Published in final edited form as:

J Am Chem Soc. 2020 January 29; 142(4): 1882–1894. doi:10.1021/jacs.9b10716.

Directed Evolution Using Stabilized Bacterial Peptide Display

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Abstract

Chemically stabilized peptides have attracted intense interest by academics and pharmaceutical companies due to their potential to hit currently ‘undruggable’ targets. However, engineering an optimal sequence, stabilizing linker location, and physicochemical properties is a slow and arduous process. By pairing non-natural amino acid incorporation and cell surface click chemistry in bacteria with high-throughput sorting, we developed a method to quantitatively select high affinity ligands and applied the SPEED (Stabilized Peptide Evolution by *E. coli* Display) technique to develop disrupters of the therapeutically relevant MDM2-p53 interface. Through *in situ* stabilization on the bacterial surface, we demonstrate rapid isolation of stabilized peptides with improved affinity and novel structures. Several peptides evolved a second loop including one sequence ($K_d = 1.8$ nM) containing an *i, i+4* disulfide bond. NMR structural determination indicated a bent helix in solution and bound to MDM2. The bicyclic peptide had improved protease stability, and we demonstrated that protease resistance could be measured both on the bacterial surface and in solution, enabling the method to test and/or screen for additional drug-like properties critical for biologically active compounds.

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

A list of primers used, mass spectrometry data, NMR chemical shifts, additional sequence data, CD plots, BLI plots, additional bulk library labeling data, exendin display flow cytometry data, and surface reaction modeling discussion is located in the supporting information file. The Supporting Information is available free of charge on the ACS Publications website.

Keywords

directed evolution; stapled peptides; non-natural amino acid incorporation; bacterial surface display; bio-orthogonal chemistry; molecular engineering

Introduction

Estimates based on the human genome indicate that approximately two-thirds of all disease-associated genes are 'undruggable' by current therapeutics¹. They reside inside cells, out of the reach of biologics, but lack a small hydrophobic binding pocket necessary for small molecule therapeutics^{2,3}. This has led researchers to push the limits of intracellular therapeutics to larger sizes, capable of binding and disrupting intracellular protein function. Peptides, by mimicking protein binding epitopes, are an attractive option for target binding, but small linear peptides typically suffer from several liabilities including rapid proteolysis and low binding affinity. Covalent modification of peptides, however, can endow these agents with drug-like properties, such as oral delivery, high affinity, and increased stability⁴⁻⁶.

One approach to improving drug properties involves introducing a covalent linker between two amino acids spanning 1 or 2 turns of the helix, which can improve affinity, *in vivo* stability, and cellular uptake⁷⁻¹⁰. A vast and diverse literature exists on chemical methods to modify peptides and peptide libraries to connect these residues, including disulfide and amide bond formation, olefin metathesis, azide-alkyne cycloaddition, aryl-cysteine bond formation, and cysteine Michael addition, among others¹¹⁻¹⁸. These approaches are nicely reviewed in Derda and Jafari 2018¹⁹. Some of the pioneering work was done by Roberts and colleagues using mRNA display²⁰ and demonstrated the power of stabilized libraries, and these technologies have been advanced to screen for peptides with multiple modifications²¹, bicyclic peptides¹⁷, and specific secondary structures such as alpha helices²². These stabilized alpha helices in particular have multiple applications including molecular imaging and intracellular therapeutics to disrupt protein-protein interactions²³. For example, the p53-MDM2 complex involves a helix on p53 interacting with a groove on MDM2. This therapeutically important interaction causes downregulation of the central tumor suppressor protein p53. Due to the high frequency of dysregulation in many cancer types²⁴, there is a strong and multi-decade effort underway to develop better therapeutic inhibitors of this interaction. By disrupting the complex, p53 can no longer be downregulated by MDM2, increasing its tumor suppressor activity. Challenges remain for these and other stabilized peptides in this nascent field, with improvements to affinity, for instance, being sensitive to

the positioning of the two modified amino acids and the linker identity. Improper peptide design can result in abrogation of binding²⁵. Manual optimization of the staple position and flanking residues requires time-consuming solid phase peptide synthesis and affinity measurements, which results in low sampling of the available design space (typically > 10¹² peptide sequences) and a detrimental impact on peptide development.

The inherently vast chemical and sequence spaces have led to the development of multiple high-throughput techniques for stabilized peptide discovery. For example, DNA barcoded chemical-peptide libraries with reactive cysteine bridging have been developed to identify molecules that bind in the sub- and low nanomolar dissociation constant (K_d) range. However, relatively small library sizes are typically achieved with DNA barcoded and one-bead-one-compound libraries. In contrast, phage display allows for much larger library sizes²⁶. Phage display can suffer from difficulties in achieving the highly efficient non-natural amino acid incorporation that is required for certain chemistries²⁷. The phage display selection process of panning also does not yield precise values of affinity or stability, measurements that are accessible via cell surface display techniques. Phage therefore requires separate solid phase peptide synthesis and affinity quantification for validation, similar to the staple optimization challenges described above. However, due to concerns over undesirable reactivity with existing ubiquitous amino acid residues (e.g. cysteine and lysine) involved in alkylation or amide bond forming stabilization techniques, these chemistries are not suitable for selective reaction on the surface of cells. Therefore, a bio-orthogonal stabilization chemistry (i.e. one which doesn't react with common biological functional groups) was chosen for on-cell peptide stabilization and directed evolution.

The demand for next-generation peptide screening approaches led us to develop bio-orthogonal Stabilized Peptide Evolution by E. coli Display (SPEED), an approach involving display of stabilized peptides on the surface of methionine auxotrophic *E. coli*. *E. coli* can display a large number of peptides on the cell surface, enabling quantitative on-cell screening (e.g. by fluorescence activated cell sorting). Using bio-orthogonal chemistry, directed evolution can be performed with the stabilizing linker in place, which often makes important target contacts in addition to stabilizing the structure^{9,28}. Tirrell and coworkers demonstrated robust and highly efficient substitution of methionine by azidohomoalanine (AHA) in metE knockout *E. coli*²⁹, and several groups, including our own, have demonstrated the utility of incorporating multiple azides for cross-linking and helix stabilization^{12,13,15}. By modifying the bacterial display eCPX scaffold³⁰, we conducted directed evolution of a cell-surface stabilized MDM2-binding peptide library of > 10⁸ mutants. Using a randomization scheme that fixed the position of the cross-linking side chains but permitted the incorporation of natural amino acids including cysteine residues, we selected a novel bicyclic sequence with high affinity (1 – 2 orders of magnitude higher binding affinity than Nutlin 3a, a potent small molecular inhibitor of the intracellular p53-MDM2 interaction) with increased protease stability.

