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Note

Effects of C-Terminal B-Chain Modifications in a Relaxin 3 Agonist Analogue

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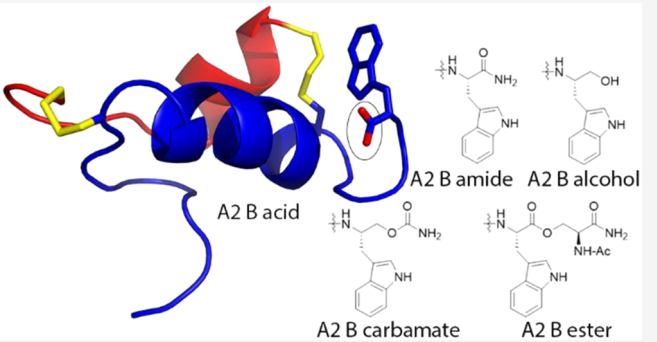
ABSTRACT: The receptor for the neuropeptide relaxin 3, relaxin family peptide 3 (RXFP3) receptor, is an attractive pharmacological target for the control of eating, addictive, and psychiatric behaviors. Several structure−activity relationship studies on both human relaxin 3 (containing 3 disulfide bonds) and its analogue A2 (two disulfide bonds) suggest that the C-terminal carboxylic acid of the tryptophan residue in the B-chain is important for RXFP3 activity. In this study, we have added amide, alcohol, carbamate, and ester functionalities to the C-terminus of A2 and compared their structures and functions. As expected, the C-terminal amide form of A2 showed lower binding affinity for RXFP3 while ester and alcohol substitutions also demonstrated lower affinity. However, while these analogues showed slightly lower binding affinity, there was no significant difference in activation of RXFP3 compared to A2 bearing a C-terminal carboxylic acid, suggesting the binding pocket is able to accommodate additional atoms.



KEYWORDS: *Insulin-like peptide, relaxin 3, H3 relaxin, GPCR, solid phase peptide synthesis, peptidomimetic*

Human relaxin 3, H3 relaxin, is a member of the relaxin hormone family.[1−4] While H3 relaxin interacts with the endogenous relaxin family peptide 3 (RXFP3) receptor, it can also bind to and activate related peptide receptors, RXFP1 and RXFP4.[3] Current literature suggests that RXFP3 is a potential therapeutic target for psychiatric disorders such as anxiety and depression.[5] Central administration of RXFP3 agonists into rats increases feeding behaviors and that of antagonists inhibits food consumption and addictive behaviors.[6] These data suggest that RXFP3 is also a potential target for pharmacological control of eating and addictive disorders.

H3 relaxin is structurally similar to insulin with two chains (A and B) and three disulfide bonds.[1,7] H3 relaxin structure−activity relationship (SAR) data suggest that the B-chain of H3 relaxin is the sole determinant of RXFP3 activity.[8,9] Briefly, R8, R12, I15, R16, and F20 are involved in receptor binding and R26 and W27 are involved in receptor activation (Figure 1).[10] A recombinant approach has been developed for efficient preparation of H3 relaxin by the Guo group.[11] However, the efficient production of full-length H3 relaxin by chemical means for SAR studies remains challenging because of its complex three disulfide bonded structure. Therefore, we simplified the structure by removing the disulfide bond from the A-chain and truncating 10 residues from the N-terminus of the A-chain. This truncation study resulted in a minimized analogue, A2.[12] This high yielding analogue exhibits H3 relaxin-like activity, both in vitro and in vivo.[12] Our SAR studies on H3 relaxin and the related INSL5 peptide revealed that a free C-terminus carboxylic acid (COOH) of the B-chain is favored for RXFP3[13] and RXFP4[14,15] receptor activity. However, this is not the case for H2 relaxin as C-terminally amidated H2 relaxin was found to be as potent as a free acid peptide in terms of RXFP1 activity.[16] Thus, the effects of C-terminal modifications are peptide and receptor-dependent.

The aim of this study was to investigate the effects of C-terminal modifications on the high yielding A2 analogue that is more easily acquired and in much higher overall yield.[12] We designed and synthesized four novel A2 analogues. Some of the modifications at the carboxylic acid level might improve the A2 peptide's stability with regard to carboxypeptidases while others were designed to reversibly hide the C-terminal to explore the possibility of a prodrug strategy (Scheme 1).

