Non-Covalent Peptide Stapling Using Alpha-Methyl-L-Phenylalanine for Alpha-Helical Peptidomimetics

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ABSTRACT: Peptides and peptidomimetics are attractive drug candidates because of their high target specificity and low toxicity profiles. Developing peptidomimetics using hydrocarbon (HC)-stapling or other stapling strategies has gained momentum because of their high stability and resistance to proteases, however, they have limitations. Here, we take advantage of the α–methyl group and an aromatic phenyl ring in a unique unnatural amino acid, α–methyl-L-phenylalanine (αF), and propose a novel, non-covalent stapling strategy to stabilize peptides. We utilized this strategy to create an α-helical B-chain mimetic of a complex insulin-like peptide, human relaxin-3 (H3 relaxin). Our comprehensive dataset (*in vitro*, *ex vivo* and *in vivo*) confirmed that the new high-yielding B-chain mimetic, H3B10-27(13/17αF), is remarkably stable in serum and fully mimics the biological function of H3 relaxin. H3B10-27(13/17αF) is an excellent scaffold for further development as a drug lead and an important tool to decipher the physiological functions of the neuropeptide G protein-coupled receptor, RXFP3.

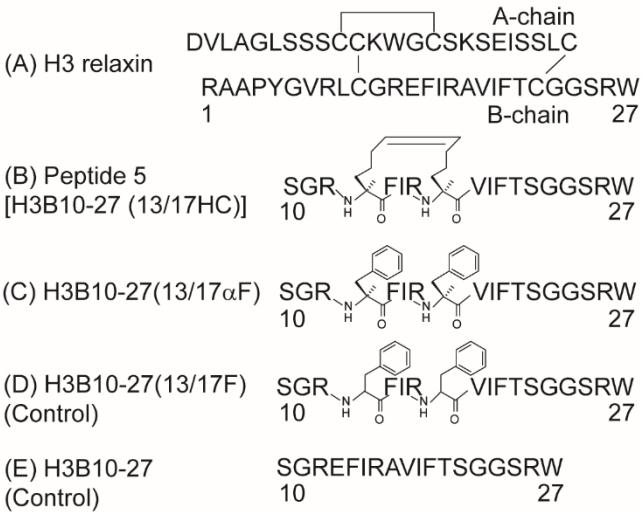
INTRODUCTION

The α–helix is the most abundant peptide structure at protein interaction interfaces involved in controlling cellular functions[1]. When these α–helical peptides are excised from proteins, they typically lose their structure and as a result, are unable to target protein interfaces. Various techniques have enabled chemists to reconstruct peptides into their natural α–helical conformation by chemical stapling[2-4] including lactam, triazole, thioether, and thioacetal bridges[3, 5]. Recently, all-hydrocarbon (HC)-stapling has been widely used[6-9] and the first HC-stapled peptides are now in clinical trials[10]. HC-stapled peptides are generated following ruthenium-catalyzed (Grubb's catalyst) ring-closing metathesis (RCM) reactions[6, 11-13]. We recently employed this method to create minimized mimetics of insulin-like relaxin family peptides that in their native forms have two chains (A and B) and three disulfide bridges[14-17] (Scheme 1A). Remarkably, an HC-stapled single-B-chain peptide (Peptide 5, Scheme 1B[17]) was able to mimic the function of the neuropeptide H3 relaxin (Scheme 1A). However, there are limitations to the HC-stapling method; the yield of the RCM product can be low if the side-chain orientation of the alkene pair is not in close proximity, and the separation of Grubb's catalyst from the product by high-performance liquid chromatography (HPLC) is difficult[14-16]. It is also challenging to control *E/Z* isomerism and distinguish between the isomers by solution NMR spectroscopy[14-16]. Other covalent stapling methods can also be low yielding as they require two-step synthesis and purification. Additionally, the rigid nature of covalent stapling may also disrupt the network of stabilizing intramolecular interactions present in the bound state of native peptides and thus may disrupt high-affinity peptidomimetic binding with the target[18]. Therefore, a novel, high-yielding non-covalent stapling strategy with one-step synthesis and purification is required to accelerate peptide-based drug development.