Results

In Situ Stabilization and Directed Evolution Using Chemical Biology

Due to the complex milieu of biomolecules on the surface of bacteria compared to phage, we selected a bio-orthogonal chemistry for in situ conjugation and peptide stabilization with a linker. The use of two identical non-natural amino acids in the peptide sequence requires an efficient and robust system for incorporation in large libraries (due to the dependence on the square of the incorporation efficiency). The residue replacement strategy of azidohomoalanine for methionine in bacterial auxotrophs satisfies all these criteria as >95% incorporation^{31,32} is achievable. Once displayed, the peptide was stabilized with a bis-alkyne linker using copper(I)-catalyzed azide-alkyne cycloaddition (Scheme 1A). After surface reaction, the bacteria were incubated with the target protein (scheme 1B) to enable selection of binders in the presence of the stabilizing cross-linker on a library of $> 10^8$ compounds. The cell-surface screen also allowed rapid monitoring of affinity maturation without the need for sequencing, peptide synthesis, and solution phase binding measurements³³. The use of a strong promoter (T5) and removal of methionine residues in the eCPX protein resulted in surface expression of $\sim 10^4$ azide-containing peptides per cell as quantified by fluorescent beads (Figure 1A) that could be specifically reacted (Figure 1B), highlighting the utility of a bio-orthogonal reaction. By titrating the linker concentration to balance the surface reaction yield versus doubly reacted (bis-adduct) peptide (Figure 1C), we were able to achieve an estimated 75% stabilization on the surface (Figure 1D). The extent of stabilization was calculated based on measurements detailed in the methods and supplementary file since there is no mass change upon the 2nd cycloaddition reaction (precluding mass spectrometry analysis).

Library Design and Selection

p53-derived peptides provided an excellent model system and medically relevant target to develop a cell surface display screening method for stabilized alpha helices. In the native p53 – MDM2 interaction, residues F19, W23, and L26 are involved in key hydrophobic contacts³⁴ and mutagenesis studies have shown the importance of these for binding³⁵. In fact, mutating either the phenylalanine or tryptophan to alanine (F19A or W23A) resulted in no detectable binding on the bacterial cell surface (Figure S1B). The individual contributions and interactions of and between other residues is less understood, and the formation of helix-stabilizing salt bridges and hydrogen bonding can influence binding^{10,36}. In the context of chemical stabilization, we sought to investigate the roles of the less critical residues in native-like p53 interacting sequence (ETFXDLWRLXEN)^{12,37} by creating a library with NNC codons in the place of these amino acids while keeping the highly conserved F19, W23, L26, and azidohomoalanine positions 20 and 27 fixed (Figure 2A). The library generated had a size of 3×10^8 transformants.

One round of magnetic-activated cell sorting (MACS) and serial rounds of fluorescence-activated cell sorting (FACS, up to seven) on the stabilized peptide library were conducted to select for improved affinity to MDM2 as assessed with the bacterial surface-displayed stabilized peptides (Figure 2B,C). After each round of selection, we isolated the plasmids and directly transformed these into fresh cells (due to low viability after surface reaction) for

sequencing or additional rounds of selection. Several positions had a high degree of residue conservation during selection, especially Y22 and D25 (Figure 2B, S2). After round 3, the apparent binding affinity for MDM2 by stabilized peptides displayed on the bacteria surface was measured for several clones to verify the method was selecting higher affinity sequences. We measured the affinity of two clones, SPD-M3-G1 and SPD-M3-V1, and found apparent K_d values of 2.0 and 9.8 nM respectively on the bacterial cell surface, which appeared promising (Table 1, Figure 2C). Bulk-library labeling by MDM2 showed consistent improvement in signal over sorts (Figures 2A, S3). We also characterized several clones from very early in the sorting campaign (post-MACS and post-two rounds of FACS) to demonstrate significant affinity improvements (Figure S4). After seven rounds of sorting, we found that all clones measured show significantly improved apparent affinity over SPD-M0-E(-2) on the bacterial cell surface (Figure S5).

To confirm these affinities corresponded to higher binding when the peptide is in solution and validate the technique, we synthesized several sequences by solid-phase peptide synthesis (>95% purity, Table S2) and measured solution phase peptide binding by bio-layer interferometry (BLI) with immobilized MDM2. Selected clones were identified for solution phase characterization and comparison with bacterial surface displayed apparent binding affinity values (Table 1). Sequences were named according to the convention SPD (stabilized peptide display)-M (sort round)-first amino acid and unique number. These included the original p53-like peptide sequence (-2 formal charge, SPD-M0-E(-2)) and two site-directed charge mutants (E17Q mutant with a -1 formal charge, SPD-M0-E(-1), and E17Q and E28Q double mutant SPD-M0-E(0)) to evaluate the ability to characterize charge mutants given the importance for intracellular access^{8,9}. SPD-M3-G1 was chosen as a higher affinity mutant from early in the screening process and SPD-M3-V1 due to the large increase in apparent binding affinity upon stabilization on the bacterial surface. Sequencing of 40 clones from round 7 of FACS showed enrichment of the SPD-M6-V1 sequence (13 of 40 clones, Figure S5), which contained a pair of *i, i+4* cysteine residues. Mutagenesis of either cysteine residue to serine resulted in a greater than two-fold loss in binding (Figure 2D), indicating a direct role for the pair and likely disulfide bond formation. While this was within our design space, the evolution of an *i, i+4* cysteine-containing sequence was unexpected from a topological perspective. Mass spectrometry and NMR confirmed disulfide bond formation for the synthesized peptide (Tables S2 and S3). The F19A mutant of the original starting sequence (SPD-M0-E(-2)) was included as a negative control (SPD-M0-E(-2)-F19A).

Development of peptide binders using bacterial surface display methods has sometimes led to a disconnect between the affinity of loop-insertional fusions versus solution phase binding³⁸. To test the relationship between cell surface affinity measurements and solution phase binding, the affinity (dissociation constant) of stabilized and non-stabilized alpha helices was measured both on-cell (by bacterial surface display, BSD) and in solution using BLI (Table 1 and Figure S6). We found that all 7 peptides for which we determined affinities exhibited improvements in binding in solution upon stabilization with propargyl ether, ranging from 4-fold for SPD-M6-V1 to 9.4-fold for SPD-M3-V1 (Table 1 and Figure S6). The differences in apparent affinity upon stabilization for the bacterial surface displayed peptides were more modest. The stabilized peptide affinity on the bacteria surface was

similar to the stabilized peptide affinity in solution as expected, but the non-stabilized form on-surface also had similar affinity as the stabilized forms for most peptides (Table 1). Binding affinity measurements and selection on the bacterial cell surface were performed using fluorescently tagged MDM2-GST because of the spurious loss of binding for some clones when incubated with smaller (truncated) MDM2-fluorescent dye conjugates. These same clones bound biotinylated MDM2 truncate (Figure S4A) and fluorescent MDM2-GST fusion protein (Table 1) with high affinity, indicating the dye size/charge on the smaller truncate was likely mediating the effect. All BLI measurements used biotinylated MDM2 truncate. Avidity is a concern for accurate measurement of affinities on a crowded surface like the *E. coli* outer membrane. To verify the GST tag did not induce confounding effects from dimerization and impact affinity measurements, we measured K_d values with biotinylated MDM2 truncate (e.g. SPD-M6-V1 had an apparent K_d of 0.4 nM, figure S4A). As it did not appear that avidity effects were playing a strong role in explaining the closer measured affinities of stabilized and non-stabilized peptides on the bacterial cell surface, we hypothesize that the secondary structure of the non-stabilized peptides may be more helical on the surface, potentially from molecular crowding effects from the membrane or lipopolysaccharide coating³⁹. Molecular crowding can increase peptide helicity, but even weak intermolecular interactions with the crowding agents can dramatically reduce the effect^{40,41}, which could explain the sequence dependence of the phenomenon (Table 1). Negative control peptides SPD-M0-E(-2)-F19A and SPD-M0-E(-2)-W23A did not exhibit measurable binding as displayed on the bacterial cell surface (Figure S1). Secondary structure (helicity) as examined by circular dichroism (CD) did not seem to correlate strongly with binding (Table 1, Figure S7), indicating a more complex relationship of entropic and enthalpic effects than simply high helix pre-organization resulting in higher binding affinity^{42,43}.