To study the effects of C-terminal modifications of the A2 peptide, we chemically synthesized four novel analogues (A2 B-alcohol, A2 B-ester, A2 B-carbamate, and A2 B-amide)

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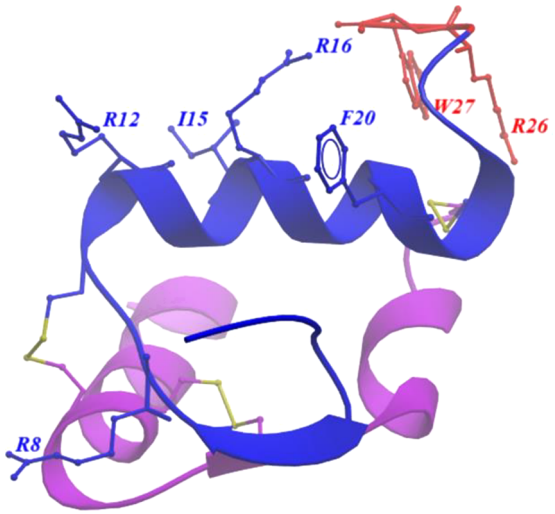
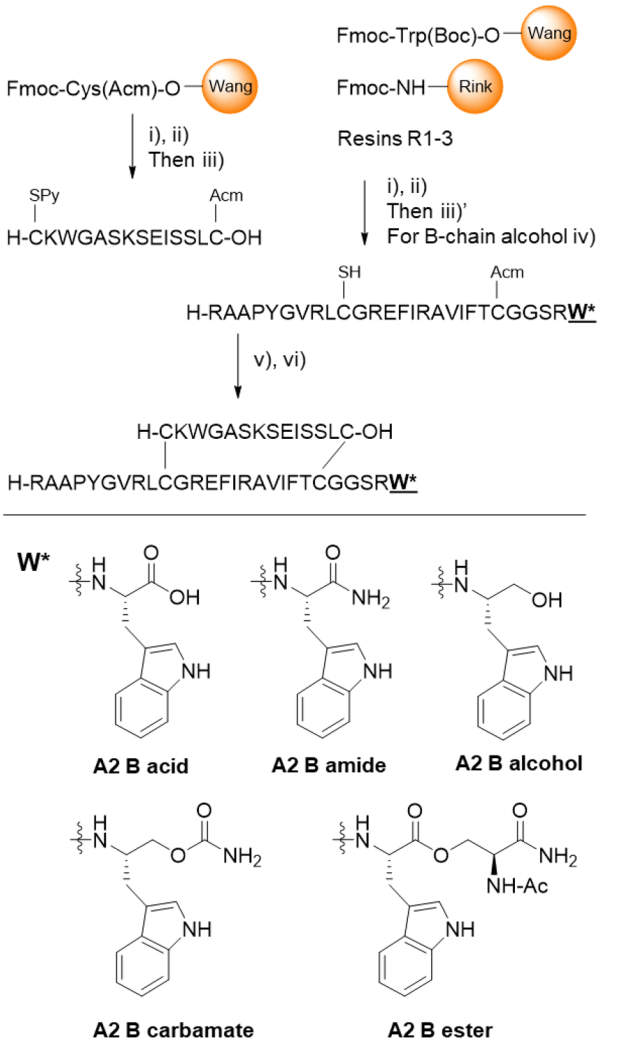


Figure 1. Human relaxin-3 representation based on the NMR data highlighting all the key residues. Purple, A-chain; blue, B-chain highlighting the amino acids involved in RXFP3 binding; red, the C-terminal dipeptide important in RXFP3 activation; yellow, disulfide bridges.

together with the parent A2 B-carboxylic acid (A2 B-acid) as control (Scheme 1). For this study, two orthogonal thiol-protecting groups (Acm and Trt) were used for each A- and B-chains.[12] The C-terminus of the B-chain was modified with different functionality while the C-terminus of the A-chain for all analogues was achieved with a carboxylic acid.