The α,α-disubstituted amino acids (e.g., α-aminoisobutyric acid; Aib) are known to stabilize helical peptides[5, 19]. We recently used them to create high-yielding B-chain analogues of H3 relaxin[20]. However, this approach only partially induced α–helicity and we were unable to mimic the activity of HC-stapled Peptide 5[20]. We hypothesized that the incorporation of a unique α,α–disubstituted amino acid, α–methyl-L-phenylalanine (αF), would fully mimic the α–helical structure of Peptide 5. In addition to the α–methyl group in αF that places steric constraints on the peptide backbone that favors helical dihedral angles, there should be additional hydrophobic π–π interactions between the two aromatic phenyl rings oriented towards each other on the same side of the peptide, leading to a non-covalently stapled helical peptide.

Stabilizing helical structures using (i) hydrophobic interactions[5, 21-22] or (ii) α,α–disubstituted amino acids[5, 19, 23-24] has been reported for *de novo* designed or specific peptide sequences. However, the combination of both these (i and ii) strategies to stabilize the α–helix, to our knowledge, has not been examined or reported. We therefore, synthesized peptide variants based on Peptide 5 (Scheme 1C-E). H3B10-27(13/17αF) contains two αF residues at positions 13 and 17 (Scheme 1C) and can be regarded as a "non-covalently stapled" or "π–π stapled" version of Peptide 5. Control peptides were H3B10-27(13/17F) which contains L-phenylalanine at positions 13 and 17 (Scheme 1D) and native H3B10-27 (Scheme 1E).

Primary structures of human relaxin 3 (H3 relaxin) and its B-chain analogues



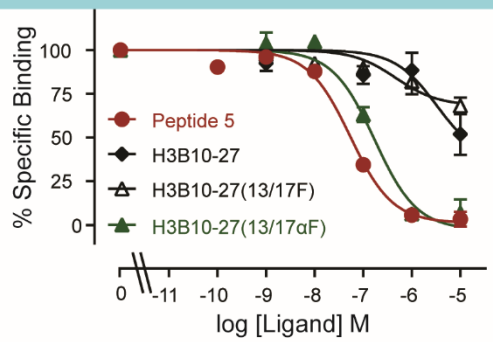
Scheme 1. Amino acid sequences of H3 relaxin (A), HC-stapled Peptide 5 (B), H3B10-27(13/17αF) (C), H3B10-27(13/17F) (D), and H3B10-27 (E).

RESULTS AND DISCUSSION

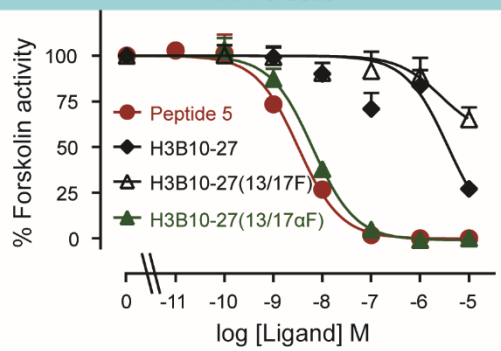
We used standard Fmoc-SPPS[25] to synthesize the peptides and purified them by reversed-phase HPLC (RP-HPLC (see S2 section in SI). They were tested in cell-based assays where H3B10-27(13/17αF) exhibited very strong RXFP3 (Figure S1) binding affinity (Figure 1A,C). In contrast, control peptides, H3B10-27(13/17F) and H3B10-27, exhibited very poor binding affinity (Figure 1A,C). Since RXFP3 is coupled to $G_{i/o}$ proteins, these analogues were tested for their ability to inhibit forskolin-stimulated cAMP activity in CHO-K1-RXFP3 cells (Figure 1B,C). Consistent with the binding data, H3B10-27(13/17αF) activated RXFP3 with similar potency to Peptide 5 while the control peptides had very low activity (Figure 1B,C). Since H3 relaxin also binds to and activates the INSL5 receptor, RXFP4, we tested RXFP4 binding and activity (see S3 section in SI). We also assessed if H3B10-27(13/17αF) retained the ability to recruit β-arrestin2[26-27] and therefore display full agonist activity at RXFP3 and RXFP4, in comparison to Peptide 5 (see S3 section in SI). Together, the binding and activity data suggest our novel analogue H3B10-27(13/17αF) can mimic the biological actions of HC-stapled Peptide 5 in both RXFP3- and RXFP4-expressing cells. However, the lower affinity and potency of both peptides on RXFP4 correlates with a lower potency in β-arrestin2 recruitment (see S3 section in SI).