Protease stability

For efficacy *in vivo*, peptides must survive protease exposure in a variety of biological milieus such as in the bloodstream and at the site of administration (i.e. for subcutaneously administered drugs⁷ or for oral delivery⁴⁴). We assessed the stability of several peptides on the bacterial surface and in solution using chymotrypsin to determine if cell surface display would enable measurement or screening for protease stability. The three stabilized peptide sequences tested, SPD-M3-G1, SPD-M0-E(-2), and SPD-M6-V1 showed the same trends on the bacterial surface (Figure 3A) as in solution (Figure 3B). Likewise, the non-stabilized peptides were much less protease-resistant on the surface (Figure 3C) and in solution (Figure 3D) as expected. Note that the time, temperature, and enzyme concentrations were optimized separately (surface versus solution) to ensure a good dynamic range for each. The disulfide loop in the SPD-M6-V1 likely contributes to its overall higher stability properties.

Structure determination

The directed evolution of a disulfide bond was surprising, since disulfide bonds generally destabilize alpha helices as seen by the low helicity signature (4%) from CD spectroscopy for SPD-M6-V1, non-stabilized (Table 1 and Figure S7). Therefore, we calculated the structure by solution NMR to investigate the mechanism(s) that gave this sequence a selective advantage. Homonuclear (¹H-¹H) NOESY (nuclear Overhauser effect

spectroscopy) and TOCSY (total correlation spectroscopy) experiments were conducted to determine the free solution structure of the SPD-M6-V1 peptide (Figure 4, S8). The observed peaks implied a predominantly alpha-helical conformation for the stabilized structure with an interesting bend conferred by the bis-triazole linker (Figure 4A, Figure S7C). ^1H NMR spectrum indicated no significant structural changes in the peptide upon binding to MDM2, implying that the molecule is highly ordered in solution, owing to the stabilization conferred by both the overlapping *i, i+4* disulfide bond and the *i, i+7* bis-triazole linker (Figure 4B,C).

To understand the interaction of SPD-M6-V1 with MDM2, one-dimensional saturation transfer difference (STD) NMR experiments were performed to identify peptide atoms interacting with the MDM2 binding interface. These data indicate close interactions between the target and Val16, Cys17, and several aromatic residues. There is also an interaction between the bis-triazole linker and MDM2 (Figure 4D). This interaction highlights a potential enthalpic contribution from the linker in addition to the entropic benefit of pre-organization, another advantage of screening in the context of the linker. The linker-target interaction is consistent with crystallographic structures reported for other stabilized peptide binders of MDM2²⁸ and other proteins⁹.

Computational docking using Autodock Vina software was consistent with the STD data. In the calculated docking poses (Figure 4B), the three key hydrophobic residues F19, W23, and L26 are oriented towards the binding pocket as expected from the canonical p53-MDM2 interaction. The highly conserved Y22 residue appears to lie in a shallow notch defined by residues 93-96 of MDM2 and may be involved in π -CH interactions with K94. D17, which was observed in 100% of *i, i+4* binders (Figure 2B) may play a key role in enforcing binding conformation, together with the disulfide bond.

Discussion

Small, helical peptides are under intense investigation for applications in molecular imaging¹⁵ and therapeutics^{4,10,26} due to their unique physicochemical properties and the possibility of hitting currently ‘undruggable’ targets. Specifically, their potential to overcome limitations of large biologics by entering cells and ability to target intracellular proteins lacking small molecule binding pockets would dramatically increase the number of available drug targets in the human genome. Antibody-based biologics excel at targeting proteins and have achieved major clinical success in oncology, immune system disorders, and other diseases but are limited to extracellular targets owing to their large size. Small molecule drugs can readily access cytosolic targets. However, intracellular protein-protein interactions involve large surface areas and typically lack small hydrophobic binding pockets, making a small molecule approach intractable for many cases^{1,3}. These physical factors can also result in low potency and off target effects by small molecules, as they lack the physicochemical properties to maintain high affinity and high selectivity. Helical peptides lie at the intersection of these two fields, where their size is small enough for potential cytosolic access but large enough to specifically disrupt protein-protein interactions prevalent in cell signaling to enable targeting of new pathways in disease. Intracellular

delivery is still a major hurdle, and high affinity is critical to efficacious targeting since intracellular peptide concentrations are likely to be low.

The complexity of protein-protein and protein-solvent interactions, particularly with dynamic binding interfaces, makes computational and rational design of high affinity binders challenging. Indeed, the design of stabilized helical peptides has largely been limited to targeting interactions with a solved X-ray crystal structure where an inhibitor can be rationally obtained^{45–48}, prompting us to develop a directed evolution platform for stabilized peptide engineering of novel drug leads on the cell surface, with several advantages over current techniques.

As ‘guardian of the genome,’ the critical p53 tumor suppressor protein is often disrupted in cancer, including through downregulation by the ubiquitin ligase MDM2²⁴. Recent peptide efforts toward targeting the p53-MDM2 interaction have centered around hydrocarbon stapling, a promising approach with one candidate ALRN-6924 currently in phase II clinical trials⁵. Stapling is thought to confer improvements in binding from locking one or more turns of a peptide, resulting in helical preorganization. However, current technologies for engineering these structures have several drawbacks including limited library size, multi-step characterization, or sequential sequence/linker optimization that can miss synergistic interactions. Given the importance of the therapeutic target and knowledge base around this protein interface^{10,34,35}, we used the p53-like peptide as a model system for engineering high affinity alpha helices using in situ stabilization.

In this approach, we applied non-natural amino acid incorporation and bio-orthogonal chemistry for in situ stabilization of surface displayed peptides followed by directed evolution using *E. coli* to engineer high affinity molecules (Scheme 1). Bacterial display allows rapid identification and decoding of high affinity binders through surface-enabled affinity measurements (in contrast to phage display, mRNA display, and bead-based libraries) and high throughput screening, enabled by larger libraries than typically generated with peptide arrays, one-bead-one-compound, yeast surface and mammalian surface display. Furthermore, we could characterize binding of several thousand clones by flow cytometry after every round of sorting (Figure 2A), allowing us to monitor selection progress in a straightforward manner. Engineering the display and stabilization reaction on the surface of bacteria using chemical biology techniques (Figure 1) resulted in the selection of high affinity sequences (low single-digit nanomolar dissociation constants) with improved binding affinity in the stabilized form (Figure 2).