Briefly, the A-chain was generated from the preloaded Wang resin after solid phase peptide synthesis (SPPS). The sequence contains two Cys residues with different protecting groups: Cys(Trt) and Cys (Acm group). The TFA cleavage with 2,2-dipyridyl disulfide (DPDS) supplement generated the N-terminal Cys(SPy) which is ready to be conjugated with the B-chain which contains one Cys with free thiol and one Cys with Acm group. B-Chains with C-terminal amide and carboxylic acid were obtained from commercially available resin after SPPS and TFA cleavage to keep a free cysteine. Other C-terminally modified B-chains were obtained after preparing Fmoc-L-Trp(Boc)ol and the resins R1−3 (Supporting Information). Briefly, the B-chain with a C-terminus alcohol was obtained through a methodology in which the L-tryptophanol (side chain Boc protected) is loaded onto the 2-chlorotrityl resin by its amino function; the acylation of the hydroxyl group with Fmoc-L-Arg(Pbf)-OH (R1) and the SPPS afforded after TFA cleavage the iso- or depsi- peptide.[17] Then, an intramolecular O-to-N acyl transfer[18] allowed the formation of the desired product. The use of acetonitrile/PBS mixture (20:80) was proven to be efficient in order to avoid the precipitation of the final B-chain alcohol as previously used with hydrophobic sequences.[19] The C-terminus with a serinyl ester was obtained after acylation of a supported Acetyl-L-Serine by Fmoc-L-Trp(Boc)-OH (R2). After SPPS and TFA cleavage, the presence of the acyl group on the serine blocks the intramolecular O-to-N acyl transfer[18] which consequently leads to a stable ester bond in this regard. Lastly, the C-terminal carbamate was generated by loading on an unprotected Rink amide resin the carbonate obtained by reacting Fmoc-L-Trp(Boc)ol with 4-nitrophenyl chloroformate (R3).[20,21] The SPPS of the separate, selectively S-protected A- and B-chains was followed by RP-HPLC. Subsequent stepwise

Scheme 1. Solid Phase Peptide Synthesis and Regioselective Disulfide Bridge Formation to Generate A2 Analogues (Yields 3-15%; Table S1)[a]



[a]Synthetic conditions: (i) 20% piperidine in DMF, 20 min, RT; (ii) Fmoc-AA-OH, HCTU, DIEA, DMF, 1 h, RT; (iii) TFA/DODT/TIS/H_2O with 10 equiv of DSPS, 2 h, RT; (iii)′ TFA/DODT/TIS/H_2O, 2h, RT; (iv) PBS/ACN (80:20) at pH 7.4, 2 h, RT; (v) addition of A-chain in 6 M GnHCl (pH 8.5) to B-chain in 6 M GnHCl (pH 5.5), 15 min, RT; (vi) I_2, AcOH, 1 h, RT.

disulfide formation was performed by thioloysis and iodolysis leading to the successful preparation of A2 analogues. The analysis and purification of the peptides (A-chain, B-chain and two-chain peptides) were carried out by using reversed-phase high performance liquid chromatography (RP-HPLC). The peptides were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The molecular weights of the final products were as follows: A2 B-acid (A2) m/z 4546.541 $[M + H]^+$, calcd 4536.312; A2 B-ester m/z 4668.35 $[M + H]^+$, calcd 4662.249; A2 B-alcohol m/z 4540.4907 $[M + H]^+$, calcd 4543.249; A2 B-carbamate: 4569.5468 $[M + H]^+$, calcd 4563.249; A2 B-amide: m/z 4539.4366 $[M + H]$+, calcd 4535.312. The purity of these peptides (≥98%) was determined by using the peak area integration of RP-HPLC traces. The detailed characterization (trace of analytical HPLC and MALDI MS, purity, and yield)

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of these analogues is provided in the Supporting Information (Figures S1−S6; Table S1).

The analogues were tested in cell-based assays for RXFP3 binding (Figure 2). The parent A2 analogue (A2 B-acid) with a

