

# **Developing peptidomimetic ligands for relaxin family peptide receptors**

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## **Abstract**

Relaxin family peptides that target G protein-coupled receptors (GPCRs), have important therapeutic applications (e.g., heart failure, fibrosis, cancer, and disorders related to psychiatry, drug addiction, eating, or colon motility). However, these family peptides have an insulin-like complex heterodimeric structure that is difficult to produce and modify to improve their pharmacokinetic properties (e.g., half-life). I have carried out structure-activity relationship (SAR) studies on these peptides leading to the generation of simplified agonists and/antagonists for the GPCRs, RXFP1, RXFP3, and RXFP4. These novel peptides are easier to prepare in larger yields and retain high affinity for their receptors. They are important research tools, and potential drug leads for the treatment of human diseases as mentioned above.

**Chapter 1** summarises the literature on the SAR studies of H2 relaxin, H3 relaxin and human INSL5 leading to the design and development of simplified agonist and antagonist.

**Chapter 2** describes all the materials and methods used in this study.

**Chapter 3** summarises the SAR studies on H2 relaxin. H2 relaxin is known to have strong anti-fibrotic, vasoprotective, angiogenic, and vasodilatory effects. The receptor for H2 relaxin, RXFP1, is a potential target for the treatment of heart failure, fibrosis and related disorders, including liver cirrhosis, and preeclampsia. Our laboratory has designed a B-chain-only analogue, B7-33, which was shown to be a potent RXFP1 agonist in cells endogenously expressing RXFP1 and in several animal models of fibrosis (mice vs rats). B7-33 is 27 residues long and has short circulation time in serum ( $t_{1/2} = \sim 6$  min). I have shown that B7-33 can be further truncated up to 4 residues, and it's *in vitro* half-life in human serum can be

increased from 6 minutes to 60 minutes. I also have developed a B7-33-based single-chain RXFP1 antagonist for the first time. These analogues are important research tools, and drug leads for the treatment of human diseases (e.g. fibrosis-related diseases, prostate, and other cancers).

**Chapter 4** summarises the SAR studies on the neuropeptide H3 relaxin. The receptor for the H3 relaxin hormone, RXFP3, is an attractive pharmacological target for the control of eating, addictive, and psychiatric behaviours. This chapter has two parts.

In the first part, I have developed four novel analogues based on two chains, and two disulfides bonded H3 relaxin analogue, also known as A2 analogue. I have introduced amide, alcohol, carbamate, and ester functionality at the C-terminus of the B-chain of A2 analogue. In terms of binding to RXFP3, the carboxylic acid remains to be the most suitable functionality. However, the cAMP response seems to be better with the C-terminal ester function. Such results highlight the possibility of generating drug candidates, as applied to H3 relaxin, to slow down the release or generating a long-acting analogue of H3 relaxin.

In the second part, I have developed an  $\alpha$ -helix stabilising chemical method. Using this method, I have produced a high yielding single-chain RXFP3 agonist and antagonist. The agonist peptide, H3B10-27-13/17 $\alpha$ F, is 20-fold more stable *in vitro* in human serum compared with the control peptides (H3B10-27-13/17EA and H3B10-27-13/17FF). The novel RXFP3 antagonist, H3B10-22R-13/17 $\alpha$ F, is only 14 amino acid long compared with the previously reported 23 residues antagonist. These analogues will facilitate the identification of physiological roles of RXFP3 and drug leads for the treatment of neurological disorders such as stress and anxiety.

**Chapter 5** summarises the SAR of insulin-like peptide 5 (INSL5), a gut hormone. Its receptor RXFP4 is a potential target for the treatment of anorexia, obesity, and colon motility disorders (e.g. constipation and diarrhoea). Our laboratory was the first to design and develop an RXFP4-specific agonist peptide known as analogue 13. However, there was no RXFP4-specific antagonist reported in the literature. The focus of this study was to utilise the non-specific RXFP3/RXFP4 antagonist,  $\Delta R3/I5$ , as a template to design an RXFP4 specific antagonist rationally. Based on analogue  $\Delta R3/I5$ , I have developed an analogue 17, which is RXFP4-selective. Analogue 17 is an ideal template for further development into a specific high-affinity RXFP4 antagonist as tool and drug leads.

