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How to cite

SABALE, Pramod Madhavrao et al. Suprastapled Peptides: Hybridization-Enhanced Peptide Ligation and Enforced α-Helical Conformation for Affinity Selection of Combinatorial Libraries. In: Journal of the American Chemical Society, 2021, vol. 143, n° 45, p. 18932–18940. doi: 10.1021/jacs.1c07013

This publication URL: https://archive-ouverte.unige.ch/unige:156465
Publication DOI: 10.1021/jacs.1c07013

Suprastapled Peptides: Hybridization Enhanced Peptide Ligation and Enforced α-helical Conformation for Affinity Selection of Combinatorial Libraries

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ABSTRACT: Stapled peptides with an enforced α-helical conformation have been shown to overcome major limitations in the development of short peptides targeting protein-protein interactions (PPI). While the growing arsenal of methodologies to staple peptides facilitates their preparation, stapling methodologies are not broadly embraced in synthetic library screening. Herein, we report a strategy leveraged on hybridization of short PNA-peptide conjugates wherein nucleobase driven assembly facilitates ligation of peptide fragments and constrains the peptide's conformation into an α-helix. Using native chemical ligation, we show that a mixture of peptide fragments can be combinatorially ligated and used directly in affinity selection against a target of interest. This approach was exemplified with a focused library targeting the p-53 / MDM2 interaction. One hundred peptides were obtained in a one-pot ligation reaction, selected by affinity against MDM2 immobilized on beads and the best binders were identified by mass spectrometry.

INTRODUCTION

The α-helical conformation of peptide is a central motif in protein folds and numerous protein-protein interactions are the product of an α-helix interacting with a shallow groove of its interacting partner.[1-2] While short peptides do not often adopt a stable α-helical conformation, many technologies have now been reported to stabilize this conformation by crosslinking the side chains of two residues on the same face of the helix (*i* and *i*+4 or *i*+7, Figure 1).[3-8] This so-called peptide stapling approach not only improves binding affinity by locking the peptide in the appropriate binding conformation but also reduces the proteolytic susceptibility of the stapled peptide by inhibiting the adoption of the non-helical conformation required for protease binding. Accordingly, stapled peptides are increasingly used in chemical biology and drug discovery. Following the pioneering work establishing the utility of stapled peptides,[9-12] there has been continued interest to advance the stapling chemistry with a particular emphasis on methodologies (for recent examples, see refs[13-21]) that can be applied to unprotected peptides that can be embraced in genetically encoded libraries.[22-25]

Herein, we report a supramolecular approach to stapling the α-helical conformation leveraged on the hybridization of short peptide nucleic acid[26-27] (PNA) sequences. Besides being efficacious in stabilizing the helical conformation, this approach also enables a simple means to generate libraries by templating native chemical ligation of two fragments.

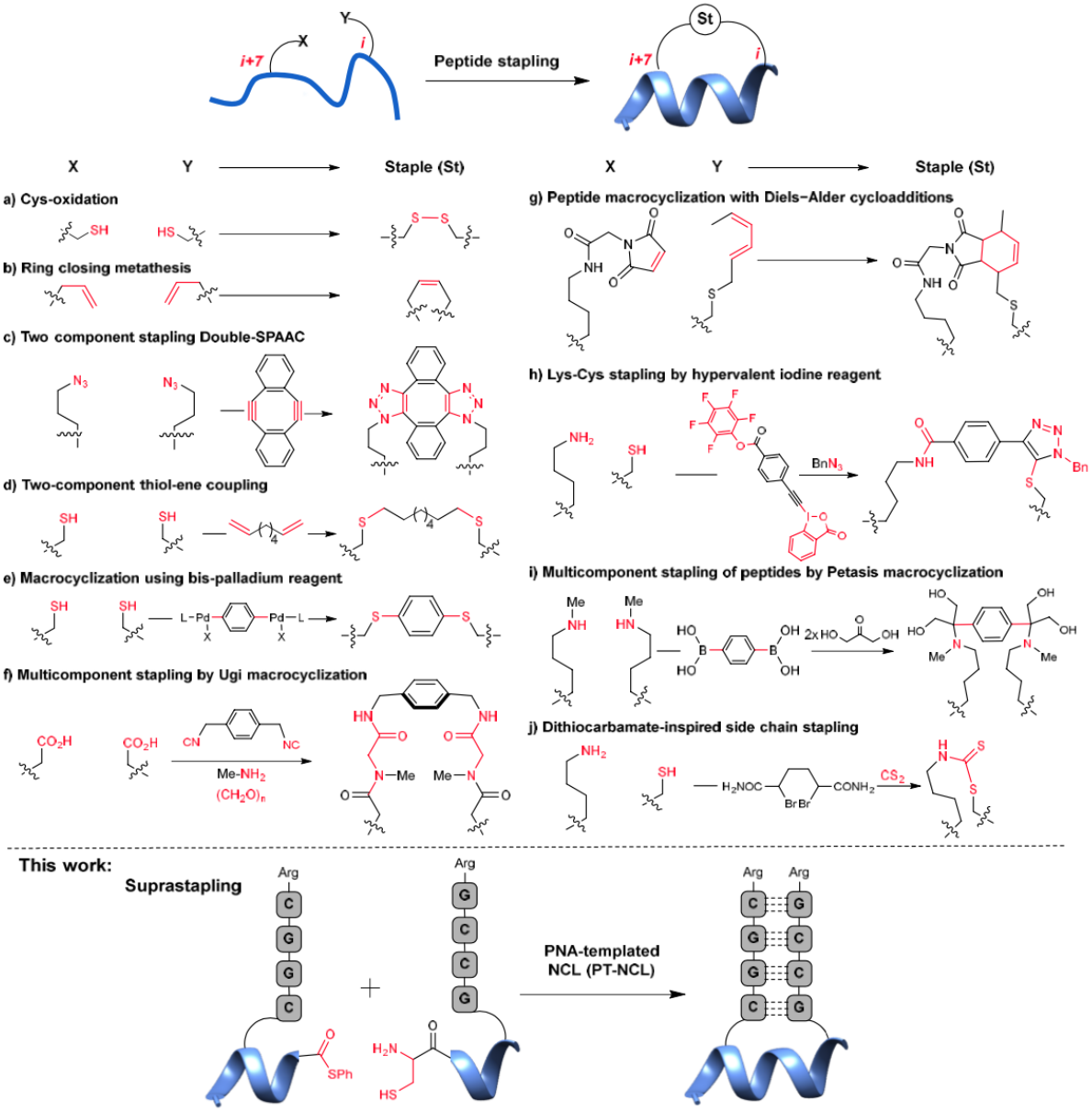


Figure 1. General scheme for peptide stapling. Previously reported methods of covalent peptide stapling. Proposed hybridization-mediated ligation and suprastapling design (This work).

