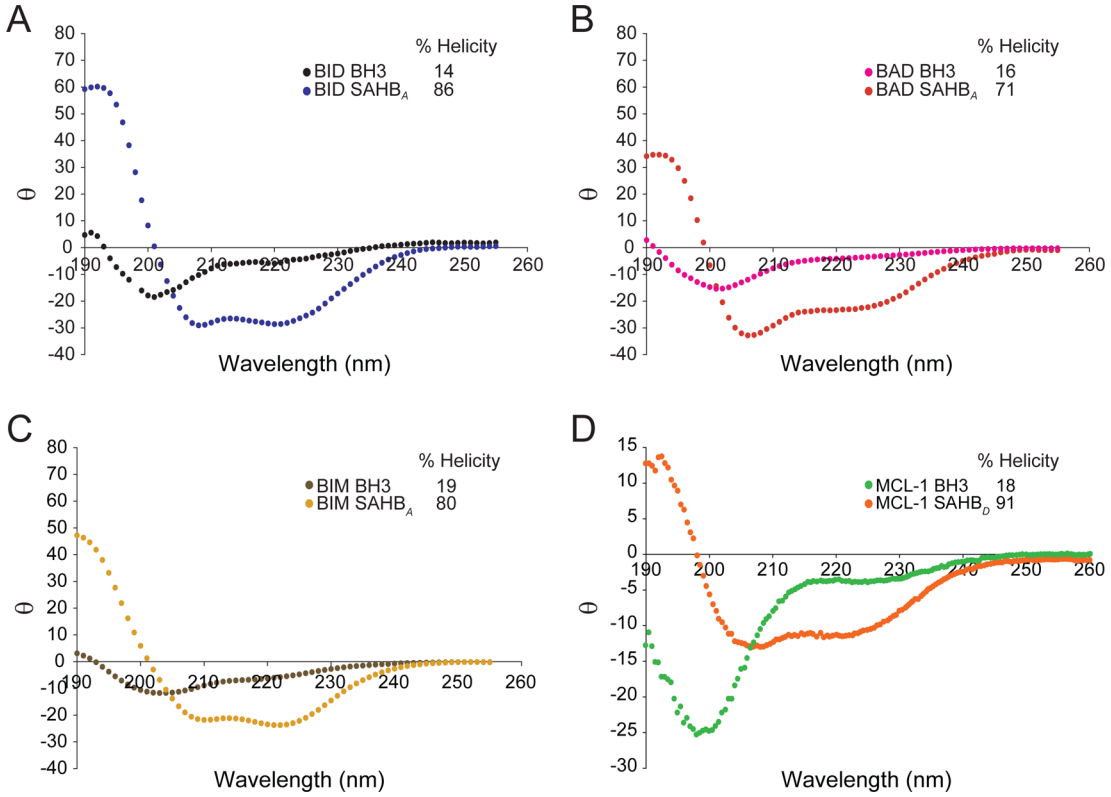

Figure 2. Enhanced α-helicity of all-hydrocarbon stapled peptides. Circular dichroism analyses of a series of BH3 peptides demonstrate that stapling can convert unfolded BID (AA 81−101) (A), BAD (aa 103−127) (B), BIM (aa 146−166) (C), and MCL-1 (208−226) (D) peptides (14−20% α-helical content) into α-helices (71−91% α-helical content) in solution (e.g., aqueous potassium phosphate, pH 7).

on the prior use of the term "staple" to reflect the organization conferred by covalent linkage of two previously independent entities, such as in stapled molecules[14] and disulfide bridge stapling,[15] and as elegantly depicted on a Blackwell and Grubbs journal cover.[16] A critically important consequence of α-helical stabilization by all-hydrocarbon stapling was the observed protease resistance,[11] a direct result of sequestering the amide bonds in the interior of the helix core and thus rendering them poor substrates for enzymatic hydrolysis.

At the same time, the late Dr. Stanley Korsmeyer, a renowned apoptosis investigator, was decoding the selective roles of BCL-2 homology 3 (BH3) domains in mediating the critical BCL-2 family protein interactions that literally regulate cellular life and death at the level of the mitochondrion. Although defined structurally as amphipathic α-helices, we found that synthetic BH3 peptides in solution were mostly unfolded but could still be categorized functionally as either inhibitors of BCL-2 family survival proteins or direct activators of BCL-2 family death proteins.[17] Dr. Korsmeyer envisioned that if these unfolded peptides could be "snapped back into shape", perhaps they could be better research tools and even become therapeutic prototypes. A postdoctoral fellow in pediatric oncology previously trained in synthetic chemistry by Dr. Edward Taylor at Princeton University and in signal transduction by Dr. Solomon Snyder at Johns Hopkins, Dr. Loren Walensky became the chemistry and biology bridge for a Korsmeyer−Verdine collaboration on generating hydrocarbon-stapled BH3 peptide helices. We found that hydrocarbon stapling reliably transformed unfolded BH3 peptides into α-helices, as measured by circular dichroism (Figure 2), and that the constructs were remarkably protease resistant in vitro and in vivo.[18] In addition, these stabilized α-helices of BCL-2 domains or SAHBs bound to their physiologic BCL-2 family targets in vitro with nanomolar affinity and via the same binding mode as unmodified peptides, as documented by NMR analysis.[18] We further observed that cancer cells treated with fluorescently labeled stapled BH3 peptides developed glowing cytoplasm, whereas the corresponding fluorescently labeled unmodified peptides showed no such effect. An exciting but unexpected result for Drs. Korsmeyer and Walensky, Dr. Verdine was less surprised: "if you were an amphipathic α-helix bearing an all-hydrocarbon staple, would you prefer to live in aqueous culture medium or head to a lipid membrane?" Indeed, the cellular uptake was dose-responsive, time-responsive, and energy-dependent and tracked with dextran-labeled pinosomes.[18] Time-dependent colocalization of FITC-SAHBs at the mitochondria correlated with induction of cell death, which was BH3 sequence-dependent in vitro and in vivo.[18] Since this original work, hydrocarbon stapling has been applied by us and independently by others to more than two dozen published peptide templates, corresponding to both extracellular and intracellular targets (Table 1). Through this body of work, we and others have encountered and overcome challenges in design, uptake, and activity and in doing so have amassed a wealth of information about how to iterate stapled peptide compositions for a host of biomedical applications. As with any new technology, we've learned that an open mind, experimental rigor, and persistence are key ingredients to making progress when applying the principles and practice of peptide stapling.

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Table 1. Applications of All-Hydrocarbon Peptide Stapling in a Diversity of Disciplines and Human Diseases, Spanning Cancer, Infectious Diseases, Metabolism, and Neuroscience