As analogues that contained α,α-disubstituted amino acids alone (e,g., Aib without phenyl rings as side-chains) were unable to mimic the biological properties of RXFP3 agonists, we hypothesized that both α–methyl groups and phenyl rings of the αF residues are essential to induce α–helical conformation and thus critical to retain their biological function. Thus, we undertook solution NMR studies (see S4 section in SI) on both the target peptide H3B10-27(13/17αF) and control peptide H3B10-27(13/17F) to confirm the presence of an α–helical conformation similar to native H3 relaxin. Both peptides produced good-quality NMR data with narrow line widths, which led to a near-complete resonance assignment using standard two-dimensional sequential assignment strategies. Figure 2A and B illustrate regions of the 2D NOESY spectra for H3B10-27(13/17αF) and H3B10-27(13/17F), respectively.

(A) Eu-H3B1-22R competition binding in RXFP3 cells



(B) Inhibition of forskolin-induced cAMP activity in RXFP3 cells



(C) Pooled binding affinity (pKi) and activity (pEC50) data on the RXFP3 receptor

Ligands	RXFP3 Eu-H3B1-22R pKi (Ki) (n)	RXFP3 cAMP pEC50 (EC50) (n)
H3 relaxin	7.64 ± 0.09 (22.09 nM) (4)	9.08 ± 0.07 (0.83 nM) (5)***
R3/I5	7.77 ± 0.16 (16.98 nM) (3)*	8.89 ± 0.09 (1.28 nM) (5)**
Peptide 5	7.38 ± 0.03 (41.68 nM) (3)	8.48 ± 0.06 (3.31 nM) (5)
H3B10-27(13/17αF)	6.83 ± 0.04 (147.91 nM) (3)***	8.16 ± 0.08 (6.91 nM) (6)**
H3B10-27(13/17F)	<5 (<10 μM) (3)	<5 (<10 μM) (3)
H3B10-27	<5 (<10 μM) (3)	<5 (<10 μM) (3)

***p<0.001, **p<0.01, *p<0.05 vs Peptide 5

Figure 1. Binding and agonist activity of peptides in CHO-K1-RXFP3 cells. (A) Competition binding curves of peptides in competition with 5 nM Eu-H3B1-22R; (B) Dose-response curves demonstrating inhibition of forskolin-induced cAMP activity. Data are mean ± SEM of 3-4 independent experiments. (C) Pooled binding affinity (pKi) and cAMP potency (pEC50) data.