RESULT AND DISCUSSION

We focused our investigation on MDM2, an oncoprotein that inhibits the p53 tumor suppressor[28-29], as a prototypical example of protein-protein interaction leveraged on a shallow cleft (located on MDM2 in the present case) interacting with an α-helical peptide (within p53 in this case). The α-helix is an amphiphilic peptide that provides steric and hydrophobic complementarity to the cleft leaving a face of the helix pointing away from the target protein amenable for stapling. The choice of MDM2 as a test bed was also motivated by the extensive medicinal chemistry research from phage display libraries,[30-32] stapled peptides[13, 33-34] and combinatorial peptide[35-36] library coupled to structural data[13, 28, 37-38] that facilitate interpretation of the results. While the proposed suprastaple is more voluminous than the one resulting from covalent stapling strategies, we reasoned that the bulk could be directed away from the important interface by selecting the appropriate residues on the helix, thus avoiding a steric clash. The distance between the terminal residues of a PNA-duplex is ca. 12 Å, which lends itself best to staple the *i* and *i*+7 residues of a peptide (Figure 2A). Based on the structural information, two different designs were envisioned, sliding the position of the suprastaple by one residue on the peptide to shift the orientation of the suprastaple by *ca.* 100° (design 1 and 2, Figure 2B). Taking the sequence of a stapled peptide reported by Spring et al.[13] and its unstapled control (**Ctrl-1**), the peptide was split in half with the objective to assemble it using a templated native chemical ligation (NCL[39]), thus replacing the alanine (Ala) at position 5 with a cysteine (Cys) (Figure 2C). While reductive procedures are known to convert Cys to Ala, we reasoned that a simple alkylation of Cys would be more practical and would also avoid

complications arising from oxidative disulfide formation. The first design would thus staple positions 2 and 9 while the second design staple positions 1 and 8. Each design was prepared with two slightly different connectivities to the suprastaple (Lys or Dap at position 1 or 2 resulting in a difference of three C-C bonds connection to the PNA). A 4-mer PNA was anticipated to be minimally sufficient based on prior examples of peptides constrained by PNA hybridization.[40-42] The control peptides were synthesized by standard SPPS, and the suprastapled peptides were prepared by NCL (*vide infra*). Testing their affinity for MDM2 by SPR showed that, as previously reported[13] **Ctrl-1** was a high-affinity binder (K_d = 32.5 nM), however, the **Ctrl-2** with the alkylated cysteine *in lieu* of Ala no longer yielded measurable affinity. While the CD spectra of both **Ctrl-1** and **-2** showed partial α-helical character (Figure S1), the SPR results indicated that modifications in **Ctrl-2** abrogated binding to MDM2. Gratifyingly, suprastapling of this **Ctrl-2** peptide could rescue its affinity and it was found to be a high affinity ligand for MDM2 (K_d = 12.1 nM, X = Lys), with even better affinity than **Ctrl-1**, provided that design-2 was used. A suprastapled peptide based on the design-1 had no measurable affinity, and we speculate that this is due to a steric clash between the PNA suprastapled and MDM2 surface. While the length of the nucleic acid component is short, measurement of the melting temperature of the suprastapled peptide afforded a T_m of 67 °C, showing that the suprastapled peptide is fully hybridized at physiological range of 20-37 °C (Figure S2). Taken together, these results indicate that the suprastaple provides a clear benefit for binding and that design-2 is sterically compatible with MDM2 binding.

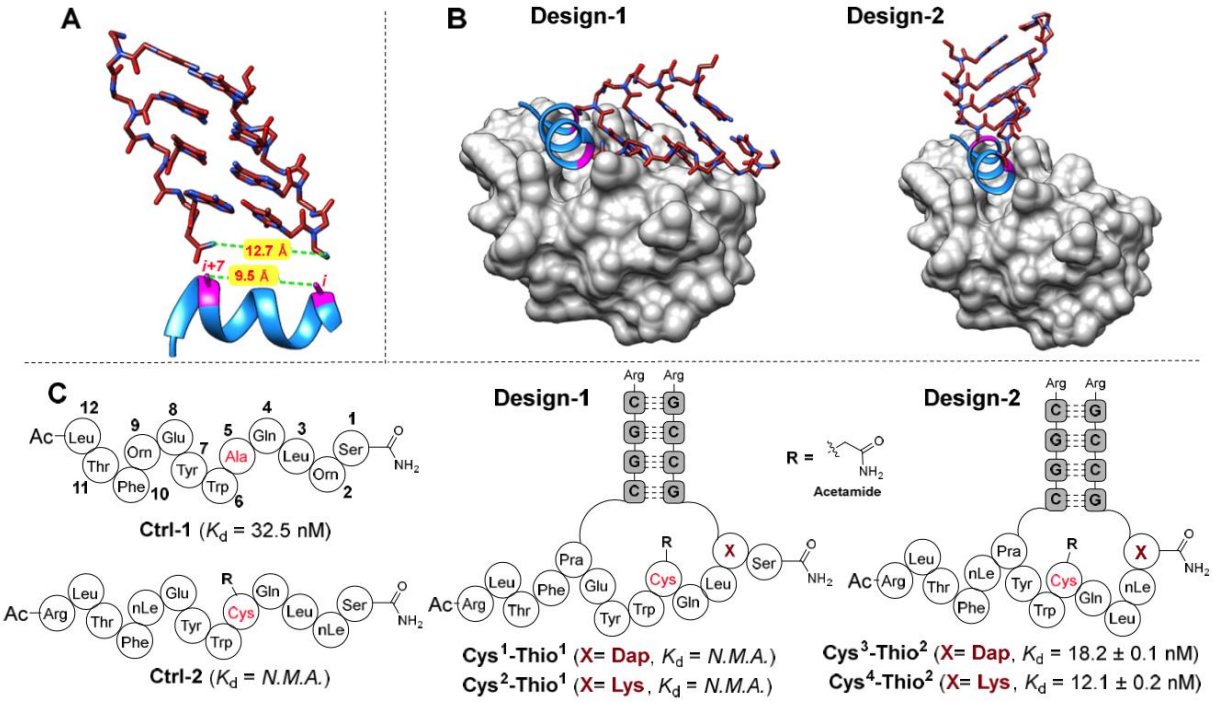


Figure 2. A) Graphical superposition of PNA-duplex (PDB: 1pup) and a 3.6_{13}-helix **B**) Graphical representation of suprastapled peptide bound to MDM2 for designs 1 and 2 generated by superposition of PNA (PDB: 1pup) aligned with the side chains extensions of design-1 and 2 based on the co-crystal structure of Ctrl-1 and MDM2 (PDB: 5afg).[13, 43] **C**) The sequences (with numbering on **Ctrl-1**) and K_d values obtained by SPR analysis for **Ctrl-1** and **Ctrl-2** peptides, and suprastapled peptides (**Cys¹-Thio¹**, **Cys²-Thio¹**, **Cys³-Thio²** and **Cys⁴-Thio²**) based on design-1 and design-2. *N.M.A.* = No measurable affinity.

We next investigated the potential of harnessing the hybridization to facilitate the ligation of the two components for the construction of the full-length peptide. It is well established that hybridization of reaction components can accelerate the rate of reactions by increasing their effective concentration, making them independent of concentration, including for a NCL.[44-48] However, the use of this reaction to ligate diverse peptides with a connectivity between *i* and *i*+7 in peptides has never been investigated. Gratifyingly, the reaction was found to be fast ($t_{1/2}$ < 3 min) and clean, affording the desired ligated product under comparable kinetics at 50 or 10 µM (Figure 3A and B). At these concentrations, the NCL unaided by hybridization of the two components yielded <10% even at prolonged reaction times.