helical ligand	protein target	target site	refs
			Cancer
BID BH3	BCL-2 family proteins	intracellular	Walensky et al. *Science* 2004; *Mol. Cell* 2006; Leshchiner et al. *Proc. Natl. Acad. Sci. U.S.A.* 2013
			Moldoveanu et al. *Nat. Struct. Mol. Biol.*, 2013
BAD BH3	BCL-2 family proteins	intracellular	Walensky et al. *Mol. Cell*, 2006; Braun et al. *Chem. Biol.* 2010
BIM BH3	BCL-2 family proteins	intracellular	Walensky et al. *Mol. Cell*, 2006; Gavathiotis et al. *Nature* 2008; Gavathiotis et al. *Mol. Cell* 2010; LaBelle et al. *J. Clin. Invest.* 2012; Okamoto et al. *ACS Chem. Biol.* 2012; Braun et al. *Chem Biol.* 2010; Bird et al. *ACS Chem. Biol.* 2014
MCL-1 BH3	MCL-1	intracellular	Stewart et al. *Nat. Chem. Biol.* 2010; Joseph et al. *PLoS One* 2012
PUMA BH3	BCL-2 family proteins	intracellular	Edwards et al. *Chem. Biol.* 2013
p53	MDM2/MDMX	intracellular	Bernal et al. *J. Am. Chem. Soc.* 2007; Bautista et al. *J. Am. Chem. Soc.* 2009; Bernal et al. *Cancer Cell* 2010; Guo et al. *Chem. Biol. Drug. Des.* 2010; Joseph et al. *Cell Cycle* 2010; Baek et al. *J. Am. Chem. Soc.* 2012; Gembarska et al. *Nat. Med.* 2012; Brown et al. *ACS Chem. Biol.* 2012; Chang et al. *Proc. Natl. Acad. Sci. U.S.A.* 2013; Wei et al. *PLoS One* 2013.
mastermind	notch	intracellular	Moellering et al. *Nature* 2009
BCL9	β-catenin	intracellular	Takada et al. *Sci. Transl. Med.* 2012
axin	β-catenin	intracellular	Grossmann et al. *Proc. Natl. Acad. Sci. U.S.A.* 2012; Cui et al. *Cell Res.* 2013
p110α	IRS1	intracellular	Hao et al. *Cancer Cell* 2013
borealin	survivin	intracellular	Shi et al. *Anal. Chem.* 2013
EZH2	EED	intracellular	Kim et al. *Nat. Chem. Biol.* 2013
eIF4G	eIF4E	intracellular	Lama et al. *Sci. Rep.* 2013
			Infectious Disease
HIV-1 capsid	Gag	intracellular	Bhattacharya et al. *J. Biol. Chem.* 2008; Zhang et al. *J. Mol. Biol.* 2008; Zhang et al. *Retrovirology* 2011; Zhang et al. *Retrovirology* 2013
HIV-1 integrase	HIV-1 integrase	intracellular	Long et al. *J. Med. Chem.* 2013
GP41 HR2 domain	GP41 six-helix bundle	extracellular	Bird et al. *Proc. Natl. Acad. Sci. U.S.A.* 2010
lasioglossin III	microbial membrane	extracellular	Chapuis et al. *Amino Acids* 2012
melectin	microbial membrane	extracellular	Chapuis et al. *Amino Acids* 2012
CD81	HCV-E2	extracellular	Cui et al. *Bioorg. Med. Chem.* 2013
esculentin-2EM	microbial membrane	extracellular	Pham et al. *Bioorg. Med. Chem. Lett.* 2013
			Metabolism/Endocrine
apolipoprotein A1	ABCA1 transporter	extracellular	Sviridov et al. *Biochem. Biophys. Res. Commun.* 2011
phospho-BAD BH3	glucokinase	intracellular	Danial et al. *Nat. Med.* 2008; Szlyk et al. *Nat. Struct. Mol. Biol.* 2014
nuclear receptor coactivator peptide 2	estrogen receptor	intracellular	Phillips et al. *J. Am. Chem. Soc.* 2011
			Neurology
conantokins	NMDA receptor	extracellular	Platt et al. *J. Biol. Chem.* 2012
galanin	galanin receptor	extracellular	Green et al. *Bioorg. Med. Chem.* 2013
neuropeptide Y	neuropeptide Y receptor	extracellular	Green et al. *Bioorg. Med. Chem.* 2013

DESIGN AND SYNTHESIS

The building blocks for hydrocarbon stapling are α,α-disubstituted non-natural amino acids bearing terminal olefin tethers of varying length. For single turn stapling, we typically employ *S*-pentenylalanine at *i*, *i* + 4 positions, and for double turn stapling, we use a combination of either *R*-octenylalanine/*S*-pentenylalanine or *S*-octenylalanine/*R*-pentenylalanine at *i*, *i* + 7 positions (Figure 3A). The same pairings can be used to install more than one staple within a given peptide template (Figure 3A). There are now multiple synthetic routes to these non-natural amino acids, such as by use of an oxazinone chiral auxiliary based on the method of Williams and colleagues[19−21] or a benzylprolylaminobenzophenone (BPB) based chiral auxiliary adapted from Belokon et al.[22] and Qiu et al.[23] (Figure 3B). We have successfully applied both synthetic routes, as previously described in detail.[24,25] For the nonchemist, these building blocks are now readily available for purchase from sources in the U.S. and abroad.

In designing stapled peptide helices, the more structural information available the better. It is especially helpful to know that the intended peptide for stapling is a bona fide α-helix in its natural context. Without this natural propensity to fold, the installed olefin groups may never juxtapose sufficiently to react. This is typically self-evident based on RCM reactions that achieve complete conversion after a few hours at room temperature compared to those that are sluggish even after prolonged heating. Our early design approach was to substitute the non-natural amino acid pair(s) on the nonbinding surface of the α-helix in order to avoid disruption of the binding interface.[18] However, with increased access to the amino acid building blocks and high throughput synthetic machinery (see below), we have since adopted a more comprehensive "staple

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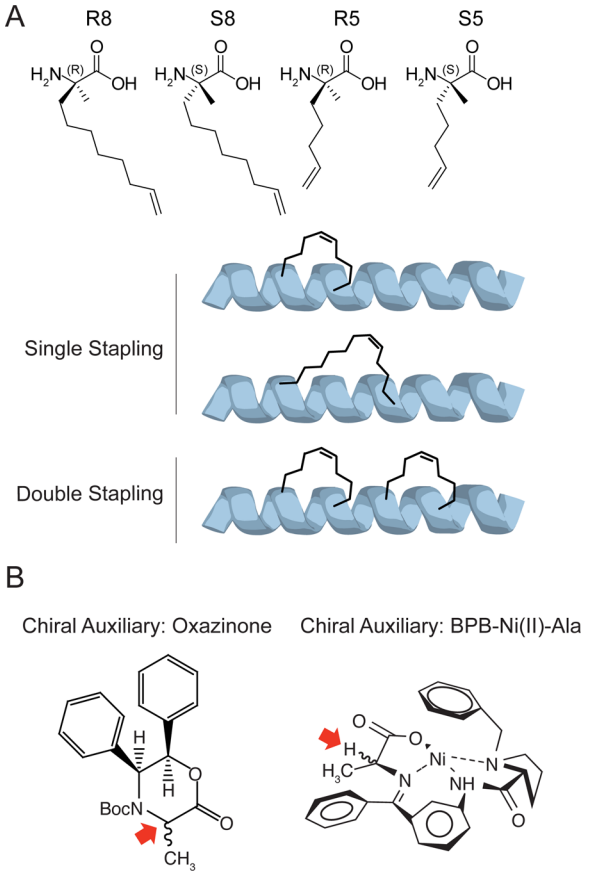

Figure 3. Building blocks of all-hydrocarbon peptide stapling. (A) A series of chiral non-natural amino acids are inserted at *i*, *i* + 4 or *i*, *i* + 7 positions and the terminal olefins cross-linked by RCM, yielding cross-links that span one or two helical turns, respectively. For example, S5–S5 pairs have been substituted at *i*, *i* + 4 positions, and S8–R5 or S5–R8 pairs have been substituted at *i*, *i* + 7 positions to generate single- or double-stapled peptides. (B) Two synthetic approaches that we have used to generate the stapling amino acids employ the oxazinone or BPB-Ni(II)-Ala chiral auxiliaries to enforce the desired stereochemistry.

scanning" approach, which essentially samples all staple positions along the length of the peptide helix. A key benefit of this strategy is the wealth of structure–activity relationship information that emerges from mutating each residue of the template and probing the various surfaces of the three-dimensional structure (Figure 4). As a result, optimally structured and biochemically efficacious constructs can be readily identified, in addition to a host of negative control compounds. However, if limited initially to a small series of constructs due to financial or synthetic constraints, structural information can guide the placement of staples away from the binding surface for positive controls and directly at the interface for negative controls.

Once designed, stapled peptides are generated using Fmoc-based peptide synthesis chemistry, as described previously.[24,25] The most frequent complication of peptide synthesis is failure to generate the full-length construct due to difficult amino acid couplings. Because the amine of the non-natural amino acid is hindered, extended deprotection and coupling times and/or double or triple couplings with fresh reagent may be required, especially after naturally bulky residues, such as arginine or β-branched amino acids (e.g., valine, isoleucine, and threonine).