**Chapter 6** summarises the findings in this study and provides future directions.



## **Declaration**

This is to certify that:

(i) The thesis comprises my original work toward the PhD except where indicated;

(ii) Due acknowledgement has been made in the text to all other material used;

and

(iii) The thesis is less than 100,000 words in length, exclusive of tables, bibliographies and appendix.

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Praveen

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## List of abbreviations

|                    |                                        |
|--------------------|----------------------------------------|
| AAD                | Allergic airways disease               |
| Ac <sub>2</sub> O  | Acetic anhydride                       |
| Aib                | Amino iso-butyric acid                 |
| Agp                | 2-amino-3-guanidino propionic acid     |
| Asp                | Aspartic acid                          |
| AT2R               | Angiotensin II type 2 receptor         |
| BSA                | Bovine serum albumin                   |
| cAMP               | Cyclic adenosine monophosphate         |
| CD                 | Circular dichroism                     |
| CDCl <sub>3</sub>  | Deuterated chloroform                  |
| CHCA               | $\alpha$ -cyano-4-hydroxycinnamic acid |
| cGMP               | Cyclic guanosine monophosphate         |
| CH <sub>3</sub> CN | Acetonitrile                           |
| CHO                | Chinese hamster ovary                  |
| CRE                | cAMP response element                  |
| CTB                | Cytotrophoblast                        |
| DBU                | 1,8-Diazabicyclo[5.4.0]undec-7-ene     |
| DCM                | Dichloromethane                        |
| DHB                | 2,5-dihydroxybenzoic acid              |
| DIC                | N,N'-Diisopropylcarbodiimide           |
| DIEA               | Diisopropylethylamine                  |
| DMAP               | Dimethylaminopyridine                  |



|            |                                                                                               |
|------------|-----------------------------------------------------------------------------------------------|
| DMF        | N, N-dimethylformamide                                                                        |
| DMR        | Dynamic mass redistribution                                                                   |
| DMSO       | Dimethyl sulfoxide                                                                            |
| DODT       | 3,6-dioxa-1,8-octanedithiol                                                                   |
| DPDS       | 2-pyridyl disulfide                                                                           |
| DTNP       | 2,2'-dithiobis(5-nitropyridine)                                                               |
| EDTA       | Ethylenediaminetetraacetic acid                                                               |
| ERK        | Extracellular signal-regulated kinase                                                         |
| EST        | Expressed sequence tag                                                                        |
| ESI        | Electrospray ionisation source                                                                |
| Eu         | Europium                                                                                      |
| Fmoc       | 9-fluorenylmethoxycarbonyl                                                                    |
| Gly        | Glycine                                                                                       |
| Glu        | Glutamic acid                                                                                 |
| GnHCL      | Guanidine hydrochloride                                                                       |
| GPCR       | G-protein coupled receptor                                                                    |
| H1 relaxin | Human relaxin 1                                                                               |
| H2 relaxin | Human relaxin 2                                                                               |
| H3 relaxin | Human relaxin 3                                                                               |
| HATU       | 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxide hexafluorophosphate |
| HBTU       | 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate                      |
| HCTU       | (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate            |