It has been previously shown that stapling significantly improves the proteolytic stability of peptides.[11] Given that a supramolecular staple may render the system less rigid as opposed to covalent macrocyclization, we next investigated the proteolytic stability of the suprastapled peptide **Cys⁴-Thio²** (12.1 nM affinity to MDM2) and compared it to linear peptides **Ctrl-1** and **Ctrl-2**. To facilitate the quantification via HPLC, the different peptides were labeled

with Cy3 to integrate the HPLC peak with a common visible range chromophore. The peptides were incubated in fetal calf serum (FCS) at low µM range (**Ctrl-1** and **Ctrl-2**; 2 µM and **Cys^4-Thio^2**; 1 µM) and the aliquots of the reaction were analyzed by HPLC. While **Ctrl-1** and **Ctrl-2** were fully degraded to multiple products after 24 h, the suprastapled **Cys^4-Thio^2** was still present >90% intact (Figure 3 C, Figure S3 for chromatograms).

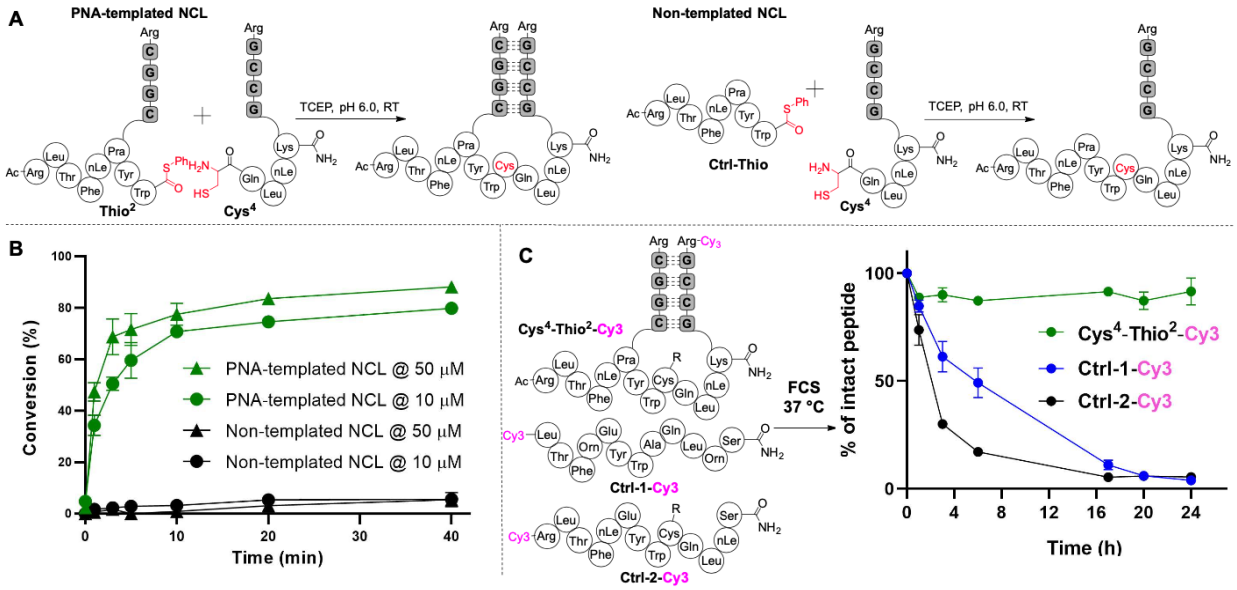
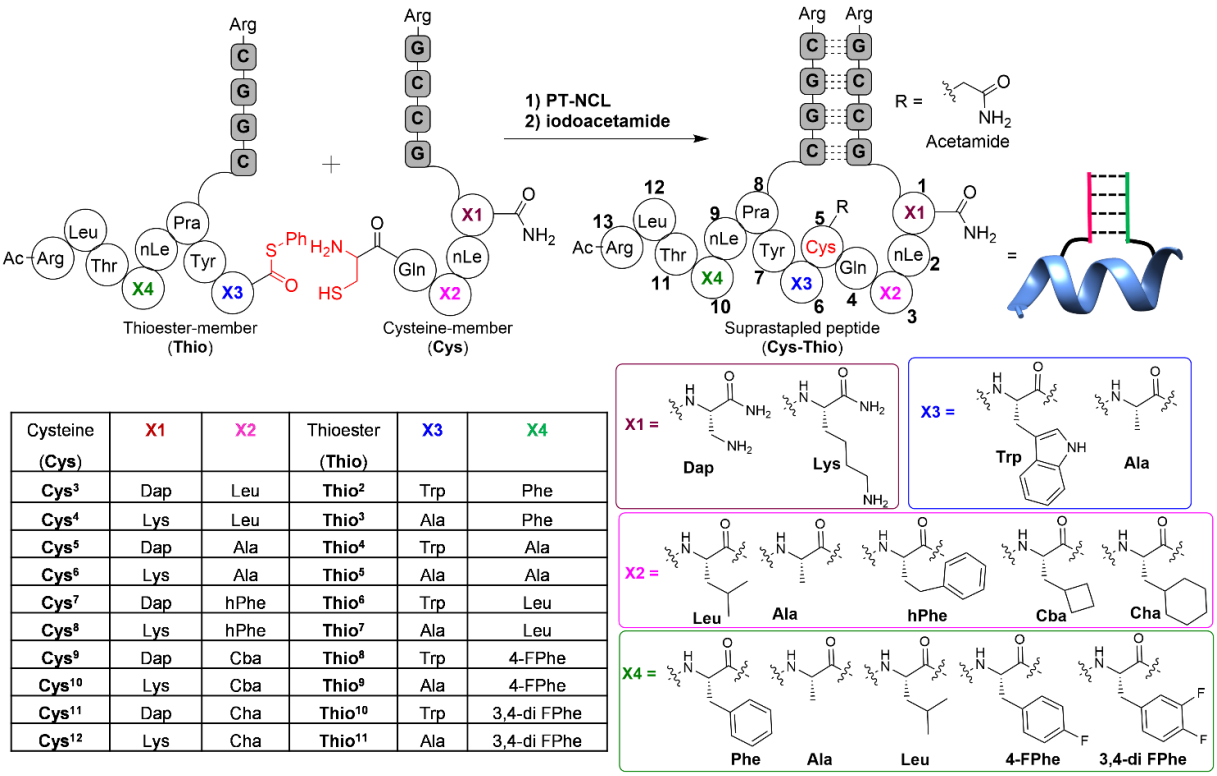


Figure 3. Model PNA-templated/non-templated NCL reaction. A) Ligation of complementary PNA-thioester (**Thio^2**) and PNA-cysteine (**Cys^4**) conjugates *vs* reaction between control thioester lacking PNA (**Ctrl-Thio**) and PNA-cysteine (**Cys^4**). B) Percentage conversion of PNA-templated **(Cys^4** and **Thio^2**) and non-templated (**Cys^4** and **Ctrl-Thio**) NCL reaction at 50 µM and at 10 µM determined by LC-MS. C) Proteolytic stability assay for **Ctrl-1-Cy3**, **Ctrl-2-Cy3** and **Cys^4-Thio^2-Cy3** analyzed by RP-HPLC. The amount of intact substrate was quantified by integration of the substrate peak observed at 545 nm (Abs of Cy3).