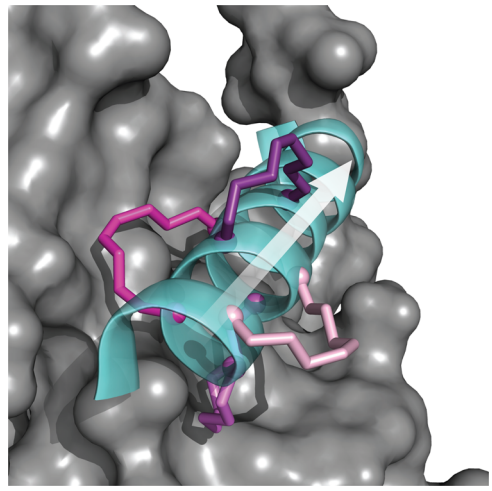

Figure 4. Generating a library of stabilized α-helices by staple scanning. Ideally, the structure of a helix-in-groove interaction can help guide the selection of staple insertion points to maximize α-helical stabilization while avoiding interference with critical, native contact points between the helix and groove. Sequential placement of staples along the entire length of the peptide sequence yields a library of constructs for structure–activity relationship analyses. We have used this staple scanning approach to identify optimal staple positions for structural stabilization, elucidate key residues and contact surfaces for ligand–target interaction, and generate negative control constructs for biological studies.

Other complications such as cross-reactions or progressive inaccessibility of the N-terminus due to on-resin aggregation can also occur. For example, Asp-Gly is the most likely amino acid pair to undergo aspartimide formation; upon repeated exposure to piperidine, the -NH- of Gly attacks the ester-protected side chain of Asp and releases *tert*-butanol to form a five-membered ring. Ring-opening by water or piperidine can yield a peptide bearing racemized Asp or a piperamide, respectively. Suppression of this unwanted reaction can be achieved by use of the commercially available side chain protected dipeptide pair, Fmoc-Asp(O^{t}Bu)-(Dmb)Gly-OH (EMD Biosciences). Progressive hindrance of the reactive N-terminus due to on-resin aggregation can also reduce synthetic yield; this occurs when the growing peptides fold as β-sheets. This complication can be avoided by lowering resin substitution, incorporating the α-helix-promoting stapling amino acids themselves, and substituting pseudoproline Ser and Thr dipeptides (EMD Biosciences) at X-Ser and X-Thr positions to produce a kink that disrupts β-sheet formation. To improve the synthetic success rate and yield of staple peptides, it is best to identify difficult sequence patterns at the outset and then modify the methodology accordingly. Peptide synthesizers that measure the fulvene deprotection product by in-line UV monitoring allow for real-time adjustments of the deprotection and subsequent coupling steps to optimize the synthetic regimen.

Originally, we synthesized stapled peptides manually using a manifold apparatus, a process that is certainly doable but time-consuming and laborious.[24,26] We and others have since employed a series of efficient peptide synthesis machines to produce large quantities of stapled peptides with excellent yields and purity, including equipment from Applied Biosystems, AAPPTec, Thuramed, CEM, and Protein Technologies. When performing automated peptide synthesis for the

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first time, we recommend optimizing the equipment and method using the unmodified template peptide first. This ensures that the standard protocol can produce the desired baseline peptide in high yield and purity before advancing to stapled peptide synthesis. Once the peptide containing the incorporated olefinic residues is complete, the RCM reaction can be performed on-resin either before or after a variety of N-terminal derivatizations (depending upon chemical compatibility) and then cleaved and deprotected using standard cleavage cocktails, as described.[24−26] In addition to N-terminal acetyl capping, we have derivatized stapled peptides with fluorophores for binding analyses and cellular imaging, biotin for affinity capture, MTSL for paramagnetic relaxation enhancement NMR, and benzophenone for photo-cross-linking and mass-spectrometry-based binding site identification (Figure 5).[18,27−29]

Stapled peptides are purified to homogeneity by HPLC/MS and then quantified. We have long preferred quantitation by amino acid analysis (AAA) because of its accuracy and consistency in assigning peptide amounts across a diversity of peptides and between lots. Because AAA can be costly and less accessible, quantitation by UV spectroscopy is also an option. However, this approach depends upon the presence of UV-active residues, which can differ significantly across a panel of distinct peptides, leading to over- or underestimating the amount of material present, which can directly impact reported EC_{50} values for biochemical and biological activity. Instead, we find that performing AAA on duplicate samples of peptide prepared at two different dilutions is the most reliable method for peptide quantitation. Aliquoted peptide is then lyophilized and stored as a powder or in 100% DMSO at −20 °C, with integrity and activity retained for years.

Stapled peptide syntheses optimized according to the above-described principles can achieve purities and yields that match the corresponding unmodified peptides. For example, a purity of 90% for the postcleavage crude material is common and can be improved to >95% by HPLC, with overall yields of 30% routinely obtained. In the absence of sequence-specific coupling challenges, unanticipated side reactions, and/or on-bead aggregation, we find that the majority of stapled peptides can be successfully generated on the first attempt.

SOLUBILITY

Because the primary goal of stapling peptides is to reinforce structure, our first characterization step is to assess secondary structure in solution by circular dichroism. However, in order to execute this and other structural analyses, soluble peptide at high micromolar concentrations is required. Therefore, it is essential to determine and optimize as necessary the solubility of stapled peptide material. Some constructs are soluble in water alone,[30] but others may need to be dissolved in 100% DMSO prior to stepwise dilution into aqueous buffers.[31] The HPLC elution profile is a useful barometer, as late-eluting, hydrophobic peptides can be more challenging to solubilize. We determine the solubility profile of newly developed stapled peptides by dissolving them in a series of aqueous buffers, varying the pH and salt concentration. Regardless of the ultimate solubilization protocol, such as dissolving the powder for experimental use in 100% aqueous or serially diluting it from a DMSO stock into aqueous buffers, it is essential to verify that the peptide is actually in solution. For example, performing a tabletop spin at maximum speed followed by inspection for the presence of a pellet can alert the user to incomplete

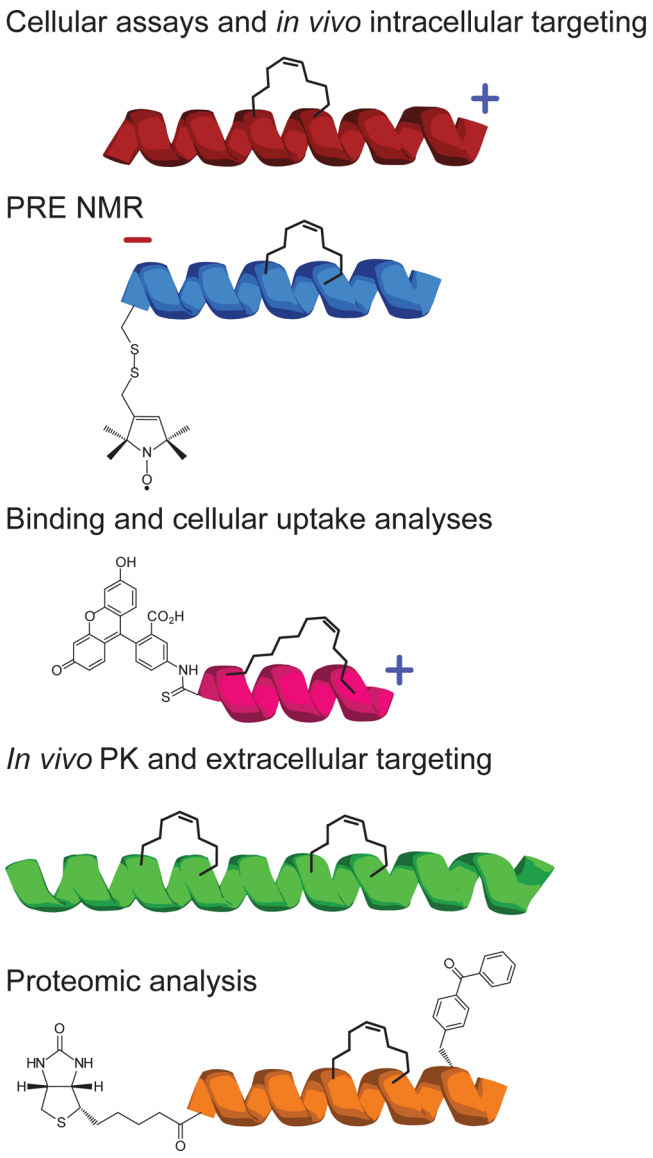

Figure 5. Design and derivatization of stapled peptides for a diversity of research applications. We have generated stapled peptides for (1) cellular studies by optimizing α-helicity and adjusting overall charge to the 0 to +2 range, (2) PRE NMR analyses by optimizing solubility with overall negative charge and appending differentially localized spin labels, (3) fluorescence polarization binding and cellular uptake analyses by N-terminal derivatization with FITC, (4) in vivo PK and extracellular targeting studies of lengthy α-helices by inserting two staples, (5) protein interaction discovery and helix binding site identification by inserting photoreactive non-natural amino acids along the length of an N-terminally biotinylated stapled peptide followed by affinity capture and mass spectrometry analysis.

solubility. If the stapled peptide is not fully dissolved in assay buffer or tissue culture medium, rigorous evaluation of its activity will be compromised. For more hydrophobic peptides, solubilization can often be achieved by iterative dilution of the DMSO stock into aqueous buffer until the goal concentration is reached. In the extreme case, insoluble peptides can be redesigned to incorporate native flanking hydrophilic or charged residues, such as Asp or Glu, to decrease overall hydrophobicity.