|           |                                                                         |
|-----------|-------------------------------------------------------------------------|
| HEK       | Human embryonic kidney                                                  |
| hINSL5    | Human like Insulin 5                                                    |
| HR        | Homoarginine                                                            |
| HSQC      | Heteronuclear single quantum coherence spectroscopy                     |
| ICV       | Intracerebroventricular                                                 |
| IGF1      | Insulin-like growth factor 1                                            |
| IGF2      | Insulin-like growth factor 2                                            |
| INSL3     | Insulin like peptide 3                                                  |
| INSL      | Insulin-like                                                            |
| ISO       | Isoproterenol                                                           |
| JNK       | c-Jun <i>N</i> -terminal kinases                                        |
| LDLa      | Low density lipoprotein class a                                         |
| LRR       | Leucine rich repeat                                                     |
| LSD       | Least significant difference                                            |
| LV        | Left ventricle                                                          |
| LVEDP     | Left ventricular end-diastolic pressure                                 |
| MALDI-TOF | Matrix assisted laser desorption ionization time-of-flight spectrometry |
| MAPK      | Mitogen-activated protein kinase                                        |
| MeOH      | Methanol                                                                |
| MI        | Myocardial infarction                                                   |
| mINSL5    | Mouse like insulin 5                                                    |
| MMP       | Matrix metalloproteinase                                                |
| MRE       | Mean residue ellipticity                                                |

|                   |                                                   |
|-------------------|---------------------------------------------------|
| MS                | Mass spectrometry                                 |
| NA                | No activity                                       |
| NaOH              | Sodium hydroxide                                  |
| NMR               | Nuclear magnetic resonance spectroscopy           |
| NOESY             | Nuclear overhauser effect spectroscopy            |
| NR                | Norarginine                                       |
| Palm              | Palmitic acid                                     |
| PB                | Phosphate buffer                                  |
| PBS               | Phosphate buffered saline                         |
| PDB               | Protein data base                                 |
| PEG               | Polyethylene glycol                               |
| pERK              | ERK 1/2 phosphorylation                           |
| RP-HPLC           | Reverse-phase high pressure liquid chromatography |
| rpm               | Revolutions per minute                            |
| RT                | Room temperature                                  |
| RXFP              | Relaxin family peptide receptor                   |
| SAR               | Structure activity relationship                   |
| SEM               | Standard error of mean                            |
| SPPS              | Solid phase peptide synthesis                     |
| SOCl <sub>2</sub> | Thionyl chloride                                  |
| tBu               | Tertiary butyl                                    |
| TFA               | Trifluoroacetic acid                              |
| TFE               | Trifluoroethanol                                  |

|       |                                    |
|-------|------------------------------------|
| TFMSA | Trifluoromethanesulfonic acid      |
| TGF   | Transforming growth factor         |
| TIPS  | Triisopropylsilane                 |
| TM    | Transmembrane                      |
| TOCSY | Total correlation spectroscopy     |
| Trt   | Trityl                             |
| VEGF  | Vascular endothelial growth factor |

## List of publications

1. **Praveen, P.**, Kocan, M., Valkovic, A., Bathgate, R. A. D. & Hossain, M. A. Single-chain peptide agonists of relaxin receptors. *Mol. Cell. Endocrinol.* **487**, 34–39 (2019).
2. **Praveen, P.**, Bathgate, R. A. D. & Hossain, M. A. Engineering of chimeric peptides as antagonists for the G protein-coupled receptor, RXFP4. *Sci. Rep.* 1–12 (2019). doi:10.1038/s41598-019-53707-z
3. Hossain, M. A., Okamoto, R., O., Karas, J., **Praveen, P.**, Liu, M., Forbes, B., Wade, J., Yasuhiro, K. Total Chemical Synthesis of a Nonfibrillating Human Glycoinsulin. *J. Am. Chem. Soc.* **142**, 1164–1169 (2020).
4. Welch, N. G., Mukherjee, S., Hossain, M. A., **Praveen, P.**, Werkmeister, J., Wade J. D., Bathgate R., Winkler D. A., Thissen H. Coatings Releasing the Relaxin Peptide Analogue B7-33 Reduce Fibrotic Encapsulation. *ACS Appl. Mater. Interfaces* **11**, 45511–45519 (2019).
5. Devarakonda, T., Mauro, A. G., Guzman, G., Hovsepian, S., Cain, C., Das, A., **Praveen, P.**, Hossain, M. A., Salloum, F. N. B7-33, a Functionally Selective Relaxin Receptor 1 Agonist, Attenuates Myocardial Infarction–Related Adverse Cardiac Remodeling in Mice. *J. Am. Heart Assoc.* **9**, (2020).
6. **Praveen, P.**, Tailhades, J., Rosengren, K., Wade, J., Bathgate, R. A. D. , Hossain, M. A. Effects of C-terminal B-chain modifications in a relaxin 3 agonist analog. *ACS Med. Chem. Lett.* **11**, 2336-2340 (2020).