We next turned our attention to affinity selection of a suprastapled library and designed a library with 10 peptides terminating with a cysteine (**Cys$^{3-12}$**) and 10 containing a thioester for NCL ligation (**Thio$^{2-11}$**), as shown in Figure 4. The synthesis of the cysteine component was achieved via standard SPPS initiating the synthesis with a Mtt-protected Lys or Dap, removal of the Mtt and synthesis of the PNA (see experimental section for full synthetic details and explicit structures).[49] The thioester component was assembled by SPPS synthesis of PNA fragment and CuAAC[50-51] coupling to an in-solution synthesized C-terminus allyl protected tripeptide. The peptide was then extended using standard SPPS by Fmoc-chemistry and finally the allyl group deprotection allowed conversion of the C-terminus to a thioester (PhS_2, PBu_3). The crude cleavage product was used directly in NCL without further purification.



Cysteine (Cys)	X1	X2	Thioester (Thio)	X3	X4
Cys^{3}	Dap	Leu	**Thio^{2}**	Trp	Phe
Cys^{4}	Lys	Leu	**Thio^{3}**	Ala	Phe
Cys^{5}	Dap	Ala	**Thio^{4}**	Trp	Ala
Cys^{6}	Lys	Ala	**Thio^{5}**	Ala	Ala
Cys^{7}	Dap	hPhe	**Thio^{6}**	Trp	Leu
Cys^{8}	Lys	hPhe	**Thio^{7}**	Ala	Leu
Cys^{9}	Dap	Cba	**Thio^{8}**	Trp	4-FPhe
Cys^{10}	Lys	Cba	**Thio^{9}**	Ala	4-FPhe
Cys^{11}	Dap	Cha	**Thio^{10}**	Trp	3,4-di FPhe
Cys^{12}	Lys	Cha	**Thio^{11}**	Ala	3,4-di FPhe

Figure 4. Design and synthesis of a 100-member suprastapled peptide focused library against MDM2.

Using a pilot library of 9-suprastapled peptides prepared and purified individually, we optimized the condition for a miniaturized affinity selection and mass spectrometry decoding (Figure 5A and B). MDM2, which was expressed as a GST fusion protein for purification, was captured on GSH magnetic beads. Excess MDM2 was washed away, and beads were blocked with BSA. The beads (10 µL) were then exposed to the library of suprastapled peptides (25-100 nM) to reach a final screening volume of 100 µL, incubated for 1 h and washed extensively to remove unbound compounds. To facilitate subsequent MALDI-TOF/MS detection, we found that it was critical to wash away the surfactant (0.1 % CHAPS) with several washes with PBS. Finally, the MDM2-bound compounds were recovered using 25% AcOH solution in water (60 µL). The resulting solution could be analyzed directly by MALDI-TOF/MS using a fraction of the solution recovered (1 µL). Deconvolution of selected compounds was possible even using concentration as low as 25 nM in the selection step suggesting efficient pulldown of tight binders in the selection step. Serving as a control, beads loaded with GST alone were instrumental to develop conditions in which non-specific binders were efficiently discarded. As shown in Figure 5B, the selection against just GST did not yield any detectable library members, while the same procedure against GST-MDM2 fusion protein afforded a clear selection of 2 out of the 9 suprastapled peptides (namely; **Cys^{4}-Thio^{2}** and **Cys^{10}-Thio^{10}**). The same experiment performed in the presence of ATP (5 mM) or crude cell lysate (0.5 mg/ml) and ATP (5 mM) afforded the same results suggesting that a complex mixture of cellular material does not interfere with the suprastapled peptide binding its target (Figure S22).

It is well established that the interaction of MDM2 with p53 relies predominantly on three large hydrophobic residues (Leu, Trp, Phe) along the same face of the helix. This pilot library was designed to have only two out of nine members that have amino acids at all three positions displaying a suitable bulky hydrophobic side chain. Serving as a positive control, **Cys^{4}-Thio^{2}** containing the three required residues was indeed selected. However, the selection also included **Cys^{10}-Thio^{10}** which has two out of the three positions with unnatural analogs (Cba in lieu of Leu and 3,4-difluoro Phe in lieu of Phe). Importantly, the screen could be performed using 2-4 µg of protein and 0.25-1 ng of compounds in a small volume (100 µL).