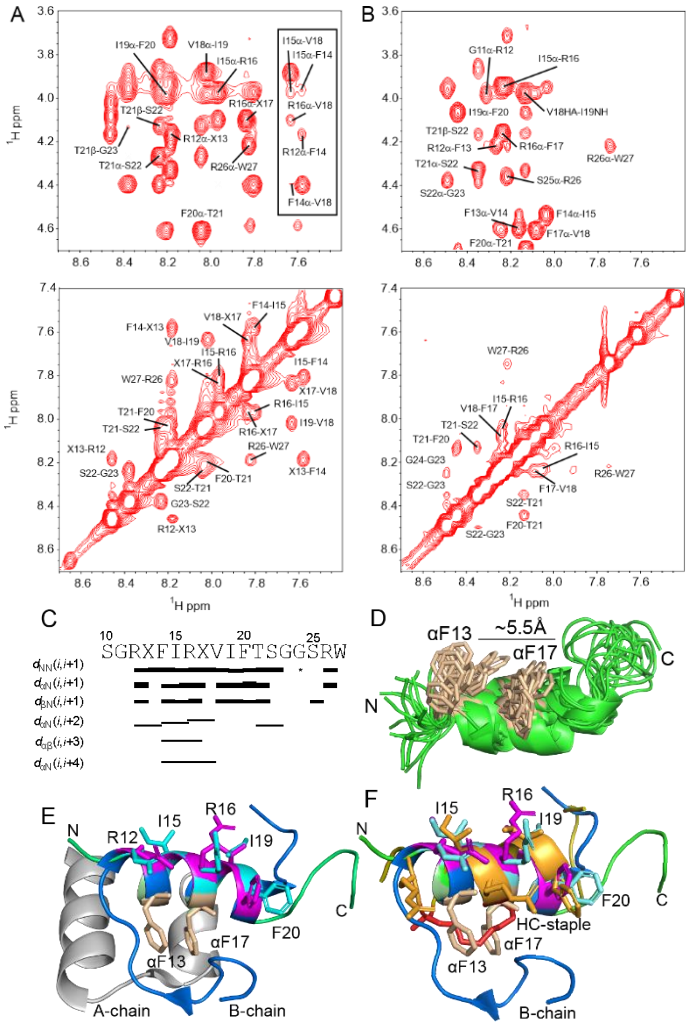


Figure 2. 2D ^{1}H ^{1}H NOESY spectra of (A) H3B10-27(13/17αF) and (B) the H3B10-27(13/17F), respectively. Boxed in (A) are assigned short-range NOEs that are observed in H3B10-27(13/17αF) but absent in the control. (C) Summary of sequential and short-range NOEs assigned for H3B10-27(13/17αF). (D) The 20 low-energy structures highlighting the orientation and proximity of the phenyl rings of αF 13 and 17 in H3B10-27(13/17αF). (E) Superposition of the NMR structure of H3 relaxin (B-chain in blue, A-chain in grey), and H3B10-27(13/17αF) (green), overlaying key residues (such as R12, I15, R16, I19 and F20) of H3 relaxin B-chain are in magenta and that of H3B10-27(13/17αF) are in cyan. (F) Superposition of the NMR structure of H3 relaxin (B-chain in blue, H3B10-27(13/17 αF) (green) and Peptide 5 (olive) overlaying key residues (R12, I15, R16, I19 and F20) of H3 relaxin B-chain are in magenta, H3B10-27(13/17αF) in cyan and Peptide 5 in light blue; αF13 and αF17 residues in H3B10-27(13/17αF) are highlighted in brown and HC stapling in Peptide 5 is highlighted in red.