Binding of multivalent ligands to the cell surface can result in avidity effects where, for example, rebinding of the second domain in a bivalent interaction slows apparent dissociation. Because GST fusion can cause dimerization of the binding domain⁴⁹ (MDM2 in this example), we designed the selection scheme to mitigate potential impacts of avidity and expression levels on the cell surface. For several sorting rounds, we ran a competition sort where we first labeled with AF647-MDM2-GST followed by a wash and AF488-MDM2-GST. The addition of the competitor AF488-tagged molecule in excess would be able to effectively compete off AF647-MDM2-GST with high avidity/low affinity by preventing rebinding but not impact high affinity interactions that lack fast dissociation and rebinding.

Because these rounds selected for high AF647 relative to AF488, only those clones with high monovalent affinity that were resistant to competition by AF488-MDM2-GST would be selected. This is seen with the lead clone selected from the sort (SPD-M6-V1) having a high monovalent affinity with similar measurements by BLI and on the bacterial surface. However, avidity could be leveraged with this technique to improve the selection of very weak binders early in the directed evolution process, such as by preloading magnetic beads with biotinylated target to increase multivalent interactions.

One of the original goals of screening peptides containing a cross-linking ‘staple’, as opposed to post-screening stapling, was to avoid steric clashes that would result in ‘false positives’ during the selection process (i.e. sequences selected from the library that lose affinity after the addition of the linker). This is indeed the case, where placing the linker in a different location lowers the affinity on the bacterial surface (data not shown). We were also able to generate a series of affinity mutants with varying charge and lipophilicity for future investigation, given the importance of physicochemical properties on cytosolic access^{8,9} and the potential to detect favorable interactions with the staple itself^{9,28}. Interestingly, there were some sequences (e.g. SPD-M3-V1) that had a low apparent binding affinity when not stabilized on the bacterial surface ($K_d = 64$ nM) but demonstrated high apparent affinity when stabilized on the bacterial surface ($K_d = 9.8$ nM) or in solution ($K_d = 14$ nM). This indicates that stabilization can also help avoid ‘false negatives’ where a sequence that exhibits high affinity when stabilized in solution (a desired sequence) might be missed when using a selection scheme without surface stabilization due to low apparent affinity (when non-stabilized) on the bacterial surface.

From a structural perspective, our work here suggests that stabilization of a single helix improves selection of high affinity ligands, but using directed evolution, we found an additional conformational constraint that further enhanced binding to the target: disulfide loops (Figures 3B and 3D). We did not anticipate the selection of disulfide bonds since they typically destabilize alpha helices and the peptides bind MDM2 in a helical conformation. However, we did allow for selection of peptides containing cysteines through the NNC degenerate codon, so disulfide bonds were within the possible evolution space. In fact, over two-thirds of the clones sequenced after round 7 of FACS had cysteines at i,i+4 or i,i+5 positions, strongly suggesting selection pressure for disulfide bonds (Figure S5A). While increased constraint within a binding partner does not necessarily result in improved affinity due to enthalpy/entropy compensation⁴³, disulfide bonds often evolve under selection to improve the binding energy of proteins/peptides⁵⁰. We obtained 3D NMR structures to get a more detailed picture of the impact of preorganization on binding. NMR data and docking analysis showed interactions between the cysteine residues and MDM2, one interaction between the linker structure and MDM2, and a rigid structure induced by both constraints (Figure 4C and 4D). Therefore, the affinity improvements observed upon linker stabilization appear to result from both entropic contributions (conformational constraint) and direct enthalpic interactions. This is similar to the interactions observed between other stapled peptides and their targets, including an MCL-1 binding molecule⁹ and a p53-based hydrocarbon stapled peptide and MDM2⁶, and it supports the strategy of directed evolution in the presence of the linker as demonstrated here. We hypothesize that the disulfide bond further constrains the structure in parallel with the bis-triazole staple. Together, we posit that

this structural rigidity confers binding improvements through a decrease in entropic penalty of binding and direct interactions further contribute to the free energy of binding.

Notably, the highest affinity sequence from the selection, the disulfide-containing peptide SPD-M6-V1, would likely not have been discovered using non-orthogonal cysteine-directed strategies such as alkylation²⁸ or arylation¹⁷. SPD-M6-V1 without stabilization exhibits poor helicity (Table 1), which is partially remedied by reaction with the bis-alkyne linker, and this results in high affinity. Its relatively poor bacterial surface affinity without click stabilization (15 nM, worse than the original template sequence) also implies that stabilization prior to sorting was necessary for discovery. The original linear starting sequence ($K_d = 113$ nM, table 1) had an affinity similar to the MDM2 inhibitor Nutlin 3a¹¹. Click-stabilization increased the affinity ~7-fold followed by directed evolution to improve the affinity ~8-fold yielding a 1.8 nM binder. This value is similar to the reported K_d of pDI (8 nM for sequence LTFEHYWAQLTS⁵¹). However, pDI is a linear peptide with negligible activity in cells⁵², likely due to proteolytic instability and reduced intramolecular hydrogen bonding for crossing membranes. Therefore, it required extensive stapling and amino acid optimization to develop ATSP-7041⁵¹, the preclinical precursor to ALRN-6924. The current platform yielded a high affinity stabilized peptide binder directly from the library and work is ongoing to investigate sequence- and linker-dependence on cellular and *in vivo* efficacy.

Despite the advantages of this approach, there are several limitations and areas for improvement. Even with library sizes of $\sim 10^9$ in bacteria, the diversity of 11 positions is still much larger at $\sim 2 \times 10^{14}$. Computational methods (eg. by Bullock 2011⁶) could be used as a starting point followed by maturation of affinity, linker location, and stability using the present approach. For three key properties of intracellular biologics – membrane permeability, binding affinity, and stability – only the latter two can be engineered with the presented approach. As knowledge around membrane permeability improves, deep sequencing of libraries⁵³ sorted for affinity and stability could be used to identify peptides with properties associated with improved membrane permeability, such as high amphiphilic moments. Finally, the proximity of helices to the bacterial surface could impact quantitative measurements. As non-natural amino acid incorporation improves in other organisms such as yeast⁵⁴, where the Aga2 mating protein involved in extracellular protein-protein interactions (flocculation) has been adapted for surface display and panning³³, additional cell-surface methods may improve the molecular engineering of stabilized alpha helices.

This work represents a unique application of helix stabilization and cell surface display to engineer new affinity ligands. The directed evolution of stabilized helices yielded the novel bicyclic peptide SPD-M6-V1 containing a disulfide bond and double-click staple for the two macrocycles, a motif that due to its high binding affinity and customizability has translational potential upon chemical optimization (such as replacing the disulfide with an intracellularly stable linker). The stability and intra-molecular hydrogen bonding from these stabilized alpha helices increase the efficiency of intracellular delivery⁵⁵ and are the subject of current investigation in our lab and others. The surface display and binding of other peptides (Figure S9) highlights the potential for using this technique against additional targets. In summary, the combination of non-natural amino acid incorporation and bio-orthogonal chemistry can be used with directed evolution for the molecular engineering of

high affinity stabilized alpha helices through in situ stabilization and screening for novel drug leads.