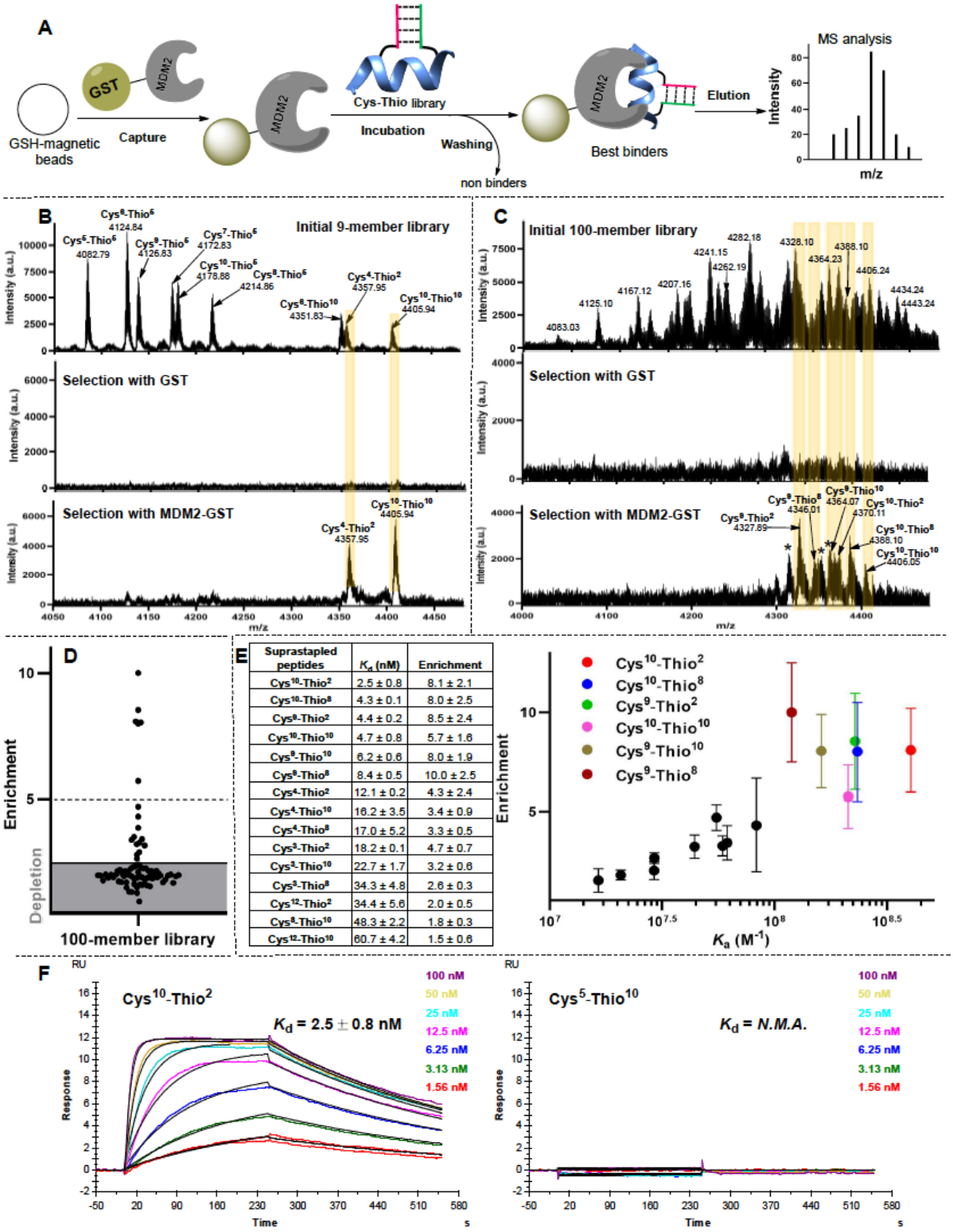


Figure 5. A) Protocol for the screening of the suprastapled library against MDM2- GST immobilized on GSH-magnetic beads. **B**-**C**) MALDI-TOF/MS analyses of suprastapled peptides selection against GST or MDM2-GST using 9-member (**B**) 100-member (**C**) libraries (*moderately enriched members [**Cys^{3}-Thio^{2}** (MW=4315.99), **Cys^{3}-Thio^{10}** (MW= 4351.98), **Cys^{4}-Thio^{2}** (MW = 4358.04)]). **D**) Scattered plot of enrichment for 100-member Suprastapled peptides determined by nanoLC-MS. **E**) Values of dissociation constant (K_d) of selected suprastapled peptides determined by SPR. Correlation of enrichment with association constant (K_a) for selected suprastapled peptides. **F**) SPR sensorgrams for best binder (**Cys^{10}-Thio^{2}**) and non-binder (**Cys^{5}-Thio^{10}**) Suprastapled peptides with K_d values. Peptide concentration for each line is given with appropriate color. *N.M.A.* = No measurable affinity.

Having validated the selection protocol, we set out to perform the selection on a library of 100 suprastapled peptides. MALDI analysis of the protein pulldown clearly showed that the selection was efficient, with no clearly distinguishable library members in the GST selection but a clear subset of library in the MDM2 positive selection

(Figure 5C). Noteworthy, some of the compounds in the library were designed not to bind MDM2. For example, **Cys^{6}-Thio^{5}** (MW: 4125) which contains Ala at all three positions of the lipophilic hotspot site, should not bind to MDM2 and it is clearly absent from the selection. The most prominent peaks correspond to **Cys^{9}-Thio^{2}** (MW: 4328), **Cys^{10}-Thio^{2}** (MW: 4370) and **Cys^{10}-Thio^{8}** (MW: 4388), which have Cba and Phe/4-F Phe in those key positions. However, screening larger library also highlighted the limitation of a MALDI-based deconvolution in which peak calling is challenging due to the limited resolution, resulting in an overlap of isotopic envelope of different members. In order to unambiguously elucidate the selected members, we took advantage of chromatic separation provided by nanoLC coupled to ESI-Orbitrap/MS that allowed us to resolve 100-member library based on their retention time and HRMS. To gain quantitative insights, the intensity of the most intense peak for each member was extracted and used to calculate relative enrichment by comparing its relative intensity in the starting library and in the selection. The result was plotted in a scattered plot, revealing 6 highly enriched suprastapled peptides followed by a group of moderately enriched suprastapled peptides (Figure 5D, and Figure S4 for the raw data), while the remaining suprastapled peptides were negatively enriched (depleted).

To unambiguously verify selected suprastapled peptides' binding affinity, the dissociation constants (K_d) were determined using SPR for all compounds showing positive enrichment (Figure 5 D). **Cys^{10}-Thio^{2}** displayed the strongest affinity (Kd = 2.5 nM, Figure 5F for sensorgram) to MDM2 which is a 5-fold improvement over the suprastapled peptide containing only naturally encoded amino acid at the key hydrophobic sites. In particular, the substitution of Leu for Cba at position 3 (gem dimethyl for a cyclobutyl) consistently led to enhanced affinity (**Cys^{10}** vs **Cys^{4}**). However, an even bulkier modification (Cha or hPhe vs Leu; **Cys^{12}** or **Cys^{8}** vs **Cys^{4}**) turned out to be detrimental. None of the peptides with Ala in lieu of Trp at position 6 (**X3**) were enriched. Position 10 (**X4**) was tolerant to fluorine substitution on the aryl ring (**Thio^{2}** vs **Thio^{8}** or **Thio^{10}**) however a Leu at that position was not tolerated with any combination at the other positions (**X1**-**X3**). The use of Lys instead of Dap as a linker to the PNA was systematically beneficial in terms of affinity across all suprastapled binders indicating that this connectivity provides a better conformational bias for an α-helix. Notably, the enrichments correlated well with the corresponding association constants (K_a) of the enriched suprastapled peptide binders for MDM2 (Figure 5E). While there is little differentiation between the top 6 hits with an affinity below 10 nM, this outcome can be expected based on modelling.[52] (Figure S5, see supporting information for the mathematical model of selection). The selection results demonstrated that positions **X4**, **X3**, and **X2** are crucial for binding to MDM2. None of the library members with an unsuitable residue at one of these sites was enriched in the screening. The affinities of 6 representative examples were nonetheless measured by SPR and indeed showed no measurable affinity. The benefit of Cba and fluorinated Phe had been previously observed by Pentelute and coworkers in libraries of unstapled peptides.[36] Taken together, these results demonstrate that suprastapled peptides can rival the conventional covalently stapled binders for MDM2, and affinity selection followed by mass spectroscopic deconvolution offers a fast-screening platform.

All suprastapled peptides discussed until this point were individually synthesized, purified, and carefully mixed to achieve an equimolar solution before being used for a selection. To test whether the proposed design is amenable for the preparation of larger suprastapled peptide libraries, we aimed to generate the library in one pot. To this end, we setup the ligation reaction between mixtures of, 10-cysteines and 10-thioesters followed by alkylation of cysteine with iodoacetamide (Figure 6A). The crude reaction mixture was desalted using a C18 Zip Tip and used directly in an affinity selection without any further purification. Importantly, this combinatorial suprastapled peptide library prepared by mixing 10 Cys peptides and 10 Thio peptide, resulted in a similar abundance of the members as with 100-member library prepared individually judged by MS analysis. No kinetic combination bias was observed, which could be the case in the absence of the templating effect of hybridization (Figure S6 and Figure S7). Finally, the selection performed with this combinatorial library against MDM2 gave comparable results to the selection with individually purified suprastapled peptides. NanoLC-MS analysis revealed the same best binders (Figure 6). Additionally, the group of moderately enriched suprastapled peptides were identified with satisfactory correlation of other affinity groups of suprastapled peptides, despite overall higher noise (Figure S8). Overall, our results suggest that a suprastapled peptide library accessible by PNA-templated NCL between mixtures of cysteine and thioester members, which are in turn readily available from split and mix combinatorial synthesis, are compatible with selection and MS analysis.