Once solubilized, we next turn to an assessment of the behavior of the stapled peptide in solution. Like many chemical

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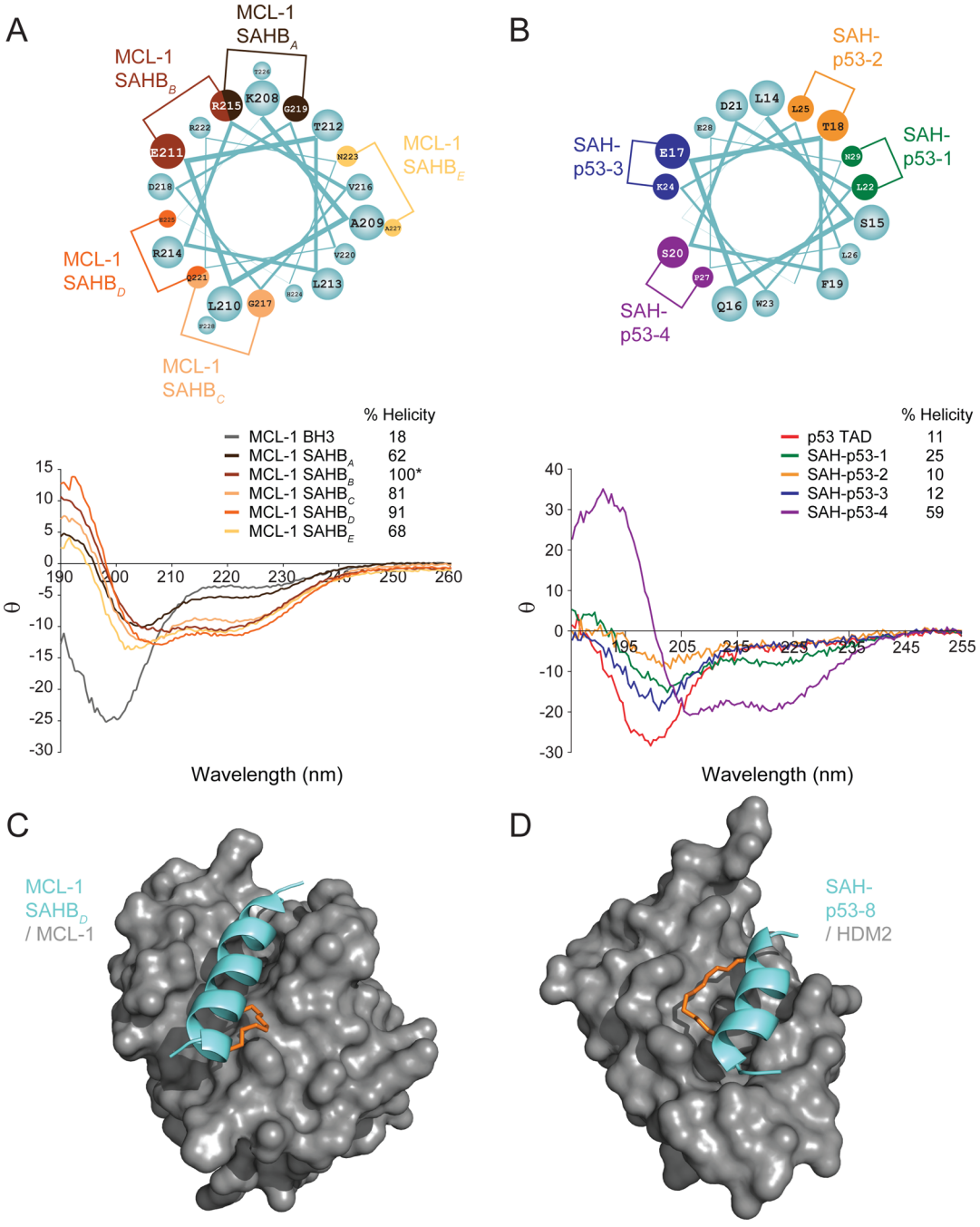

Figure 6. Structural analysis of stapled peptide helices. (A, B) Examination of a series of differentially stapled MCL-1 BH3 (aa 208−226) (A) and p53 transactivation domain (aa 14−29) (B) peptides by circular dichroism demonstrates the importance of staple position in optimizing α-helical stabilization. Whereas the majority of MCL-1 SAHB constructs manifest substantial structural stabilization compared to the unmodified MCL-1 BH3 peptide (A), only one of the sampled positions in the p53 sequence yielded a peptide with marked α-helicity. These data demonstrate that installing a hydrocarbon staple at any one location does not guarantee structural enhancement, but sampling a series of positions can typically yield a construct or a panel of constructs with the desired properties. (C, D) X-ray structures of the stapled peptide/target protein complexes MCL-1 SAHB/MCL-1 (C) and SAH-p53-8/HDM2 (D) demonstrate the reinforced α-helical structure of the peptide ligands and the capacity of the staple itself to engage the protein surface, resulting in enhancement of binding activity without compromising specificity.

compounds, peptides can aggregate depending upon the composition and concentration. Stapled peptides are typically applied in biological studies within a nanomolar to low micromolar dosing range, concentrations at which self-association is rarely observed. However, to rule out self-association, stapled peptide samples dissolved at various concentrations can be evaluated by native gel electrophoresis[32] and/or gel filtration chromatography[29] for the presence of higher order species. If aggregation is observed at a particular concentration, either the stapled peptide should be employed below this concentration in biological studies or alternative solubilization buffers explored. If all else fails, the peptide can typically be redesigned to remedy its propensity to self-associate by reducing overall hydrophobicity, as described above.