## **List of conference presentations**

1. 13<sup>th</sup> Australian Peptide Conference 2019, 8<sup>th</sup>- 13<sup>th</sup> September 2019, Port Douglas, Qld Australia (Poster presentation)
2. 7<sup>th</sup> Modern Solid Phase Peptide Synthesis & Its Application symposium, 5<sup>th</sup> -7<sup>th</sup> September 2019, Pullman Sea Temple, Palm Cove, Qld Australia (Poster presentation)
3. 2019 Florey Graduate Research Symposium, 7<sup>th</sup> -10<sup>th</sup> May 2019 (Oral presentation)
4. 8<sup>th</sup> International Conference on Relaxin and Related Peptides, 6<sup>th</sup> -10<sup>th</sup> May 2018, Riu Palace Cabo San Lucas, Mexico (Poster and Oral presentation)
5. 2018 Florey Graduate Research Symposium, 3<sup>rd</sup> -8<sup>th</sup> May 2018 (Oral presentation)
6. 6<sup>th</sup> Modern Solid Phase Peptide Synthesis & Its Applications Symposium, 12<sup>th</sup>-14<sup>th</sup> October 2017, Kingfisher Bay Resort, Fraser Island, QLD, Australia (Poster presentation)

## **List of awards**

1. Travel award to attend 7<sup>th</sup> Modern Solid Phase Peptide Synthesis & Its Applications Symposium, 5-7<sup>th</sup> September 2019
2. Geoffrey Tregear Travel Award, 31<sup>st</sup> May 2018
3. Travel award to attend 6<sup>th</sup> Modern Solid Phase Peptide Synthesis & Its Applications Symposium, 12-14<sup>th</sup> October 2017

# **Chapter 1 Introduction**

## **1.1 Abstract**

There are seven human relaxin family peptides that have two chains (A and B) and three disulfide bonds. The target receptors for four of these peptides are known as relaxin family peptide receptors, RXFP1-RXFP4. Detailed structure-activity relationship (SAR) studies of relaxin family peptides have been reported over the years that led to the design of new analogues with agonistic and antagonistic properties. This chapter briefly summarises the SAR of human relaxin 2 (H2 relaxin), human relaxin 3 (H3 relaxin) and human INSL5 (hINSL5) leading to the design and development of simplified ligands.

## 1.2 Introduction

There are seven insulin-like relaxin family peptides in humans that include human relaxin 1 (H1 relaxin), human relaxin 2 (H2 relaxin), human relaxin 3 (H3 relaxin), insulin-like peptide 3 (INSL3 also known as a relaxin-like factor or Leydig insulin-like peptide), insulin-like peptide 4 (INSL4), insulin-like peptide 5 (INSL5) and insulin-like peptide 6 (INSL6)<sup>1-12</sup>. These peptides are known to have two peptide chains (A and B) linked by two inter-chain disulfide bonds and one intra-A-chain disulfide bond<sup>1,13,14</sup>. The relaxin peptides, INSL3 and INSL5 bind to and activate G protein-coupled receptors (GPCRs) that are now known as relaxin family peptide receptors (RXFPs)<sup>15</sup>. The receptors for INSL4 and INSL6 are unknown<sup>16</sup>.