For H3B10-27(13/17αF) strong HN_i-HN_{i+1} sequential signals were observed throughout the residues ranging from Arg12 – Gly23. Importantly, several short-range NOEs, $H\alpha_i$-HN_{i+2}, $H\alpha_i$-HN_{i+4}, and $H\alpha_i$-$H\beta_{i+3}$ (Figure 2A and C) were also identified within the same region, supporting the presence of a helical structure, while for the control peptide the lack of short-range NOEs suggesting this peptide comprises an unstable and transient, if any, helical structure (Figure 2B). Using structural restraints from the NMR data we calculated the structure of H3B10-27(13/17αF). Figure 2D illustrates a family of 20 low-energy structures representing the solution conformation of H3B10-27(13/17αF) with well-defined helical structure from the αF13 to F20. This structure displays the two aromatic phenyl rings of αF13 and αF17 oriented towards each other on the same side of the peptide within an average of 5.5 Å distance, which would favor the formation of hydrophobic π–π interactions that can stabilize a helical structure. Literature reports suggest that, in he specific ring arrangements and angles, the π–π stacking interactions are possible when the distance is within 7 Å[28]. However, as such interactions are also possible in the control peptide with two phenyl moieties, they are clearly insufficient to stabilize the helix. These data support the notion that α–methyl substitutions are essential to restrict peptide flexibility and put a steric constraint on the peptide backbone that favors helix formation. As shown in Figure 2E, the helical structure within the target peptide overlays onto the B-chain helix of H3 relaxin[29] with an RMSD of ~1 Å, highlighting the key RXFP3 binding residues Arg12, Ile15, Arg16 and Phe20, exposed on the surface of the helix. Our target peptide is also structurally very similar to the HC-stapled Peptide 5[17] (Figure 2F). Together, this validates the formation of helical structure in H3B10-27(13/17αF) due to the α–methyl substitution of Phe residues at positions 13 and 17 and supports the high activity of H3B10-27(13/17αF) against RXFP3 (Figure S1).

The *in vitro* serum stability was examined for 8 h at 37°C (Figure 3) and H3B10-27(13/17αF) was demonstrated to be ~15-fold more stable in human serum (half-life ~300 min) than the control peptide (half-life ~20 min). The improved stability in serum is likely due to the helical structure of the peptide but as the two unnatural amino acids (αF) cannot be recognized by proteases this may also contribute (see S5 section in SI).

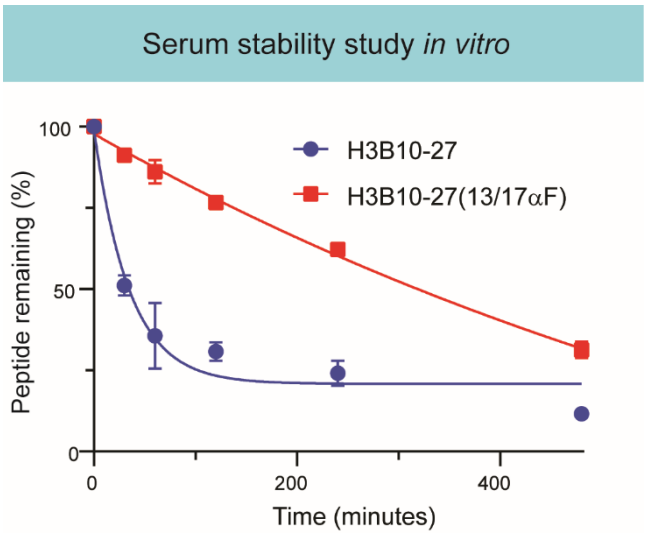


Figure 3. Our π–π stapled peptide exhibits improved stability in human serum compared with non-stapled linear peptide. There were significant differences between the peptides at all time points (P-value = 0.0088, t-test). One-phase exponential decay and paired t-test analyses were performed with GraphPad Prism 9. Data are from 3 independent experiments.

Relaxin-3 is a neuropeptide[30] and its endogenous receptor, RXFP3, is abundantly expressed in numerous regions of rodent brain consistent with innervation from relaxin-3 nerve fibres originating from the nucleus incertus, the primary site of relaxin-3 expression[31]. The physiological roles of the relaxin-3/RXFP3 system are emerging but suggest that RXFP3 agonists have potential for the treatment of neuropsychiatric disorders,

such as anxiety and depression[32-33]. To verify that H3B10-27(13/17αF) retains biological activity in animals, we examined its activity in two rat models.