Methods

Plasmid construction and library design

All primers were purchased from Integrated DNA Technologies (Coralville, IA), and all restriction enzymes were purchased from New England Biolabs (Ipswich, MA). The eCPX gene from pB33eCPX (a gift from P.S Daugherty, Addgene plasmid # 23336) was modified to avoid extraneous AHA incorporation and reaction. All non-start codon methionine sites (M99, M153, M156) were mutated⁵⁶ to leucine with primers 1 and 2 to generate pB33-eCPX(-met) without a significant difference in surface display levels. eCPX(-met) was inserted into pQE80L using *EcoRI* and *HindIII* sites with primers 3 and 4 (see Table S1).

For surface display comparisons of vector systems, exendin-4 was cloned into pB33eCPX using primers 5 and 2 with *SfiI* as described in ref. ⁵⁷. Exendin-4 was cloned into pet28A using primers 6 and 2 with pB33eCPX(-met)-exendin as the template, and restriction enzymes *NcoI* and *HindIII*.

The p53-like-peptide sequence ETFMDLWRLLMEN (SPD-M0-E(-2)) was cloned into pQE80L-eCPX(-met) using primers 7 and 8 to generate pQE80L-eCPX(-met)-SPD-M0-E(-2). For surface chemistry optimization, the HA tag was cloned adjacent and downstream of the peptide using SOE PCR with primers 9, 10, 8, and 11.

A library containing NNC degenerate codons to mutate all positions in the SPD-M0-E(-2) sequence except F19, M20, W23, L26, and M27 was generated using PCR as detailed in ref. 57 using primers 12 and 8 using pQE80L-eCPX(-met) as the template.

Charge mutants involving the mutations E17Q and E28Q were made using primers 13 and 14 using pQE80L-eCPX(-met)-SPD-M0-E(-2) as the template to generate SPD-M0-E(-1) and SPD-M0-E(0).

SfiI cut and gel extracted insert was ligated into *SfiI* cut pQE80L-eCPX(-met) and transformed into electrocompetent methionine auxotrophic *E. coli*, a generous gift from J. van Deventer with cloning procedures adapted from ref. 57.

Surface display of individual clones

Individual clones were grown overnight at 37°C in M9 medium containing 20 amino acids as described in ref. 58 and diluted 1:25 in M9 + 20 AAs. Cells were grown to OD₆₀₀ 0.6-1 (about 3 hours) at 37°C, centrifuged at 4000xg for 5 minutes, and then grown for 30 minutes in M9 medium containing 19 amino acids (no methionine) for metabolic depletion. Cells were centrifuged and then induced with 0.5 mM IPTG in M9 medium with 19 amino acids and 40 µg/mL azidohomoalanine at room temperature for 3 hours. Azidohomoalanine was synthesized inhouse according to ref. 59. Cells were then reacted as below.

For flow cytometric quantification of exendin display in various vector systems, cells with the respective plasmid were induced as above and labeled with 10 µg/mL mouse anti-

exendin (Abcam ab23407) in PBS/0.2% BSA. Cells were pelleted and resuspended in PBS/BSA containing 10 $\mu\text{g/mL}$ chicken anti-mouse AF647 (Thermo Fisher) and analyzed by flow cytometry. Median fluorescent values were compared with a standard curve made from Quantum Simply Cellular anti-Mouse beads (Bangs Laboratories) labelled with the same stock of chicken anti-mouse AF647.

Display and reaction conditions

The pBAD33 arabinose promoter system (originally utilized for bacterial surface display for tightly controllable expression and low induction-driven toxicity⁶⁰) resulted in poor expression of peptide displayed in AHA incorporation conditions (Figure 1A). Likewise, low levels of display were obtained with the pET28A T7 system commonly used for over-induction of recombinant proteins, as was similarly observed by Ayyadurai and coworkers with homopropargylglycine (HPG)⁶¹. Cloning of the gene into the pQE80L/T5 vector (Figure S10C) resulted in robust expression under both methionine and AHA incorporation conditions, and reaction and gel analysis showed promising specificity of AHA incorporation (Figure 1B). Mutation of all non-start codon methionine sites (M99, M153, M156) resulted in decreased extraneous reaction (Figure S10A).

We optimized reaction conditions to maximize stabilized peptide yield on-surface. Propargyl ether was chosen as the bis-alkyne linker to connect the *i, i+7* azides in the peptide sequences due to its helix-inducing propensity¹⁵, flexibility, and solubility. With directed evolution using cell surface display, typically selected cells are immediately cultured after sorting, but the copper catalyst needed for the azide-alkyne stabilization reaction is toxic to bacteria⁶². We therefore tested several copper chelators (based on previously reported improvements in toxicity and reactivity⁶³) and temperature/time conditions but found that in order to achieve high reaction efficiency, the viability of the bacteria was too low for continual outgrowth after sorting (data not shown). The plasmids could be 'rescued' from the sorted bacteria by polymerase chain reaction (PCR), but PCR can introduce errors and biases into the sorted libraries (especially when the diversity is high)⁶⁴. To avoid this bias, we directly transformed the plasmids into new bacteria following whole plasmid extraction with good yield⁶⁵. The transformation efficiency of this step was approximately 1/3 of the number of sorted cells, in line with ref. ⁶⁵, so we oversampled our desired stringency typically by 20x to account for sequence coverage and loss of clones from transformation. For example, with a library of diversity 1×10^6 clones where the brightest 1% of cells were desired, 2×10^7 cells were sampled and 2×10^5 cells were collected, which typically yielded 6×10^4 transformants.

We performed quantitative fluorescence analysis on western blots of cells reacted with either SulfoCy5-alkyne or SulfoCy5.5-azide dyes after reaction with the linker and normalized the signal to an anti-hemagglutinin tag (Figure 1C). From these experiments, we deduced the fraction of displayed peptide-specific azides that were reacted and the fraction of reacted peptides that were also stabilized to calculate the overall stabilization efficiency (Figure 1D) by fitting to a series of reaction equations (supplemental data) since mass spectrometry cannot track the 2nd intramolecular stabilizing reaction. The concentration of propargyl ether

that gave the best stabilization was 500 μ M; this was used for all selection and characterization experiments.

Library expression

The libraries encoding p53 peptide variants were grown from frozen stock in M9 medium containing all 20 amino acids at 5-10x sequence coverage at starting OD₆₀₀ of approximately 0.1. At OD₆₀₀ 0.6-1, cells were centrifuged at 4000xg and methionine depleted for 30 minutes in M9 medium containing 19 amino acids (without methionine). Cells were centrifuged and then induced with 0.5 mM IPTG at room temperature in M9 medium containing 19 amino acids and 40 μ g/mL azidohomoalanine for 4 hours at room temperature. Cells were then reacted as below.