Relaxin family peptides have potential therapeutic applications for treating many human diseases, including heart failure, fibrosis, stress-anxiety, and eating and colon motility disorders<sup>5,6</sup>. Despite their enormous promise as drugs, as they are peptides, they have short serum half-lives<sup>17-19</sup> which limits their ability to be used for chronic treatment. Each of the peptides can also cross-react with other RXFP receptors with various affinities<sup>5,6</sup> which, while probably not related to their biological function, has unknown consequences for drug targeting. Additionally, their complex, two-chain (A and B chains), three disulfide-bonded structure (with ~50 amino acids) make them expensive to manufacture and difficult to easily modify to improve their pharmacokinetic properties (e.g., bioavailability, half-life).

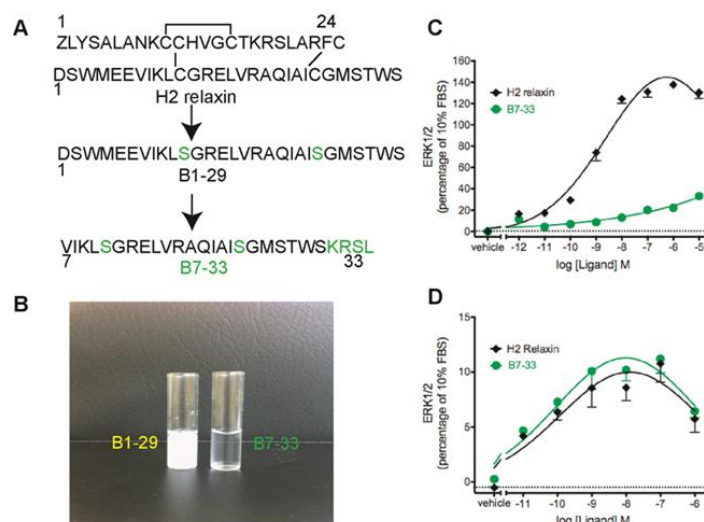
It would be ideal to develop single chain variants of these peptides which were receptor-specific and would, therefore, be more easily modified to improve their pharmacokinetic properties. Developing a receptor-specific simple peptide scaffold requires an understanding of the mechanism of peptide binding and activation. This is complicated for relaxin peptides and receptors due to the complexity of both the peptide and receptor. Extensive SAR studies on H2 relaxin/RXFP1, H3 relaxin/RXFP3 and INSL5/RXFP4 carried out over the years have led to a better understanding of these interactions and the development of simplified agonists for RXFP1<sup>20</sup>, RXFP3<sup>21</sup> and RXFP4<sup>22</sup>, single-chain antagonists for RXFP2<sup>23</sup>, RXFP3<sup>24</sup> and



non-selective antagonist for RXFP4<sup>25</sup>. However, this chapter summarises the current knowledge on the development of H2 relaxin, H3 relaxin and INSL5 based simplified agonist and antagonist<sup>20–22,24–26</sup>.

### 1.3 H2 relaxin and RXFP1

The H2 relaxin peptide contains a 24 residue A-chain and a 29 residue B-chain (**Figure 1.1A**). The endogenous receptor for H2 relaxin is RXFP1 (formerly called LGR7<sup>27</sup>) that consist of classical 7-transmembrane spanning region (TM), a large extracellular domain containing ten leucine-rich repeats (LRRs) and an *N*-terminal low-density lipoprotein type A (LDL<sub>A</sub>) module. In addition to RXFP1, H2 relaxin can also bind and activate RXFP2 (LGR8)<sup>28</sup>, the native receptor for insulin-like peptide 3 (INSL3) and has also been reported to interact with RXFP3 (GPCR 135)<sup>14</sup>, the receptor for relaxin-3.



**Figure 1.1:** Design, solubility and *in vitro* activity of B7-33. (A) Design of B7-33. Truncation of six residues from the *N*-terminus and elongation of 4 residues (KRSL) at the *C*-terminus of B1-29 resulted in a soluble B-chain derivative of H2 relaxin, B7-33. (B) The native H2 B-chain (B1-29) is insoluble (left vial) whereas the B7-33 is soluble (right vial) at concentration 4 mg mL<sup>-1</sup> H<sub>2</sub>O. (C) B7-33 weakly activated pERK1/2 in stably expressing RXFP1 (HEK-293T) cells but (D) strongly activated pERK1/2 in cells endogenously expressing RXFP1 (rat renal myofibroblasts) as shown by concentration-response curves. Figure adapted from Hossain et al. Chem Sci 2016.