We recently demonstrated that injections of an RXFP3 agonist (A2 analogue, modified form of H3 relaxin with two chains[34]) into the caudal dorsal medulla oblongata modulated respiratory rate and the arterial chemoreceptor reflex in an *in situ* perfused rat brainstem preparation[35]. Because H3B10-27(13/17αF) displayed high serum stability (Figure 3), we examined whether systemic application in the perfusate (blood substitute) could replicate these results (see S6 section in SI). Similar to centrally injected A2,[35] systemic application of H3B10-27(13/17αF) significantly increased phrenic nerve activity (PNA) 17.8 ± 1.6 to 23.0 ± 2.9 PNA-bursts/min (Figure 4; +27.7 ± 5.4%, p = 0.014, n = 6). In addition, systemic application of H3B10-27(13/17αF) also significantly enhanced the arterial chemoreceptor reflex-mediated tachypnea from 4.0 ± 0.4 tachypneic PNA bursts to 7.8 ± 0.5 PN bursts (Figure 4; p< 0.001, n = 6). This *ex vivo* data, together with *in vitro* data (Figure 1) confirmed that H3B10-27(13/17αF) mimics the pharmacological action of the RXFP3 agonists, Peptide 5 and A2.

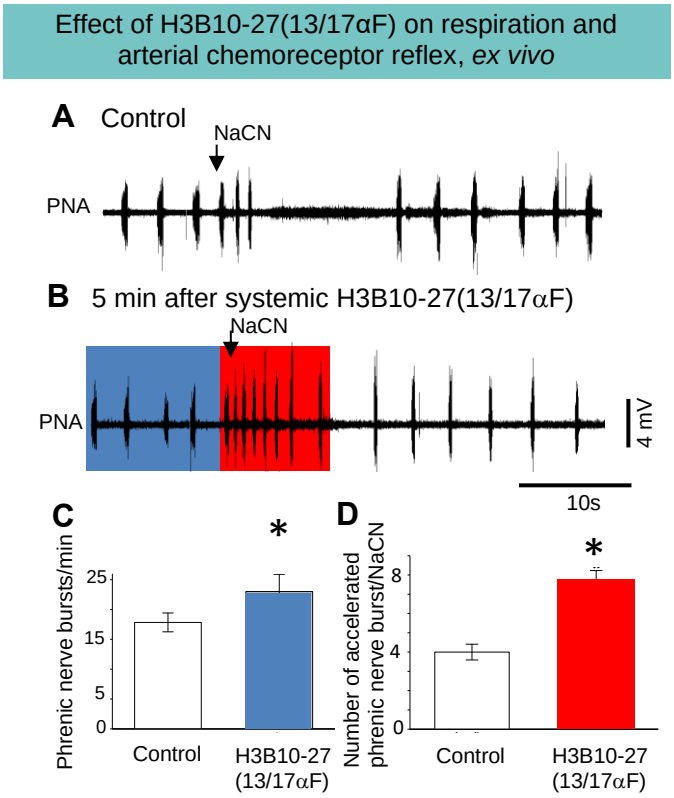


Figure 4. Effect of H3B10-27(13/17αF) on respiratory rate and the arterial chemoreceptor reflex in an *in situ* perfused rat brainstem preparation. (A) Control recording of phrenic nerve activity (PNA) before and after evoking the arterial chemoreceptor reflex with a bolus injection of sodium cyanide (NaCN, 0.2 ml, 0.01%) in the perfusion circuit (black arrow, asterisks). (B) Effect of systemic application of 2 μM H3B10-27(13/17αF) on PNA burst rate (blue) and NaCN tachypnea (red). (C,D) Group data, n = 6; **p*≤0.01.