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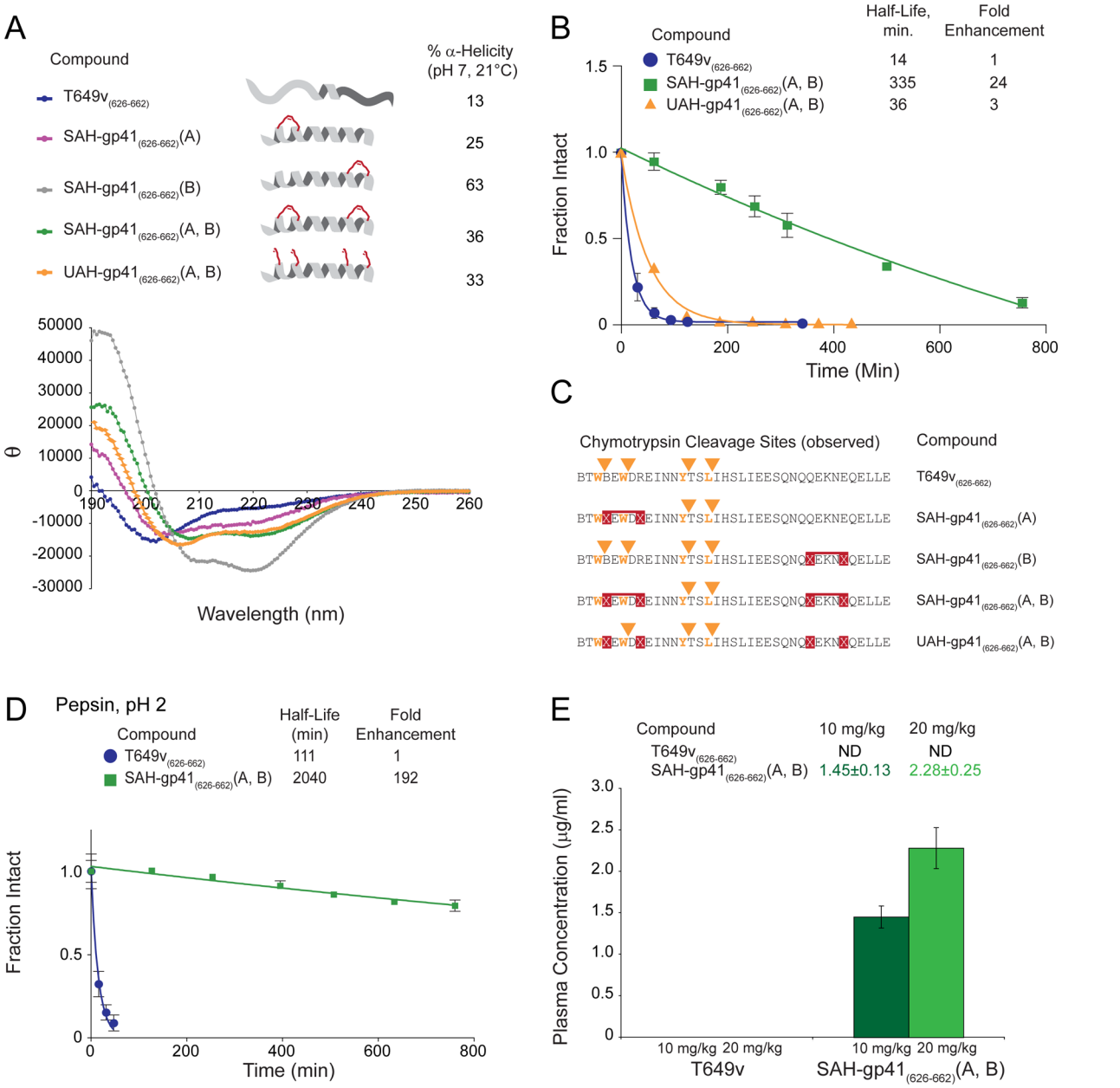

Figure 7. Protease resistance of hydrocarbon-stapled peptides. (A, B) A mechanistic analysis of peptide fortification by hydrocarbon stapling revealed that the average α-helicity in solution of a doubly stapled lengthy peptide SAH-gp41(A,B) vs the corresponding tetrasubstituted but unstapled analogue UAH-gp41(A,B) was the same (A), yet the proteolytic half-life of the doubly stapled construct was prolonged by 24-fold compared to the unmodified peptide, whereas the tetrasubstituted but unstapled analogue showed only a 3-fold difference (B). (C) Proteomic analysis of the digestion products revealed that peptide double stapling slowed the kinetics of proteolysis at sites distal to the staple and completely prevented hydrolysis at sites flanked by or immediately adjacent to the staple. Notably, the tetrasubstituted but unstapled analogue was unable to achieve this degree of protection. (D) The dramatic antiproteolysis effect of hydrocarbon double stapling was reflected by a 192-fold enhancement in half-life of SAH-gp41(A,B) compared to the corresponding unmodified peptide in the presence of pepsin at pH 2. (E) The striking in vitro proteolytic stability of SAH-gp41(A,B) at acidic pH prompted us to explore its oral bioavailability after administration to mice by oral gavage. SAH-gp41(A,B) achieved measurable and dose-dependent plasma concentrations, in fully intact form, within 30 min of oral administration, whereas the corresponding unmodified construct was not detectable (ND).

STRUCTURAL ANALYSIS

Circular dichroism provides a rapid assessment of average α-helical content of stapled peptides in solution. The relative benefit of installing a particular hydrocarbon staple is determined by comparison with the corresponding unmodified peptide. Screening a library of differentially stapled peptides often identifies the optimal staple position(s) for maximizing α-helical stabilization (Figure 6A and Figure 6B). The degree of staple-induced structural stabilization can also be comparatively assessed by NMR, as previously described.[33] It is important to note that (1) inserting a staple at any given position does not guarantee structural reinforcement[33] and (2) maximizing α-helicity does not guarantee optimal biochemical or biological activity. For example, in the case of hydrocarbon-stapled gp41 HR2 domains, constructs of intermediate helicity were optimal for biological activity, with excessive helicity from a single C-

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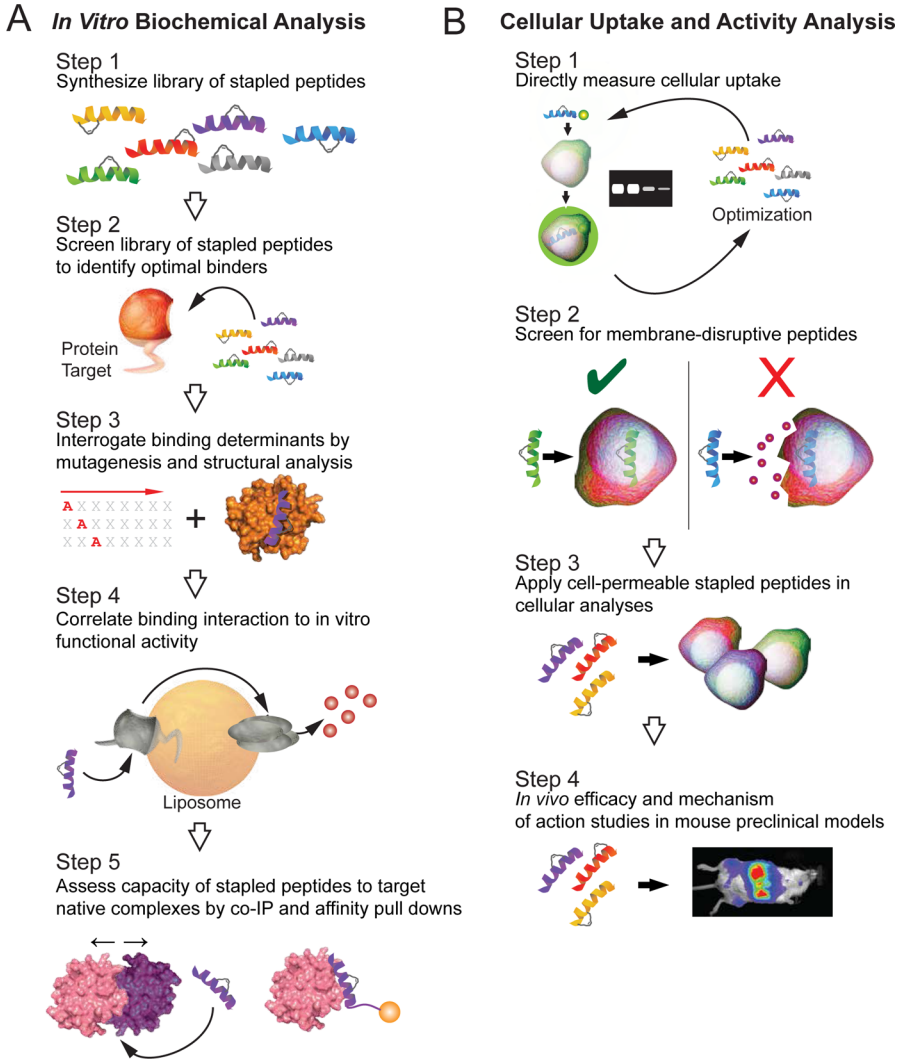

Figure 8. Deploying stapled peptides for biological investigation. Workflows for using stapled peptides in (A) in vitro biochemical, structural, and functional studies and (B) cellular and in vivo analyses.

terminal constraint actually reducing antiviral activity.[32] Ultimately, the topography and plasticity of the receptor surface will select for the optimally structured stapled peptide ligand. Thus, advancing a spectrum of structurally stabilized peptide α-helices to biochemical and biological testing is advised in order to determine which construct is best for a given target.