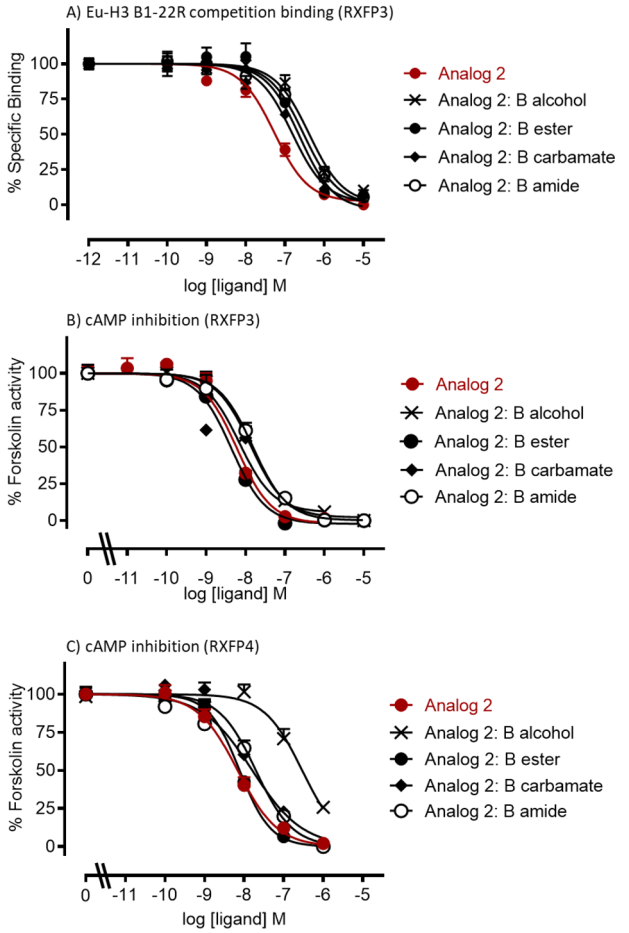

Figure 2. Competition binding (A) and cAMP activity assays of A2 and its analogues in (B) CHO-RXFP3 cells and (C) CHO-RXFP4 cells. Europium-labeled H3 B1-22R was used as the labeled ligand in the competition binding assays.

free B-chain C-terminal carboxylic acid showed higher affinity compared A2 B-amide (Figure 2A, Table 1) consistent with our previous observation that amidated B-chain analogues of H3 relaxin exhibited a lower binding affinity for RXFP3 compared with B-chain acid analogues. However, while A2 B-alcohol and A2 B-ester also showed lower binding affinity the affinity of A2 B-carbamate was not significantly different to A2 B-acid. The analogues were then tested for RXFP3 potency. As RXFP3 couples to the inhibitory G proteins Gi/o, the analogues were tested for their ability to inhibit forskolin-stimulated cAMP activation in CHO-RXFP3 cells. Interestingly, unlike H3 relaxin B-chain modified peptides,[13] while some analogues appeared to show lower potency the difference to the activity of A2 B-acid was not statistically significant (Figure 2B, Table 1). The high potency of the ester peptide (A2 B-ester) can likely be explained in terms of the prodrug principle. The reporter gene assay used to measure cAMP activity requires a 6 h stimulation during which we hypothesized that the CHO-K1 cells are liberating esterases into the media that will generate the native A2 B-acid as a metabolite from the A2 B-ester (Figure 2B). The conversion of A2 B-ester was confirmed by MALDI TOF MS spectrometric analysis of cell media at the end of stimulation which showed that a high proportion of the peptide (~30%) was converted to A2 B-acid (Supporting Information; Figure S6). This prodrug strategy can have useful application for generating long-lasting molecules in which the parent drug or at least one of its metabolites is activating RXFP3. The slightly higher potency of the A2 B-alcohol and A2 B-ester compared to their reduced affinity is also possibly due to slight differences in the stability of the peptides over the 6 h activation time of the reporter assays. The A2 B-carbamate analogue demonstrated similar potency to A2 acid, in line with its similar affinity for RXFP3. Overall, it is interesting to note that in comparison with H3 relaxin where C-terminal amide substitution resulted in 10 times lower binding and activity at RXFP3[13], the modification at the C-terminal of the B-chain is less detrimental for A2. We therefore also tested the peptides activity at the related RXFP4 receptor. The results reflected the peptides activity at RXFP3 such that all the analogues, other than the B-alcohol, showed similar activity at RXFP4 (Figure 2C, Table 1).

It is possible that the less restrained structure of the truncated peptide, which lacks a large portion of the chain and one disulfide bond, allows A2 to have a more adaptable binding mode. Similar effects are seen for linear and unstructured antagonists of RXFP3.[22] RXFP3 mutagenesis and modeling data[23] suggest that the aromatic ring of TrpB27 is stacked against the aromatic ring of RXFP3 Trp138 while the C-terminal acid is in a position to form a salt bridge across the binding pocket to the extended side chain of Lys271.[23,24] The modifications introduced here removes the negative charge of the C-terminus but retains hydrogen bond acceptors that may still interact with Lys271. The dual oxygens and the flexibility around the C−O−C bonds in the carbamate version could potentially provide two hydrogen bond acceptors in the correct arrangement for coordinating the amine group, similar to a free carboxylic acid.