Whether such suprastapled peptides will prove to be efficiently taken up by cells across a broad peptide sequence space remains to be investigated.[53] Preliminary experiments with a Cy3-conjugate of the best binder (**Cys^{10}-Thio^{2}**) showed cellular uptake albeit with significant amounts of the fluorescence appearing in puncta (Figure S24), indicative of endosomal trapping, concurring prior observations with a covalently stapled peptide targeting MDM2.[13] The suprastapled peptide used in this pilot study made use of unmodified PNA and could benefit from functionalized PNA that have been shown to enhance cellular permeability such as GPNA.[54-55]

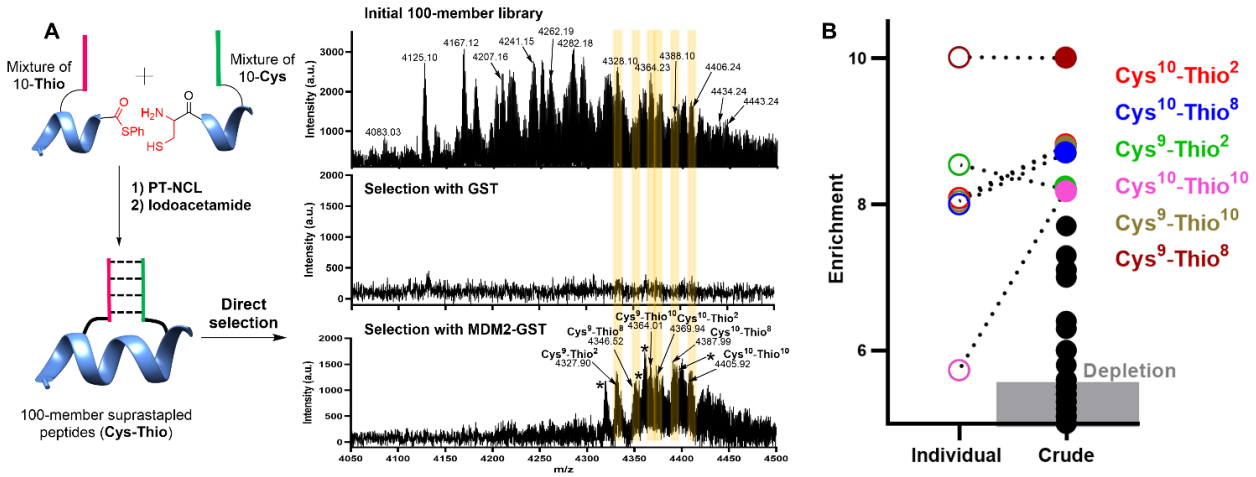


Figure 6. A) Direct selection of 100-member library, prepared by NCL reaction between mixtures of 10-cysteines and 10-thioesters without any purification, analyzed by MALDI-TOF/MS (*moderately enriched members [**Cys^3-Thio^2** (MW=4315.99), **Cys^3-Thio^{10}** (MW= 4351.98), **Cys^4-Thio^2** (MW = 4358.04), **Cys^4-Thio^{10}** (MW = 4394.02)]). **B**) Comparison of the 6-highly enriched suprastapled peptides for a 100-member library prepared individually and by mixture of 10-cysteines and 10-thioesters analyzed by nano-LCMS.

CONCLUSION

In summary, we have developed a novel supramolecular peptide stapling methodology (suprastapling) based on PNA-hybridization and validated the system by selecting binders for MDM2, a clinically relevant target. From a supramolecular stand point, the work supplements previous examples demonstrating the formation of peptide loops[40] or defensin mimics enforced by hybridization,[56] and we anticipate that this strategy can be extended to other important biological motifs such as β-sheets and hairpins.[57] From a molecular diversity perspective, the library synthesis is facilitated by hybridization-enhanced NCL between PNA-peptide cysteine and PNA-peptide thioester conjugates. To the best of our knowledge, this is the first example for the stitching and stabilization of α-helical peptides by hybridization. Importantly, the suprastapled peptide (**Cys^4-Thio^2**) exhibited remarkable stability in serum. The suprastapled peptides targeting MDM2 could be selected from libraries of 100-members using mass spectrometry to identify the tightest binders. The selections are performed in a highly miniaturized format and can be carried out with crude ligation products. We anticipate that larger libraries could be screened using this technology. To further enhance the mass spectrometry quantification, the thiol alkylation following NCL could be performed with isotopically differentiated iodoacetamides in order to screen the isotopically differentiated library in the positive and negative selection. While this was not necessary at this stage due to the chromatographic separation of mass redundant compounds, MS/MS analysis could be used to resolve the identity of co-eluting compounds. Last but not least, the cysteine could be maintained unalkylated to endow cellular permeability using emerging disulfide exchange mechanisms for cellular uptake.[58-60] Collectively, our results demonstrate that the suprastapling methodology described here is useful to synthesize peptide libraries via PNA-templated ligation reactions (e.g. NCL). While this suprastapled is larger than traditional covalent staples, we show that at least in the case of peptides targeting MDM2, this can be accommodated.

ASSOCIATED CONTENT

Supplementary figures, detailed procedures and physical characterization of compounds reported This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no conflict of interest. The raw data have been deposited on Zenodo (https://zenodo.org: **DOI**: 10.5281/zenodo.5559594).

ACKNOWLEDGMENT

This work was supported by the Swiss National Science Foundation (188406) and NCCR Chemical Biology (185898).

REFERENCES

(1) Azzarito, V.; Long, K.; Murphy, N. S.; Wilson, A. J., Inhibition of alpha-helix-mediated protein-protein interactions using designed molecules. *Nat. Chem.* **2013,** *5*, 161-173.

(2) Bullock, B. N.; Jochim, A. L.; Arora, P. S., Assessing Helical Protein Interfaces for Inhibitor Design. *J. Am. Chem. Soc.* **2011,** *133*, 14220-14223.

(3) White, C. J.; Yudin, A. K., Contemporary strategies for peptide macrocyclization. *Nat. Chem.* **2011,** *3*, 509-524.

(4) Lau, Y. H.; De Andrade, P.; Wu, Y. T.; Spring, D. R., Peptide stapling techniques based on different macrocyclisation chemistries. *Chem. Soc. Rev.* **2015,** *44*, 91-102.

(5) Cromm, P. M.; Spiegel, J.; Grossmann, T. N., Hydrocarbon Stapled Peptides as Modulators of Biological Function. *ACS Chem. Biol.* **2015,** *10*, 1362-1375.

(6) Guarracino, D. A.; Riordan, J. A.; Barreto, G. M.; Oldfield, A. L.; Kouba, C. M.; Agrinsoni, D., Macrocyclic Control in Helix Mimetics. *Chem. Rev.* **2019,** *119*, 9915-9949.

(7) Li, X.; Chen, S.; Zhang, W. D.; Hu, H. G., Stapled Helical Peptides Bearing Different Anchoring Residues. *Chem. Rev.* **2020,** *120*, 10079-10144.

(8) Bluntzer, M. T. J.; O'Connell, J.; Baker, T. S.; Michel, J.; Hulme, A. N., Designing stapled peptides to inhibit protein-protein interactions: An analysis of successes in a rapidly changing field. *Peptide Sci.* **2021,** *113*, e24191.

(9) Jackson, D. Y.; King, D. S.; Chmielewski, J.; Singh, S.; Schultz, P. G., General-Approach to the Synthesis of Short Alpha-Helical Peptides. *J. Am. Chem. Soc.* **1991,** *113*, 9391-9392.