A variety of stapled peptides in complex with their biological targets have now been visualized by computer simulation,[34,35] NMR,[28,36−38] and X-ray crystallography.[30,39−41] In each case and as anticipated, the peptide is observed in α-helical conformation. Whereas the staple is typically oriented away from the binding interface,[34,35,37] staples inserted at the amphipathic border can engage in complementary hydrophobic interactions with the binding surface itself[30,39,41] (Figure 6C and Figure 6D). In the case of a stapled MCL-1 BH3 helix bound to antiapoptotic MCL-1, the specific residues engaged by the hydrocarbon staple are actually employed in physiologic interactions with other natural BH3 domain residues.[30] A similar phenomenon was observed for the SAH-p53-8/hDM2 interaction.[39] Importantly, the binding specificities of such staple-interacting constructs were not disturbed by these fortuitous interactions,[30,33,39,41,42] which instead enhanced affinity and showcase the opportunity to actually harness the staple for medicinal-chemistry-based optimization of target binding.

■ PROTEASE RESISTANCE

One of the most striking features of hydrocarbon-stapled peptides is their proteolytic resistance,[11,18,33] which correlates with both the degree of α-helical stabilization and the number of inserted staples.[32] To assess the relative contribution of the staple itself to the observed protease resistance, we previously compared the induced α-helicity and in vitro protease resistance of a 36 amino acid long peptide containing single staples, double staples, or substituted but not stapled non-natural amino acids. Importantly, each of the peptides had the same number of chymotrypsin sites. The most telling comparison was between the double stapled and tetrasubstituted-but-unstapled constructs, which showed similar average α-helical stabilization in solution but a 9-fold difference in half-life (Figure 7A and Figure 7B). From a mechanistic standpoint, the double staples not only slowed the kinetics of proteolytic digestion but completely eliminated cleavage of two chymotryptic sites that either localized within the protective umbrella of the staple or was immediately adjacent to it (Figure 7C). In

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an acidic environment, the double stapled peptide manifested further enhancement of α-helical structure and a 192-fold prolongation in half-life compared to the corresponding unmodified peptide (Figure 7D). Strikingly, the construct was detected in full-length form in blood withdrawn from mice 30 min after oral gavage treatment,[32] demonstrating the capacity of this doubly stapled peptide to withstand the acidic environment of the stomach and achieve intestinal absorption into the blood in full-length form (Figure 7E). Importantly, even though insertion of α,α-disubstituted non-natural amino acids without olefin metathesis can achieve α-helical induction,[32] and for some templates even cellular uptake,[33] closure of the staple is essential for developing protease-resistant constructs for in vivo application.

In addition to the remarkable difference between stapled and unstapled/unmodified peptides upon exposure to proteolytic enzymes in vitro and in vivo, we also found that stapled peptides have greater intracellular stability. We recently compared the intracellular levels of a stapled BCL-9 peptide with the corresponding unmodified version bearing a cell-penetrating TAT sequence at the N-terminus.[43] Whereas equivalent uptake kinetics was observed for the two fluorescently labeled peptides, the TAT-BCL-9 peptide exhibited time-dependent elimination and was undetectable by 12 h, while the stapled BCL-9 construct maintained similar, high level peptide throughout the time course. Taken together, these data highlight that hydrocarbon stapling can remedy a major liability of peptide therapeutics, namely, susceptibility to rapid proteolytic degradation in vivo. What's more, awareness of specific sequence vulnerabilities to extra- or intracellular proteases can guide the placement of staples and thereby potentially eliminate key sites of proteolysis.

BIOCHEMICAL TESTING

To carefully establish the structure−activity relationship for a pilot panel of stapled peptides, it is ideal to have both a direct target binding assay and an in vitro biochemical assay to link binding with functional consequences. Fluorescence polarization, surface plasmon resonance, ELISA, FRET, BRET, isothermal calorimetry, and other measures of ligand−protein interaction allow for calculation of binding constants and assessment of comparative binding efficacy for both positive and negative control stapled peptides. With lead constructs in hand, including mutant controls, optimal binders can then be advanced to functional testing. For example, we have studied the comparative binding activity of SAHBs for a series of recombinant BCL-2 family member proteins and examined their biochemical activity in modulating death channel formation in liposomal and mitochondrial release assays.[29−31] The capacity of specific, high affinity SAHBs to disrupt the corresponding protein−protein interactions, for example, can be assessed further by co-immunoprecipitation of native complexes from cellular lysates. Such biochemical assays are routinely used to vet iterative panels of stapled peptides, optimized based on sequence composition, staple composition and placement, measured α-helicity, overall charge, and compound solubility (Figure 8A). In advance of cellular application, protein targeting in cellular lysates can also be examined by stapled peptide pull-down assays that employ FITC-tagged, biotinylated, and/or photoreactive constructs, followed by protein detection by Western blotting and/or proteomic analyses.[27,31,42] In our view, in vitro biochemical testing of stapled peptides is an essential step toward their development for cellular and in vivo application. Advancing stapled peptides directly from synthesis to cellular testing is a treacherous path because without validating and optimizing constructs for specific, high affinity biochemical activity first, we believe there is little chance that cellular work will succeed. Instead, we strongly endorse a stepwise approach that starts with SAR-driven biochemical optimization of stapled peptide design, followed by direct measurement of cellular uptake and synthetic adjustment as needed to maximize penetrance, all before advancing lead constructs to cellular and in vivo analyses.

CELLULAR UPTAKE

Designing peptides for cellular delivery is one of the most exciting yet challenging frontiers of peptide therapeutics. Traditionally, unfolded and polar peptides have shown little propensity for cellular uptake except when cell penetrating tags such as TAT, antennapedia, and poly-Arg are appended. The explicit mechanisms of uptake remain active areas of investigation but appear to involve energy-dependent pinocytosis[44] and perhaps in some circumstances direct penetration.[45] We have observed cellular uptake of stapled peptides in a time-, temperature-, and ATP-dependent manner, consistent with a pinocytotic mechanism.[18] Egress from pinosomes to intracellular sites of biological activity (e.g., mitochondria, nucleus, cytosol) has been observed by live confocal microscopy performed over time;[18,29,46] the explicit mechanism(s) of pinosomal export and opportunities to facilitate this process are active areas of investigation. We have also found that, depending on their sequence, some (but not all) stapled peptides manifest reduced cellular uptake in the presence of serum. For those constructs impeded by serum, dose-responsive reduction of cellular uptake by serum has been observed, with consequent impairment in biological activity.[46] This could derive from either direct serum component binding, which we have measured for albumin,[27] or other mechanisms, such as competing with natural serum-containing substrates for pinocytosis.[47]

To eliminate the potential for variable, serum-based reduction of cellular uptake for specific constructs, we have traditionally initiated our uptake analyses using serum-free medium (e.g., Opti-MEM) or by treating cells in the absence of serum for a 1−4 h period followed by serum replacement.[18] We evaluate cellular uptake of FITC-stapled peptides by live confocal microscopy, FACS analysis of treated cells, and fluorescence scan of electrophoresed lysates from treated cells[18,29,33,46] (Figure 8B). Before analysis, the cells are washed to remove stapled peptide-containing medium. For the FACS and cell lysate evaluations, cells are also treated with trypsin to digest surface protein and thus eliminate any nonspecifically bound peptide. On the basis of our development and analysis of many series of stapled peptides, we find that their capacity for cellular penetrance depends on a combination of factors that include charge, hydrophobicity, and α-helical structure, with negatively charged and less structured constructs often requiring sequence modification to achieve cellular uptake.[24,25] For example, substituting Asn for Asp and/or Gln for Glu, or adding native or non-native charged residues at the N- or C-termini to adjust the overall charge to 0 to +2, can often enhance the cell permeability of stapled peptides. Producing constructs with greater α-helical content through differential staple placement has also been a successful intervention. Of the published stapled peptide constructs successfully applied in

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cells to date, over one-third are efficacious in the presence of serum,[40,41,43,48−52] whereas the remainder benefit from at least a serum-free window to facilitate cellular loading.[18,28−30,33,42,53−58] 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.[18,32]

It is also important to be aware that certain peptides, such as cationic antimicrobial peptides,[59] can perturb membranes as a result of their amino acid composition. Therefore, in advance of treating cells (or purified organelles) with stapled peptides, it is important to perform a maximally tolerated dose titration to screen for constructs that disrupt membranes based on composition or dose range. Monitoring cells by light microscopy (e.g., trypan blue exclusion) and performing LDH release assays immediately after treatment are facile approaches for identifying disruptive peptides (Figure 8B). Stapled peptides should only be applied at tolerated doses, and redesigned when necessary to eliminated unwanted biophysical properties, so that on-target, sequence-dependent biological activity can be achieved.