On surface reaction of displayed peptide

For reaction characterization and binding measurements of unique sequences, typically 1 mL induced cells displaying AHA-incorporated peptide were centrifuged at 4000xg for 2 min and washed twice with ice-cold PBS (155 mM NaCl, 1 mM potassium phosphate, 3 mM sodium phosphate pH 7.4). The pellet was then reacted in 1.8 mL ice-cold PBS containing 100 μ M CuSO₄, 500 μ M Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 5 mM sodium ascorbate, and 500 μ M propargyl ether for 4 h at 4°C. After the reaction, the cells were pelleted and resuspended in 1 mL PBS containing 0.2% w/v bovine serum albumin (PBS/BSA). For library selections, these volumes were scaled up accordingly.

For surface reaction optimization, cells displaying AHA-incorporated SPD-M0-E(-2)-HA were washed once in PBS following propargyl ether reaction and then reacted further with 50 μ M SCy5-alkyne or SCy5.5-azide with 100 μ M CuSO₄, 500 μ M THPTA, and 5 mM sodium ascorbate overnight. Cells were then pelleted, washed 1X with cold PBS, and then resuspended in 1 mL PBS. For each well, 5 μ L cells were lysed in a total volume of 20 μ L containing 50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% beta-mercaptoethanol and 12.5 mM EDTA, and loaded onto a Bolt 4–12% Bis-Tris Plus gel (Invitrogen). Bands were transferred to a PVDF membrane using the iBlot system (Thermo Fisher), blocked overnight at 4°C with gentle shaking with 2% BSA in Tris-buffered saline with 0.1% Tween 20 (TBST), and probed with 2 μ g/mL anti-HA in TBST (Thermo Fisher, clone 2-2.2.14) with 0.2% BSA for 4 hours at room temperature. After 3 washes in TBST, the membrane was probed with 1 μ g/mL goat anti-mouse-IRDye800CW (Licor) for 1 hour at room temperature. The blot was scanned on a Licor Odyssey CLx scanning fluorescence imager following 3 washes in TBST and bands quantitated. The ratio of 700 nm channel fluorescence (for SCy5 and SCy5.5 dyes) to 800 nm channel (for anti-HA, loading normalization) was reported.

Protein expression and purification

pGEX-4T MDM2 WT was a gift from Mien-Chie Hung (Addgene plasmid # 16237) and was transformed into BL21DE3 cells and induced by 0.5 mM IPTG at 30°C for 5 hours after reaching OD₆₀₀ = 1.0. Expressed GST-tagged MDM2 was purified as described by ref 66.

Magnetic selection with MDM2

MDM2-GST was biotinylated by reaction with NHS-PEG4-Biotin (Thermo Fisher). Library-expressing and reacted cells were labelled with 2 nM biotin-MDM2 at 4°C for 1 hour in 15 mL PBS/BSA and cells were washed once with PBS/BSA to remove weakly-bound and residual biotin-MDM2. Labelled cells were incubated with Dynabeads MyOne Streptavidin T1 (Thermo Fisher) in a 1:1 ratio with end-over-end rotation at 30 rpm using MACSmix (Miltenyi Biotec). Following incubation, magnetic beads were isolated by application of a magnetic field and beads washed twice with 15 mL PBS/BSA. DNA from bound cells was isolated as follows: beads were resuspended in 250 µL buffer P1 from the Qiaprep spin miniprep kit (Qiagen), bound cells lysed with 250 µL buffer P2, and neutralized with 350 µL buffer. After centrifugation, the supernatant was applied to a Qiaprep column and after washing with buffer PE, DNA was eluted with 30 µL H₂O (yielding approximately 100 ng DNA) and re-transformed into electrocompetent methionine auxotrophic *E. coli*.

Fluorescence activated selection with MDM2

Following library expression and surface stabilization, the MACS sorted library underwent selection by increasingly stringent rounds of FACS on a MoFlo Astrios instrument as follows: FACS round 1: labeled with 1.5 nM MDM2-AF647, brightest 1.2% of events collected, 3×10^5 transformants obtained; round 2: labeled with 0.2 nM MDM2-AF647, brightest 1.2% of events collected, 1.1×10^5 transformants obtained; round 3: labeled with 20 nM MDM2-AF647 for 30 min, washed 2x, labeled with MDM2-AF488 at 200 nM. Top 2.5% of events positive for AF647 and low for AF488 collected, 1.1×10^5 transformants obtained; round 4: similar scheme as round 3, brightest 15% of cells collected, 2.3×10^5 transformants obtained; round 5: similar scheme as round 3, brightest 2% of events collected, 1.8×10^5 transformants obtained; round 6: labeled with 0.5 nM MDM2-AF647, brightest 1% of events collected, 3.0×10^4 transformants obtained; round 7: labeled with 0.5 nM MDM2-AF647, brightest 0.05% of events collected, 3.2×10^3 transformants obtained. All cell labeling steps with primary MDM2-AF647 were done for a minimum of four hours prior to sorting, and all labeling steps with secondary MDM2-AF488 were done for a minimum of one hour.

The plasmid contents of selected cells were extracted as detailed in ref. 65 and re-transformed into electrocompetent methionine auxotrophic *E. coli* for further selection and/or binding characterization.

Characterization of selected clones

From each round of selection, typically 8 clones were sequenced by Sanger sequencing (32 clones were sequenced after FACS round 7) using primer 11. For characterization of individual clones, each clone was grown up and reacted as detailed previously. For binding titrations, appropriate concentrations and volumes (10-fold excess) of AlexaFluor647 tagged MDM2-GST diluted with PBS with 0.2% BSA was added to approximately 1×10^6 cells and incubated on ice for 3 hours. Labeled cells were centrifuged at 4000xg for 2 minutes, washed once with 300 µL PBS with 0.2% BSA, and resuspended in 200 µL PBS for analysis by flow cytometry (Attune Focusing Cytometer). Median fluorescence intensities were

normalized to the highest concentration and fit to a one-site binding model in GraphPad Prism v. 6.

Peptide Synthesis and Stabilization

Solid phase peptide synthesis of *i,i*+7 diazido peptides was carried out using a CEM Liberty Blue Microwave Peptide Synthesizer with 0.3 mmol/g loading Rink amide resin and Fmoc amino acids in dimethylformamide (DMF). The peptides were synthesized at 0.05 mmol scale and cleaved by addition of a cocktail composed of 93% (v/v) trifluoroacetic acid (TFA), 5% (v/v) H₂O, 5% (w/v) phenol, and 2% (v/v) triisopropylsilane (TIPS). The resulting solution was evaporated under nitrogen to a small volume and precipitated via dropwise addition to tert-butyl methyl ether. The precipitate was then collected, lyophilized, and purified by preparative reverse phase gradient HPLC with water and acetonitrile mobile phases buffered with 0.1% TFA for all peptides except for SPD-M6-V1, which was purified with 25 mM triethylammonium acetate (TEAA) in H₂O/MeCN. The resin and Fmoc amino acids were purchased from ChemPep Inc. Fmoc-Azidohomoalanine was synthesized as in the method outlined by Lau⁶⁷ or purchased from ChemPep, Inc. All other reagents were purchased from Sigma Aldrich.