(10) Blackwell, H. E.; Grubbs, R. H., Highly efficient synthesis of covalently cross-linked peptide helices by ring-closing metathesis. *Angew. Chem. Int. Ed.* **1998,** *37*, 3281-3284.

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

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

(13) Lau, Y. H.; Wu, Y. T.; Rossmann, M.; Tan, B. X.; de Andrade, P.; Tan, Y. S.; Verma, C.; McKenzie, G. J.; Venkitaraman, A. R.; Hyvonen, M.; Spring, D. R., Double Strain-Promoted Macrocyclization for the Rapid Selection of Cell-Active Stapled Peptides. *Angew. Chem. Int. Ed.* **2015,** *54*, 15410-15413.

(14) Assem, N.; Ferreira, D. J.; Wolan, D. W.; Dawson, P. E., Acetone-Linked Peptides: A Convergent Approach for Peptide Macrocyclization and Labeling. *Angew. Chem. Int. Ed.* **2015,** *54*, 8665-8668.

(15) Wang, Y. X.; Chou, D. H. C., A Thiol-Ene Coupling Approach to Native Peptide Stapling and Macrocyclization. *Angew. Chem. Int. Ed.* **2015,** *54*, 10931-10934.

(16) Rojas, A. J.; Zhang, C.; Vinogradova, E. V.; Buchwald, N. H.; Reilly, J.; Pentelute, B. L.; Buchwald, S. L., Divergent unprotected peptide macrocyclisation by palladium-mediated cysteine arylation. *Chem. Sci.* **2017,** *8*, 4257-4263.

(17) Li, X.; Tolbert, W. D.; Hu, H. G.; Gohain, N.; Zou, Y.; Niu, F.; He, W. X.; Yuan, W. R.; Su, J. C.; Pazgier, M.; Lu, W. Y., Dithiocarbamate-inspired side chain stapling chemistry for peptide drug design. *Chem. Sci.* **2019,** *10*, 1522-1530.

(18) Montgomery, J. E.; Donnelly, J. A.; Fanning, S. W.; Speltz, T. E.; Shangguan, X. H.; Coukos, J. S.; Greene, G. L.; Moellering, R. E., Versatile Peptide Macrocyclization with Diels-Alder Cycloadditions. *J. Am. Chem. Soc.* **2019,** *141*, 16374-16381.

(19) Ricardo, M. G.; Llanes, D.; Wessjohann, L. A.; Rivera, D. G., Introducing the Petasis Reaction for Late-Stage Multicomponent Diversification, Labeling, and Stapling of Peptides. *Angew. Chem. Int. Ed.* **2019,** *58*, 2700-2704.

(20) Ricardo, M. G.; Ali, A. M.; Plewka, J.; Surmiak, E.; Labuzek, B.; Neochoritis, C. G.; Atmaj, J.; Skalniak, L.; Zhang, R.; Holak, T. A.; Groves, M.; Rivera, D. G.; Domling, A., Multicomponent Peptide Stapling as a Diversity-Driven Tool for the Development of Inhibitors of Protein-Protein Interactions. *Angew. Chem. Int. Ed.* **2020,** *59*, 5235-5241.

(21) Ceballos, J.; Grinhagena, E.; Sangouard, G.; Heinis, C.; Waser, J., Cys-Cys and Cys-Lys Stapling of Unprotected Peptides Enabled by Hypervalent Iodine Reagents. *Angew. Chem. Int. Ed.* **2021,** *60*, 9022-9031.

(22) Diderich, P.; Bertoldo, D.; Dessen, P.; Khan, M. M.; Pizzitola, I.; Held, W.; Huelsken, J.; Heinis, C., Phage Selection of Chemically Stabilized alpha-Helical Peptide Ligands. *ACS Chem. Biol.* **2016,** *11*, 1422-1427.

(23) Iqbal, E. S.; Richardson, S. L.; Abrigo, N. A.; Dods, K. K.; Franco, H. E. O.; Gerrish, H. S.; Kotapati, H. K.; Morgan, I. M.; Masterson, D. S.; Hartman, M. C. T., A new strategy for the in vitro selection of stapled peptide inhibitors by mRNA display. *Chem. Commun.* **2019,** *55*, 8959-8962.

(24) Navaratna, T.; Atangcho, L.; Mahajan, M.; Subramanian, V.; Case, M.; Min, A.; Tresnak, D.; Thurber, G. M., Directed Evolution Using Stabilized Bacterial Peptide Display. *J. Am. Chem. Soc.* **2020,** *142*, 1882-1894.

(25) Wong, J. Y. K.; Mukherjee, R.; Miao, J. Y.; Bilyk, O.; Triana, V.; Miskolzie, M.; Henninot, A.; Dwyer, J. J.; Kharchenko, S.; Iampolska, A.; Volochnyuk, D. M.; Lin, Y. S.; Postovit, L. M.; Derda, R., Genetically-encoded discovery of proteolytically stable bicyclic inhibitors for morphogen NODAL. *Chem. Sci.* **2021**, *12*, 9694-9703.

(26) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E., Pna Hybridizes to Complementary Oligonucleotides Obeying the Watson-Crick Hydrogen-Bonding Rules. *Nature* **1993,** *365*, 566-568.

(27) Barluenga, S.; Winssinger, N., PNA as a Biosupramolecular Tag for Programmable Assemblies and Reactions. *Acc. Chem. Res.* **2015,** *48*, 1319-1331.

(28) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P., Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **1996,** *274*, 948-953.

(29) Haupt, Y.; Maya, R.; Kazaz, A.; Oren, M., Mdm2 promotes the rapid degradation of p53. *Nature* **1997,** *387*, 296-299.

(30) Brown, C. J.; Quah, S. T.; Jong, J.; Goh, A. M.; Chiam, P. C.; Khoo, K. H.; Choong, M. L.; Lee, M. A.; Yurlova, L.; Zolghadr, K.; Joseph, T. L.; Verma, C. S.; Lane, D. P., Stapled Peptides with Improved Potency and Specificity That Activate p53. *ACS Chem. Biol.* **2013,** *8*, 506-512.

(31) Phan, J.; Li, Z. Y.; Kasprzak, A.; Li, B. Z.; Sebti, S.; Guida, W.; Schonbrunn, E.; Chen, J. D., Structure-based Design of High Affinity Peptides Inhibiting the Interaction of p53 with MDM2 and MDMX. *J. Biol. Chem.* **2010,** *285*, 2174-2183.

(32) Bottger, V.; Bottger, A.; Howard, S. F.; Picksley, S. M.; Chene, P.; GarciaEcheverria, C.; Hochkeppel, H. K.; Lane, D. P., Identification of novel mdm2 binding peptides by phage display. *Oncogene* **1996,** *13*, 2141-2147.

(33) Bernal, F.; Tyler, A. F.; Korsmeyer, S. J.; Walensky, L. D.; Verdine, G. L., Reactivation of the p53 tumor suppressor pathway by a stapled p53 peptide. *J. Am. Chem. Soc.* **2007,** *129*, 2456-2457.

(34) Lau, Y. H.; de Andrade, P.; Quah, S. T.; Rossmann, M.; Laraia, L.; Skold, N.; Sum, T. J.; Rowling, P. J. E.; Joseph, T. L.; Verma, C.; Hyvonen, M.; Itzhaki, L. S.; Venkitaraman, A. R.; Brown, C. J.; Lane, D. P.; Spring, D. R., Functionalised staple linkages for modulating the cellular activity of stapled peptides. *Chem. Sci.* **2014,** *5*, 1804-1809.