Central (*icv*) administration of RXFP3 agonists in rats is known to increase food intake[17, 34]. Therefore, we evaluated H3B10-27(13/17αF) in adult male rats and compared its effect on food intake with A2[34] (Figure 5) (see S7 section in SI). An *icv* infusion of vehicle (aCSF) did not produce feeding or any marked ingestion of chow (Figure 5). Infusion of 0.1 nmol of H3B10-27(13/17αF) (n = 10), produced a trend towards increased food intake, relative to aCSF (n = 8), however, infusion of higher amounts of H3B10-27(13/17αF) produced significant, dose-dependent increases in food intake relative to aCSF [i.e., differences between aCSF and 0.5 nmol H3B10-27(13/17αF) (n = 10; *p* = 0.019, Mann-Whitney U test), aCSF and 1 nmol H3B10-27(13/17αF) (n = 14; *p* = 0.019, Mann-Whitney U test); aCSF and 4 nmol H3B10-27(13/17αF); p = 0.014, Mann-Whitney U test)]; or aCSF and A2 (n = 15) (p = 0.003, Mann-Whitney U test). In a further comparison, a significant difference was observed between the 0.1 nmol and 1 nmol H3B10-27(13/17αF) (*p* = 0.042; t = 2.162, df = 22; D'Agostino and Pearson normality test and unpaired t-test) (Figure 5). The maximum effect observed was comparable to that of an infusion of A2 (1 nmol A2 in 5 μl).

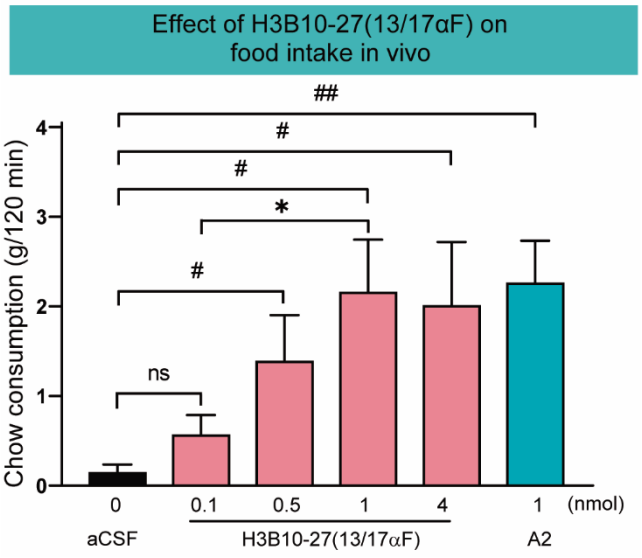


Figure 5. Effect of *icv* infusion of increasing concentrations of H3B10-27(13/17αF) on chow consumption in adult, male rats, within a 120 min period post-treatment. An *icv* infusion of A2 peptide[34] is illustrated for comparative purposes. Values are mean ± SEM. ns, non-significant; #*p*<0.05, and ##*p*<0.01, Mann-Whitney test, **p*<0.05, t-test.

Chemical synthesis of H3B10-27(13/17αF) (18 residues) was very high yielding (~60%) compared with both H3 relaxin (51 residues, yield less than 2% starting from the A-chain, 5.7% starting from the B-chain[36]) and HC-stapled Peptide 5 (18 residues, yield, ~10%). (see S8 section in SI).

In conclusion, we report a novel, non-covalent stapling strategy that utilizes the helix-inducing properties of both the α–methyl and phenyl moieties in αF. We successfully applied this by employing a pair of unique unnatural amino acids αF, to engineer an α–helical B-chain mimetic of H3 relaxin, H3B10-27(13/17αF), which demonstrated improved serum stability and full agonist activity at RXFP3 in both cell-based and animal (*ex vivo* and *in vivo*) studies. It is a much simpler scaffold, compared with H3 relaxin and Peptide 5, to improve its drug properties (e.g., *in vivo* half-life, CNS delivery). It is also an important tool to elucidate the physiological roles of H3 relaxin/RXFP3 neurocircuits. Our non-covalent stapling method, unlike covalent stapling methods, is not likely to disrupt the network of stabilizing intramolecular interactions present in the bound state of peptides, and therefore, it may have general utility in stabilizing a wide range of biologically important peptide targets.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/

Materials and methods and experimental details are provided, including protocols for peptide synthesis, purification and analysis, cell-based characterization studies, solution NMR spectroscopy, and *in vitro*, *ex vivo* and *in vivo* assays.

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