### 1.3.1 Structure-activity relationship studies

Extensive SAR data<sup>29–33</sup> suggests multiple interactions between the peptide A and B-chains and the receptor necessary for RXFP1 activation. Such a complex mechanism of H2 relaxin/RXFP1 interaction poses considerable challenges for modifying and minimising the size of the peptide without altering its biological activity. However, as the B-chain of H2 relaxin contains the primary binding site (R<sup>B13</sup>, R<sup>B17</sup>, I<sup>B20</sup>), and the A-chain is believed to contain a weaker secondary binding site, there were several attempts to engineer B-chain only ligands for RXFP1<sup>20,34</sup>.

### 1.3.2 Design of a single B-chain mimetic of H2 relaxin, B7-33

The earlier attempts to mimic the helical B-chain of H2 relaxin was not successful in that all the single-B-chain analogues were found to be inactive despite some of them exhibited helical structure<sup>34</sup>. The native H2 B-chain with 29 residues (B1-29) and its cyclic derivatives were found to be insoluble (**Figure 1.1B**). Based on SAR studies data<sup>31,32</sup> an *N*-terminus truncated, and *C*-terminus elongated B-chain specific peptide, B7-33 was developed (**Figure 1.1A**)<sup>35</sup>. The highly positively charged peptide B7-33 was freely water-soluble, unlike B1-29 and its derivatives (**Figure 1.1B**). B7-33 was then tested in several cell and animal models in comparison to H2 relaxin as outlined below.

### 1.3.3 The activity of H2 relaxin and B7-33 in cells or *ex vivo*

Upon activation by H2 relaxin, RXFP1 increases activity at a variety of intracellular signalling pathways including cyclic adenosine monophosphate (cAMP) and extracellular signal-regulated kinases 1 and 2 (ERK1/2). However, the particular pathways activated depend on the type of cell being stimulated. For example, HEK293T cells expressing RXFP1 (HEK-RXFP1 cells) show robust increases in cAMP activity after relaxin stimulation<sup>27,36,37</sup>, whereas fibroblasts expressing RXFP1 show little or no cAMP activity after relaxin stimulation<sup>38,39</sup>. Additionally, chronic (48 hours or more) treatment of fibroblasts with relaxin impedes the signalling of transforming growth factor-beta 1 (TGF-β1) in order to abrogate

fibrosis, such as by suppressing the activation of fibroblasts into myofibroblasts, and up-regulating the collagen-degrading matrix metalloproteinases (MMPs)<sup>40</sup>. In rat renal myofibroblasts, H2 relaxin-induced upregulation of MMPs is dependent on RXFP1, ERK1/2, nitric oxide, and cyclic guanosine monophosphate signalling<sup>41</sup>. The anti-fibrotic effects of relaxin are also dependent on complexes formed between RXFP1 and the angiotensin II type 2 receptor (AT2R)<sup>42</sup>.

H2 relaxin and B7-33 were initially compared for activity in recombinant HEK-RXFP1 cells<sup>35</sup>. Whereas H2 relaxin potently increased cAMP accumulation, B7-33 had a substantially weaker potency (H2 relaxin pEC<sub>50</sub>: 10.49 ± 0.13; B7-33 pEC<sub>50</sub>: 5.12 ± 0.06), which is consistent with the weak affinity of B7-33 in these cells, although it was still a full agonist<sup>35</sup>. Similarly, B7-33 was also a weak full agonist for cAMP accumulation in THP-1 cells. Unlike H2 relaxin, which binds at RXFP2<sup>27</sup>, albeit with low affinity, B7-33 did not bind to HEK-RXFP2 cells even at high micromolar concentration<sup>35</sup>.