Diazido peptides were stabilized in solution via copper catalyzed azide alkyne cycloaddition. Peptides were stabilized in 1:1 water:*tert*-butanol at 1 mM concentration (1 equiv) with propargyl ether (1 equiv) by addition of copper (II) sulfate (10 equiv), tris(3-hydroxypropyltriazolylmethyl)amine (10 equiv) and sodium ascorbate (50 equiv) at room temperature overnight. The reaction mixtures were purified by HPLC in water and acetonitrile mobile phases buffered with 0.1% TFA (except for SPD-M6-V1 which was purified in triethylammonium acetate (25 mM) in H₂O/MeCN) and the product fractions were lyophilized and characterized via electrospray ionization mass spectrometry (ESI-MS) and reported in Table S2.

Solution phase binding kinetics

Binding affinities with the peptide in solution were measured via biolayer interferometry (BLI) using an Octet RED96 system (Pall ForteBio). MDM2 truncate (residues 10–118, provided by Prof. Jeanne Stuckey) was biotinylated with NHS-PEG4-Biotin (Thermo Fisher). The resulting protein was diluted to 500 nM in the assay buffer (0.3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS)) and loaded onto Super Streptavidin Biosensors. Peptide samples were prepared in the same assay buffer and each measurement was done in a 96 well plate using 10 concentrations per peptide. Resulting binding curves were fitted using Graphpad Prism v. 6. For each peptide, dissociation data was globally fit for all concentrations using a one-phase exponential decay model to yield k_{off} . The association data were fit for each concentration to yield values for k_{on} , and K_d is reported as $k_{\text{off}} / k_{\text{on}}$. The reported standard deviations of K_d are between values from all data sets (~8 association/dissociation curves per peptide each day measured on 3 separate days).

Circular Dichroism

Solution phase peptide helicity values were estimated by circular dichroism on a JASCO J-815 spectropolarimeter. Samples were diluted in 1:1 water:acetonitrile to 10 μ M and transferred to a 1 mm path length quartz cell. Measurements were recorded in three accumulations from 250 nm to 190 nm. Percent helicity values were determined from molar ellipticity at 222 nm divided by the calculated maximum molar ellipticity for 13 and 14-mer peptides.

NMR Spectroscopy, Data Processing, and Structure Calculations

High-resolution NMR spectra of free SPD-M6-V1 peptide were obtained in sodium phosphate buffer, pH 6.8. Lyophilized peptide (SPD-M6-V1) was dissolved in 10 mM aqueous sodium phosphate buffer (pH 6.8) with 10% D₂O to a final concentration of 300 μ M. All NMR spectral measurements were acquired at room temperature (298K) in a Bruker AVANCE II 600 MHz spectrometer equipped with a cryoprobe and water signal was suppressed using WATERGATE. For resonance assignment and NOE contacts, 2-D homonuclear TOCSY (mixing time 80 ms with 32 scans) & NOESY (mixing time 200 ms with 88 scans) experiments were acquired using spectral width of 13 ppm with 2 K $\times$ 512 complex points and the States-TPPI for quadrature detection at the t₁ dimension. To probe the interactions of SPD-M6-V1 with MDM2, a saturation transfer difference (STD) experiment was conducted by addition of MDM2(10–118) to a final concentration of 9 μ M, pH 6.8. MDM2(10-118) was saturated at 0.15 ppm with a cascade of 40 selective Gaussian-shaped pulses (49 ms each) in an interval of 1 ms resulting in total saturation time of 2 s. An identical experiment one-dimensional STD experiment (as a control) was conducted with only SPD-M6-V1 peptide at same concentration. All NMR data were processed using TopSpin 2.6 (Bruker), and the chemical shifts were referenced directly to the frequency of water (4.7 ppm). After zero filling along the t₁ dimension, 2 K (t₂) $\times$ 1 K (t₁) data matrices were obtained. All the spin system assignment and spectral analysis were done using SPARKY 3.113⁶⁸.

Sequence-specific resonance assignments of peptide was achieved by analyses of two-dimensional ¹H–¹H TOCSY and NOESY spectra⁶⁹. Most of the resonances of SPD-M6-V1 are unambiguously identified (Figure S8). Analyses of ¹H–¹H NOESY spectra reveal backbone/backbone, backbone/sidechain, and sidechain/sidechain NOE contacts for the peptide. SPD-M6-V1 peptide contains 4 aromatic amino acids and a number of NOEs could be identified involving aromatic ring protons with the backbone and side chain protons of aliphatic residues (Figure S8C). NOE contacts detected for peptide folding are summarized in Figure S8B and characterized by sequential and medium range interactions.

Assigned NOESY peaks were classified as weak, medium or strong and translated to an upper bound distance restraints: 5.0, 3.5 and 2.8 Å, respectively. Backbone dihedral angles ϕ and ψ were predicted using TALOS using ¹H α chemical shifts. The structure of SPD-M6-V1 was calculated using CYANA 2.1. The CYANA library for the unnatural amino acid was created using the algorithm cylib developed by the Guntert group⁷⁰ (Table S5). During the CYANA runs, both amino acids (X20 and X27) were combined through an oxo group (Figure 4A) in side chains of the unnatural amino acids. The structure calculation was done

in a stepwise manner to get the final ensemble with low RMSD and low violation of distance and dihedral angle constraints.

Protease digests

On-surface peptides were expressed as mentioned above and approximately 10^5 displaying bacteria were incubated with 1 $\mu\text{g/mL}$ chymotrypsin in 80 μL PBS on ice. At each time point, samples were quenched by addition to 2% BSA in PBS, centrifuged and resuspended in 0.2% BSA in PBS. Resulting cells were labelled with 50 nM MDM2-GST-AF647 on ice for 2 h in 0.2% PBS/BSA, washed, and analyzed by flow cytometry (Biorad ZE5). Median fluorescent intensities were analyzed to obtain the curves in Figures 3A and 3C. For in solution measurements of protease degradation, stabilized and non-stabilized peptides were incubated with chymotrypsin at 5 $\mu\text{g/mL}$ in PBS. Peptides were first diluted to 50 μM in PBS before adding the enzyme and incubating at 37 C. After each time point, samples were flash-frozen in liquid nitrogen to stop digestion and stored at -80 C until analysis. Samples were analyzed via reverse-phase HPLC at 214 nm and 280 nm wavelengths. The fraction of intact peptide remaining at each time point was quantified by the AUC of the intact peptide peak on the chromatograph at 214 nm. Exponential decay curves and half-lives were fit in GraphPad Prism v.8.

Structure-guided docking

AutoDock Vina⁷¹ was used to dock the calculated solution structure of SPD-M6-V1 to the MDM2 crystal structures in 3EQS⁵¹. Alignment of several crystal structures (2GV2, 1T4F, 1YRC, 3V3B, 4HFZ, 3G03, 3EQS) MDM2 showed similar binding groove composition and amino acid side chain conformation, so 3EQS was selected for structure-based docking. MDM2 and peptide were pH-adjusted to physiological pH (7.4) using the PDB2PQR server⁷². AutoDockTools (ADT) was used to prepare the protein and peptide PDB files and determine the search space for conformational flexibility. MDM2 residue side chains on the periphery of the binding pocket (L54, F55, Y100) were allowed to flex using ADT. Polar hydrogen atoms were added, non-polar ones were removed, and Gasteiger partial atom charges were calculated. Structures were visualized using PyMOL (Schrödinger, Delano Scientific, LLC, New York, NY, USA). The selected structure (Figure 4B) was chosen based on agreement with experimental data (Figure 4C) showing interactions between MDM2 and V16, C18, F19, Y22, and W23.