Finally, it is important to underscore that overlooking the above-described, previously reported[24,25,60] design and characterization principles can lead to misapplication of stapled peptides in cellular assays and, as a consequence, misleading conclusions. A prominent example is a recently reported collaborative Walter and Eliza Hall Institute (WEHI) and Genentech study[61] that examined the structural and biochemical features and cellular activity of a stapled peptide modeled after the death domain of the proapoptotic protein BIM. The authors analyzed our weakened-by-design construct (developed to capture a transient ligand−protein interaction by HSQC NMR)[28] that has suboptimal α-helical stabilization and overall negative charge of −2, both of which limit cellular uptake, and "unexpectedly" found that stapling BIM BH3 peptides "does not necessarily enhance affinity or biological activity".[61] Without performing any direct measure of cellular uptake, the authors further concluded from their negative results, which were predictable based on the prior literature,[18,28,29,31,62] that stapled BIM BH3 peptides are "not inherently cell permeable". Curiously, the WEHI/Genentech team chose not to focus their study on our original stapled BIM BH3 construct,[31] which manifests robust α-helicity, nanomolar binding affinity to the broad spectrum of BCL-2 family targets, and most importantly, cell permeability and sequence-specific cellular and in vivo activity[29,31] (Figure 9). In a single supplementary experiment, the authors apply the correct construct for cellular work to demonstrate lack of activity in wild-type mouse embryo fibroblasts (MEFs) and again point to a lack of cellular penetrance (without analyzing cellular uptake).[61] However, we had already reported that this potent, cell permeable analogue showed little to no activity in adherent wild-type MEFs despite actually being cell penetrant (as measured directly) but effectively activated the apoptotic pathway in resistant hematologic cancer cells, indeed predicting a potential therapeutic window for treating cancer.[29] In our view,[63] an important lesson from the WEHI/Genentech study[61] is that lack of attention to sequence composition and biophysical properties can lead to misapplication of stapled peptides and faulty conclusions (Figure 9).

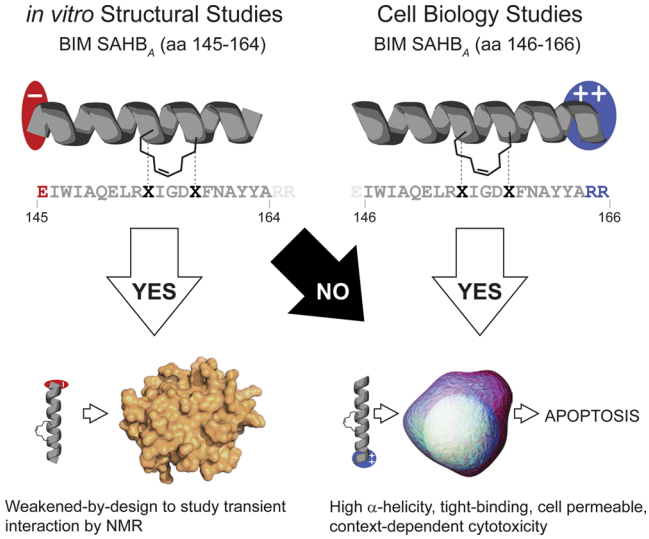

Figure 9. Stapling down the facts on BIM SAHBs. In order to accomplish a challenging NMR analysis of the hit-and-run interaction between BIM BH3 and BAX (left), we adjusted the sequence of our prototype high α-helicity, high affinity, and cell permeable BIM SAHB$_A$ (146−166) peptide[29,31] (right) to enhance its solubility and weaken (i.e., slow down) its BAX-activating capability[28] (left). Czabotar and colleagues from WEHI and Genentech[61] also successfully applied this refashioned BIM SAHB$_A$ (145−164) peptide for structural determination (left) ("Yes" arrow). However, the authors misapplied the weakened-by-design BIM SAHB$_A$ (145−164) construct in binding and cellular studies ("No" arrow) and predictably observed no cellular activity, yet broadly concluded that stapling BIM BH3 does not enhance its binding affinity or biological activity. In response to our recent correspondence,[63] Czabotar and co-authors have now tested the correct BIM peptide (right) in leukemia cells and successfully reproduced our published results.To facilitate the successful application of peptide stapling, a rigorous and detail-oriented approach is required and includes careful consideration of the sequence, biophysical, biochemical, structural, cellular uptake, and biological properties of discrete stapled peptide constructs[63] ("Yes" arrows).

■ CELLULAR AND IN VIVO ACTIVITY

With potent and cell permeable stapled peptide constructs in hand, a broad spectrum of cellular and in vivo studies are achievable, with exemplary studies spanning the fields of cancer, infectious disease, metabolism, and neuroscience (Table 1). Stapled peptides modeled after the BH3 domains of BID and BIM have been applied in cellular and in vivo studies to document therapeutic reactivation of the apoptotic pathway in preclinical mouse models of human leukemia.[18,29] The discovery that the stapled BH3 domain of antiapoptotic MCL-1 is an exquisitely selective inhibitor of MCL-1 revealed the utility of this agent in sensitizing apoptotic responses to proapoptotic treatments that are otherwise thwarted by MCL-1 expression.[30] Stapled p53 peptides have been designed to target the p53 antagonists HDM2 and HDMX, reactivating the p53 tumor suppressor pathway in cells and in vivo.[33,42] Next-generation analogues have recently been developed that are more effective in the presence of serum[41,48] and are showing promising activity in mouse models of solid tumors.[41] Pathologic β-catenin signaling in cancer has been targeted by stapled peptides modeled after the β-catenin-interacting domains of BCL-9[43] and axin.[40] Stapled peptides corresponding to a mutant interaction domain of p110α have been

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deployed to disrupt the oncogenic IRS1-p110α E545K interaction and thereby inhibit tumor growth in a mouse xenograft model of human colorectal carcinoma.[50] Most recently, EZH2-based stapled peptides have been shown to selectively inhibit histone-3 Lys27 trimethylation by disrupting the EZH2-EED complex, effectively suppressing PRC2-dependent cancer cell growth by targeting this epigenetic "writer".[52] In all cases, the stapled peptide-based modulation of signal transduction and induction of cell death (or, for EZH2, growth arrest and induced differentiation) was shown to be exquisitely dependent on the bioactive peptide sequence.

Outside the cancer field, stapled peptides have been used to inhibit HIV-1 infection by extracellular targeting of the gp41 fusion apparatus[32] and intracellular inhibition of capsid particle assembly[37,57,58] and viral DNA integration.[64] Proof-of-concept for effective extracellular receptor targeting by stapled peptides has been reported for the ABCA1 transporter,[65] NMDA receptor,[66] galanin receptor,[67] neuropeptide Y receptor,[67] and the HCV envelope glycoprotein 2.[68] Strikingly, a stapled peptide version of the PKA-phosphorylated BAD BH3 helix selectively targets glucokinase in pancreatic β-cells and restores glucose-stimulated insulin secretion in BAD-deficient islets, suggesting a therapeutic application in diabetes.[49,69] Indeed, the growing diversity of cellular and in vivo studies published by our group and independently by others showcases the potential broad impact of stapled peptides in dissecting and targeting extra- and intracellular proteins for therapeutic benefit (Figure 10).