(35) Quartararo, A. J.; Gates, Z. P.; Somsen, B. A.; Hartrampf, N.; Ye, X. Y.; Shimada, A.; Kajihara, Y.; Ottmann, C.; Pentelute, B. L., Ultra-large chemical libraries for the discovery of high-affinity peptide binders. *Nat. Commun.* **2020,** *11*, 3183.

(36) Touti, F.; Gates, Z. P.; Bandyopdhyay, A.; Lautrette, G.; Pentelute, B. L., In-solution enrichment identifies peptide inhibitors of protein-protein interactions. *Nat. Chem. Biol.* **2019,** *15*, 410-418.

(37) Pazgiera, M.; Liu, M.; Zou, G. Z.; Yuan, W. R.; Li, C. Q.; Li, C.; Li, J.; Monbo, J.; Zella, D.; Tarasov, S. G.; Lu, W., Structural basis for high-affinity peptide inhibition of p53 interactions with MDM2 and MDMX. *P Natl Acad Sci USA* **2009,** *106*, 4665-4670.

(38) Chang, Y. S.; Graves, B.; Guerlavais, V.; Tovar, C.; Packman, K.; To, K. H.; Olson, K. A.; Kesavan, K.; Gangurde, P.; Mukherjee, A.; Baker, T.; Darlak, K.; Elkin, C.; Filipovic, Z.; Qureshi, F. Z.; Cai, H. L.; Berry, P.; Feyfant, E.; Shi, X. G. E.; Horstick, J.; Annis, D. A.; Manning, A. M.; Fotouhi, N.; Nash, H.; Vassilev, L. T.; Sawyer, T. K., Stapled alpha-helical peptide drug development: A potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *Proc. Natl. Acad. Sci. USA* **2013,** *110*, E3445-E3454.

(39) Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H., Synthesis of Proteins by Native Chemical Ligation. *Science* **1994,** *266*, 776-779.

(40) Machida, T.; Dutt, S.; Winssinger, N., Allosterically Regulated Phosphatase Activity from Peptide-PNA Conjugates Folded Through Hybridization. *Angew. Chem. Int. Ed.* **2016,** *55*, 8595-8598.

(41) Oh, K. J.; Cash, K. J.; Lubin, A. A.; Plaxco, K. W., Chimeric peptide beacons: a direct polypeptide analog of DNA molecular beacons. *Chem. Commun.* **2007**, 4869-4871.

(42) Thurley, S.; Roglin, L.; Seitz, O., Hairpin peptide beacon: Dual-labeled PNA-peptide-hybrids for protein detection. *J. Am. Chem. Soc.* **2007,** *129*, 12693-12695.

(43) Rasmussen, H.; Kastrup, J. S.; Nielsen, J. N.; Nielsen, J. M.; Nielsen, P. E., Crystal structure of a peptide nucleic acid (PNA) duplex at 1.7 angstrom resolution. *Nat. Struct. Biol.* **1997,** *4*, 98-101.

(44) Gorska, K.; Winssinger, N., Reactions Templated by Nucleic Acids: More Ways to Translate Oligonucleotide-Based Instructions into Emerging Function. *Angew. Chem. Int. Ed.* **2013,** *52*, 6820-6843.

(45) Di Pisa, M.; Seitz, O., Nucleic Acid Templated Reactions for Chemical Biology. *ChemMedChem* **2017,** *12*, 872-882.

(46) Ficht, S.; Mattes, A.; Seitz, O., Single-nucleotide-specific PNA-peptide ligation on synthetic and PCR DNA templates. *J. Am. Chem. Soc.* **2004,** *126*, 9970-9981.

(47) Sayers, J.; Payne, R. J.; Winssinger, N., Peptide nucleic acid-templated selenocystine-selenoester ligation enables rapid miRNA detection. *Chem. Sci.* **2018,** *9*, 896-903.

(48) Middel, S.; Panse, C. H.; Nawratil, S.; Diederichsen, U., Native Chemical Ligation Directed by Photocleavable Peptide Nucleic Acid (PNA) Templates. *ChemBioChem* **2017,** *18*, 2328-2332.

(49) Zambaldo, C.; Barluenga, S.; Winssinger, N., PNA-encoded chemical libraries. *Curr. Opin. Chem. Biol.* **2015,** *26*, 8-15.

(50) Tornoe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **2002,** *67*, 3057-3064.

(51) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed.* **2002,** *41*, 2596-2599.

(52) Hulme, E. C.; Trevethick, M. A., Ligand binding assays at equilibrium: validation and interpretation. *Brit. J. Pharmacol.* **2010,** *161*, 1219-1237.

(53) Chu, Q.; Moellering, R. E.; Hilinski, G. J.; Kim, Y. W.; Grossmann, T. N.; Yeh, J. T. H.; Verdine, G. L., Towards understanding cell penetration by stapled peptides. *MedChemComm* **2015,** *6*, 111-119.

(54) Dragulescu-Andrasi, A.; Rapireddy, S.; He, G. F.; Bhattacharya, B.; Hyldig-Nielsen, J. J.; Zon, G.; Ly, D. H., Cell-permeable peptide nucleic acid designed to bind to the 5 '-untranslated region of E-cadherin transcript induces potent and sequence-specific antisense effects. *J. Am. Chem. Soc.* **2006,** *128*, 16104-16112.

(55) Saarbach, J.; Sabale, P. M.; Winssinger, N., Peptide nucleic acid (PNA) and its applications in chemical biology, diagnostics, and therapeutics. *Curr. Opin. Chem. Biol.* **2019,** *52*, 112-124.

(56) Rapireddy, S.; Nhon, L.; Meehan, R. E.; Franks, J.; Stolz, D. B.; Tran, D.; Selsted, M. E.; Ly, D. H., RID-1Mimic Containing gamma PNA Scaffold Exhibits Broad-Spectrum Antibacterial Activities. *J. Am. Chem. Soc.* **2012,** *134*, 4041-4044.

(57) Zeng, H. Q.; Yang, X. W.; Flowers, R. A.; Gong, B., A noncovalent approach to antiparallel beta-sheet formation. *J. Am. Chem. Soc.* **2002,** *124*, 2903-2910.

(58) Schneider, A. F. L.; Kithil, M.; Cardoso, M. C.; Lehmann, M.; Hackenberger, C. P. R., Cellular uptake of large biomolecules enabled by cell-surface-reactive cell-penetrating peptide additives. *Nat. Chem.* **2021**, *13*, 530-539.

(59) Gasparini, G.; Bang, E. K.; Molinard, G.; Tulumello, D. V.; Ward, S.; Kelley, S. O.; Roux, A.; Sakai, N.; Matile, S., Cellular Uptake of Substrate-Initiated Cell-Penetrating Poly(disulfide)s. *J. Am. Chem. Soc.* **2014,** *136*, 6069-6074.

(60) Laurent, Q.; Martinent, R.; Lim, B.; Pham, A. T.; Kato, T.; Lopez-Andarias, J.; Sakai, N.; Matile, S., Thiol-Mediated Uptake. *JACS Au* **2021,** *1*, 710-728.

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