Statistics

Statistical analyses for Figs. 1A, 2C, and 2D were performed in GraphPad Prism v. 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank A.-M. Deslauriers-Cox, M. Savary, and D. Adams of the University of Michigan Flow Cytometry Core for assistance with FACS. We acknowledge the help of H. Remmer of the University of Michigan Proteomics and Peptide Synthesis Core for productive discussions regarding solid phase peptide synthesis as well as J. DelProposto

from the University of Michigan Life Sciences Institute for assistance with biolayer interferometry (BLI) measurements. We thank members of the T. Scott laboratory for use and assistance with their solid phase peptide synthesizer. We thank J. van Deventer and H. Gao for helpful discussions and J. Bardwell for conversations and assistance in editing the manuscript. We also thank Peter Guntert and Sina Kazemi for helping with CYANA library generation.

Funding Sources:

This work was supported by an NSF CAREER Award CBET 1553860 (to G.M.T) and NSF Graduate Fellowships (to T.N. and L.A.). Additional support was provided by NIH Grant R35 GM128819 (to G.M.T).

ABBREVIATIONS

MDM2	Mouse Double Minute 2
FACS	Fluorescence Activated Cell Sorting
MACS	Magnetic Activated Cell Sorting
SPPS	Solid Phase Peptide Synthesis
BLI	Bio-layer interferometry
AUC	Area Under Curve

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Figure 1.

Chemical Biology Enabled Stabilization. A: Comparison of display levels conferred by different vectors with and without azidohomoalanine incorporation. In this example, the 39 amino acid exendin peptide is displayed, labeled with an anti-exendin and fluorescent secondary antibody, and quantified by flow cytometry using quantitative beads ($n = 3$ independent bacteria cultures, unpaired t test two-tailed $df = 4$; $t = 0.75$, $p = 0.50$; $t = 11.7$, $p = 0.0003$; $t = 6.61$, $p = 0.00270$ respectively). B: SPD-M0-E(-2) with an HA tag generated using primers 9 and 10 displayed and reacted with SCy5-alkyne (red) and blotted with anti-HA (blue). C: Reaction progress and fraction of reacted peptides that are also stabilized as a function of propargyl ether concentration ($n = 3$ independent blots per data point). D: Fraction of surface displayed peptides that are stabilized as a function of propargyl ether concentration ($n = 3$ independent blots per data point).

Figure 2.

Stabilized Peptide Library Design and Selection. A: top: randomization scheme for library generation showing the residues randomized (gray), kept constant (black), and stabilization sites (red). Bottom: population level and individual clone analysis by flow cytometry. Histograms are shown for approximately 12,000 events for samples labeled with 2.5 nM MDM2-GST. “No selection” is the original library containing 3×10^8 transformants. “MACS” refers to the population obtained from one round of positive selection with magnetic beads. FACS 1, 3, 5, 7 refer to the populations sorted after 1, 3, 5, and 7 rounds of fluorescence-based sorting. The even-numbered sorted populations are not shown here for clarity. B: Logo plot results of selection showing relative frequencies of amino acids in non-cysteine containing sequences and *i, i+4* containing sequences. C: SPD-M3-G1 exhibits a nearly 5-fold improvement in affinity over the starting sequence SPD-M0-E(-2) after only 3 rounds of sorting indicating the selection following surface reaction is effective ($n = 3$ independent trials of duplicates per data point, unpaired t test two-tailed, $df = 4$. $t = 8.036$, $p = 0.0013$). D: Disulfide bond formation is important for binding of SPD-M6-V1 and mutation of one of the cysteine residues results in reduced affinity on the bacteria surface ($n = 3$ independent trials, unpaired t test two-tailed, $df = 4$. $t = 4.97$, $p = 0.0076$).

Figure 3.

Chymotrypsin digest trajectories. A: Bacteria displaying stabilized peptides were treated with chymotrypsin (1 $\mu\text{g/mL}$ on ice) and labeled with MDM2-GST-AF647 for peptide detection. B: Stabilized peptides synthesized by SPPS were treated with chymotrypsin (5 $\mu\text{g/mL}$ at 37 C) and quantified by HPLC for validation. C: As in A, except with non-stabilized peptides. D: As in B, except with non-stabilized peptides.

Figure 4. Structural characterization using solution NMR A: Ensemble of 20 lowest energy structures and B: representative single structure. C: Docked model showing likely bound conformation. D: Saturation transfer difference (STD) spectrum of SPD-M6-V1 bound to MDM2.

Scheme 1.

Stabilized peptide engineering with *E. coli* display (SPEED). A: On the bacteria surface, the displayed peptides are reacted with a stabilizing, bis-alkyne molecule (shown here as propargyl ether). B: Displaying bacteria are then incubated with a ligand of interest. After display, stabilization, labeling, and selection, the plasmids of desired cells are purified and re-transformed into fresh cells, completing the directed evolution cycle.

Table 1 –

Measured dissociation constants of peptides on bacterial surface (BSD, soluble MDM2) and in solution (BLI, surface-bound MDM2) and CD-measured helicity values for peptides in this article

Name	Sequence	BSD K _d (nM)			BLI K _d (nM)			Helicity (%)		
		Stabilized (S)	Non-stabilized (NS)	Ratio (NS/S)	Stabilized (S)	Non-stabilized (NS)	Ratio (NS/S)	Stabilized (S)	Non-stabilized (NS)	Ratio (S/N S)
SPD-M0-E(-2)	ETFXDLWRLXEN	9.6 ± 1.6	8.7 ± 0.2	0.9	15 ± 5.3	113 ± 16	7.5	53	30	1.8
SPD-M0-E(-1)	QTFXDLWRLXEN	11 ± 6.2	13 ± 6.8	1.2	17 ± 3.4	157 ± 29	9.2	40	29	1.4
SPD-M0-E(0)	QTFXDLWRLXQN	12 ± 4.8	17 ± 5.5	1.5	74 ± 28	454 ± 106	6.1	41	37	1.1
SPD-M3-G1	GGTFXGYWADLXAF	2.0 ± 0.31	2.9 ± 1.0	1.4	5.1 ± 3.9	21 ± 3.7	4.1	18	17	1.1
SPD-M3-V1	VLSFXDYWNLLXGS	9.8 ± 3.4	64 ± 33	6.6	14 ± 4.3	131 ± 40	9.4	NM	NM	-
SPD-M6-V1	VCDFXCYWNDLXGY	1.4 ± 0.10	15 ± 4.8	11	1.7 ± 0.16	6.8 ± 2.8	4	28	3	9.3
SPD-M0-E(-2)-F19A	ETAXDLWRLXEN	ND	ND	ND	604 ± 177	>10,000	-	50	14	3.7