CLINICAL TRANSLATION

In the context of established drug modalities such as small molecules and antibodies, the stapled peptide technology is relatively new. Since the first all-hydrocarbon staple publication by Verdine and colleagues in 2000, remarkable progress has been made in a relatively short period of time and the number of reported studies employing stapled peptides is accelerating (Figure 10A). Synthetic protocols have been optimized and advanced to GMP production scale. Increased accessibility to the stapling amino acids and high throughput peptide synthesis equipment has enabled many laboratories in the U.S. and abroad to harness the stapled peptide technology for their individual research needs. The structures of stapled peptides in complex with their targets are being solved[30,37−41] and are revealing novel modes of interaction,[28,38,69] binding and specificity determinants,[30] and opportunities for affinity optimization. The hard work of stapled peptide iteration and optimization for a host of research and therapeutic needs is bearing fruit across disciplines (Table 1). The design principles for enhancing cellular uptake are being refined, and the explicit mechanisms of cellular uptake, and how they can be harnessed to maximize stapled peptide import, are subjects of intense investigation. The dual goals of the biochemical, cellular, and in vivo work are to advance our basic understanding of signaling pathways, both homeostatic and pathologic, and unleash the therapeutic potential of peptides for treating human disease (Figure 10B). With the first clinical trial of a stapled peptide in man successfully completed by Aileron Therapeutics in 2013 and additional trials already being planned, the early indications regarding the clinical translational potential of stapled peptides are now forthcoming. For example, ALRN-5281, a long-acting growth hormone releasing hormone (GHRH) agonist indicated for metabolic/endocrine disorders, showed no serious adverse events, dose-limiting safety findings, or tolerability issues

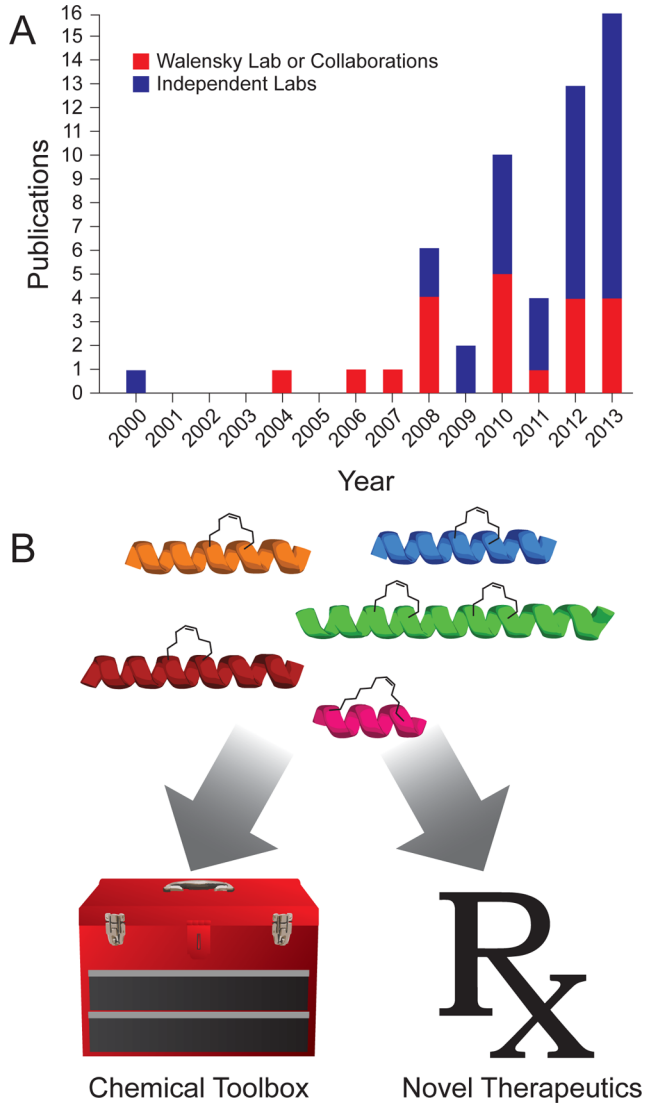

Figure 10. Growth of stapled peptide applications for biomedical discovery and drug development. (A) Since the original reports of the all-hydrocarbon cross-linked peptide helix[11] and its proof-of-concept utility for signal transduction research, cellular delivery, and therapeutic targeting,[18] there has been increased accessibility to and successful application of the technology by us and many other laboratories, as indicated by the growing number of yearly stapled peptide publications. (B) Stapled peptides serve as versatile probes for protein interaction research and as prototype therapeutics for modulating extracellular and intracellular protein targets.

leading to study withdrawal in a phase I study of 32 subjects. As with any new technology, much remains to be learned, speed bumps are inevitable, and enthusiasts and naysayers abound. Nevertheless, a focused, persistent, rigorous, stepwise, and open-minded approach will ultimately provide the greatest chance for stapled peptides to realize their full potential.

AUTHOR INFORMATION

Corresponding Authors

*L.D.W.: (address) Dana-Farber Cancer Institute, 450 Brookline Avenue, Mayer 664, Boston, MA 02215; (phone, office) 617-632-6307; (fax) 617-582-8240; (e-mail) loren_walensky@dfci.harvard.edu.

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*G.H.B.: (address) Dana-Farber Cancer Institute, 1 Jimmy Fund Way, Smith 758, Boston, MA 02215; (phone, office) 617-582-8517; (e-mail) greg_bird@dfci.harvard.edu.

Notes

The authors declare the following competing financial interest(s): L.D.W. is a scientific advisory board member and consultant for Aileron Therapeutics.

Biographies

Loren D. Walensky, M.D., Ph.D., is an Associate Professor of Pediatrics at Harvard Medical School, Principal Investigator and Pediatric Oncology Physician at Dana-Farber Cancer Institute/Children's Hospital Boston, Medical Director of Dana-Farber's Linde Program in Cancer Chemical Biology, and Director of the Harvard/MIT M.D.−Ph.D. Program. He graduated as valedictorian from Princeton University with a B.A. in Chemistry in 1990 and received his M.D., Ph.D. degrees in 1997 from Johns Hopkins, where he trained with Solomon H. Snyder, M.D. Dr. Walensky completed Pediatrics Residency and Hematology/Oncology Fellowship at Harvard, where his postdoctoral research was mentored by Gregory L. Verdine, Ph.D., and the late Stanley J. Korsmeyer, M.D. The Walensky laboratory develops novel chemical approaches to dissect and target pathologic protein interactions in cancer and other human diseases.

Gregory Bird, Ph.D., is a Senior Scientist in the Walensky laboratory at the Dana-Farber Cancer Institute. He received his B.S. and Ph.D. degrees in Chemistry from the University of Pittsburgh in 1999 and 2006, respectively. Dr. Bird's graduate studies were mentored by Chris Schafmeister, Ph.D., and focused on the development of a universal molecular scaffold to facilitate the design, construction, and analysis of macromolecules that orient functionality in three-dimensional space. He joined the Walensky laboratory as a Postdoctoral Fellow in 2006 and was promoted to Senior Scientist in 2012. Dr. Bird has advanced the peptide stapling technology to a diversity of in vivo applications in cancer, infectious disease, and metabolism.

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■ ABBREVIATIONS USED

ABCA1, ATP-binding cassette member 1; BAD, Bcl-2-associated death promoter; BAX, Bcl-2-associated X protein; BCL-2, B-cell lymphoma 2; BCL-9, B-cell CLL/lymphoma 9; BH3, BCL-2 homology 3; BID, BH3 interacting-domain death agonist; BIM, Bcl-2 interacting mediator of cell death; BPB, benzylprolylaminobenzophenone; BRET, bioluminescence resonance energy transfer; EED, embryonic ectoderm development; EZH2, enhancer of zeste homologue 2; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GMP, good manufacturing practice; hDM2, human homologue of mouse double minute 2; HR2, heptad repeat 2; IRS1, insulin receptor substrate 1; LDH, lactate dehydrogenase; MCL-1, myeloid cell leukemia sequence 1; MEF, mouse embryonic fibroblast; MEM, Eagle's minimum essential medium; MTSL, 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonothioate; PRC2, polycomb repressive complex 2; PRE, paramagnetic relaxation enhancement; SAH, stabilized α-helix; SAHB, stabilized α-helices of BCL-2 domains; TAT, trans-activator of transcription

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