Table 1. Pooled binding affinity (pK_i, pEC_{50}) Data[a]

	RXFP3 in vitro assays		RXFP4
analogues	Eu-H3 B1-22R pK_i (n)	cAMP pEC_{50} (n)	cAMP pEC_{50} (n)
H3 relaxin[12]	7.78 ± 0.06	9.0 ± 0.07	8.94 ± 0.13 (4)
A2: acid	7.34 ± 0.14 (3)	8.21 ± 0.08 (3)	8.16 ± 0.18 (4)
A2: B-ester	6.73 ± 0.03 (3)**[c]	8.36 ± 0.16 (3)	8.15 ± 0.17 (3)
A2: B-carbamate	6.96 ± 0.15 (3)	8.21 ± 0.17 (3)	7.71 ± 0.10 (3)
A2: B-alcohol	6.48 ± 0.16 (3)***[b]	7.88 ± 0.10 (3)	6.55 ± 0.12 (3)***[b]
A2: B-amide	6.72 ± 0.09 (4)**[c]	7.80 ± 0.13 (3)	7.84 ± 0.21 (3)

[a]RXFP3 and RXFP4 in vitro assays were carried out on A2 and the 4 analogues. [b]$p < 0.001$. [c]$p < 0.01$ vs analogue 2.

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Finally, we carried out circular dichroism (CD) studies to understand the effect of C-terminal modifications on their secondary structures. We thus analyzed the secondary structure of two analogues with reduced binding affinity, A2 B-ester and A2 B-amide and calculated the mean residual ellipticity (MRE) (Figure 3). The other analogues were not tested due to

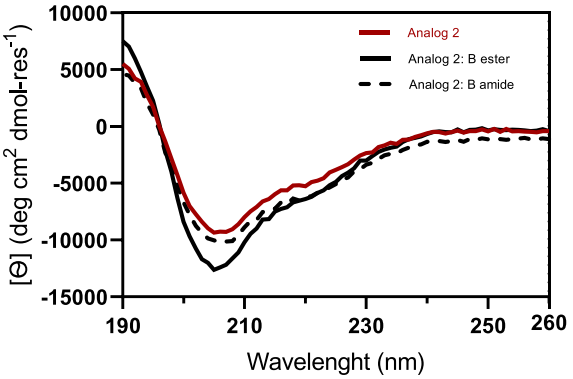

Figure 3. Circular dichroism spectroscopy overlapping showing that the overall secondary structure is maintained despite the C-terminal B-chain modification.

insufficient peptides. The CD spectra showed a typical α-helical pattern with double minima at 208 and 222 nm. There was no significant alteration in the overall secondary structure of A2 B-ester or A2 B-amide compared to A2 B-acid.

In conclusion, we prepared four novel A2 analogues bearing C-terminal modifications. We found that modifications at the C-terminus of the B-chain of A2, unlike that of parent H3 relaxin, is generally tolerable for RXFP3 cAMP potency. This result is supported by the fact that modifications did not alter the overall structure of these peptides. The serinyl ester was found to be as potent as the starting A2 analogue. Such results suggest that the A2-ester converted to A2-acid during the cell assays leading to higher potency than expected from its affinity for the receptor. This property together with the likely improved stability in serum of this modification could potentially be utilized for developing A2 analogues with longer-lasting effects for animal studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00456.

Description of peptide synthesis chemicals and reagents, amino acid and resin preparations, solid phase peptide synthesis, preparation for H3 B-chain bearing a C-terminal alcohol, regioselective disulfide bond folding, peptide characterization, in vitro assays (binding and cAMP) (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript. Conceived and designed the experiments: J.T. and M.A.H.. Performed the experiments: P.P., M.L., and J.T. Analyzed the data: R.A.D.B. and M.A.H. Contributed reagents/materials: J.D.W., R.A.D.B., and M.A.H. Wrote the paper: P.P., J.T., K.J.R., J.D.W., R.A.D.B., and M.A.H.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCR, G-protein coupled receptor; RXFP, relaxin family peptide receptor; NMR, nuclear magnetic resonance spectroscopy; SAR, structure−activity relationship; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; CD, circular dichroism; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HCTU, (2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight spectrometry; ACN, acetonitrile; AcOH, acetic acid; MRE, mean residual ellipticity; Trt, Trityl; tBu, tertiary butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; H_2O, water; DODT, 3,6-dioxa-1,8-octanedithiol; GnHCL, guanidine

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hydrochloride; PBS, phosphate buffered saline; SPPS, solid phase peptide synthesis.

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