H2 relaxin and B7-33 were also compared for their ability to induce phosphorylation of ERK1/2. In HEK-RXFP1 cells, both agonists produced rapid and transient ERK1/2 phosphorylation, but in this case, B7-33 showed substantially lower efficacy as well as potency (**Figure 1.1C**)<sup>35</sup>. However, when tested for ERK1/2 phosphorylation in rat renal myofibroblasts, B7-33 surprisingly showed comparable efficacy and potency to H2 relaxin (**Figure 1.1D**). This was unlikely to be related to differences in RXFP1 between species, as B7-33 still only showed weak cAMP activity in HEK293T cells expressing mouse or rat RXFP1<sup>35</sup>. Similarly, B7-33 induced upregulation of MMP-2 to a similar degree as H2 relaxin after a 72-hour treatment, which occurred via RXFP1 as demonstrated by using an RXFP1 antagonist<sup>35</sup>. Additionally, AT2R antagonist also blocked the ability of B7-33 to up-regulate MMP-2 in rat renal myofibroblasts which demonstrates H2-like similar mechanism of action<sup>42</sup>.

The actions of B7-33 and H2 relaxin was compared in an *ex vivo* model of vascular disease using mouse mesenteric arteries pre-incubated in placental trophoblast conditioned media to induce endothelial dysfunction, characteristic of preeclampsia<sup>43</sup>. Co-incubation of these

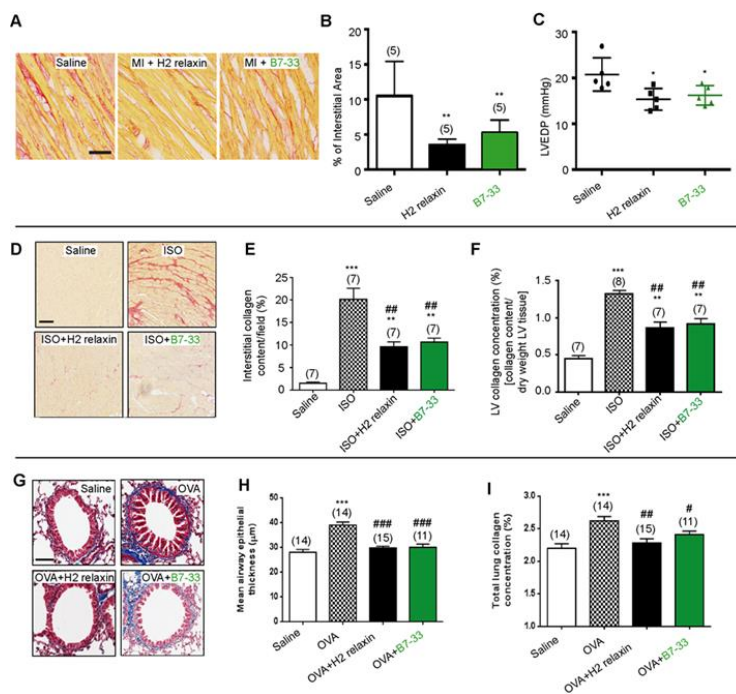
arteries in trophoblast conditioned media with B7-33 or H2 relaxin prevented the development of endothelial dysfunction<sup>43</sup>. Further studies of B7-33 signalling networks in diverse cellular backgrounds will enhance our understanding of the molecular mechanisms behind the actions of B7-33. Based on these findings in fibroblasts, and particularly its high potency in activating pERK1/2, and MMPs, the efficacy of B7-33 in several animal models was examined as outlined below.

#### **1.3.4 The activity of H2 relaxin and B7-33 in animal models**

Myocardial infarction (MI)-induced heart failure model: The efficacy of B7-33 in a pathophysiologically relevant rat model of MI-induced heart failure was examined (**Figure 1.2A-C**)<sup>35</sup>. Systemic administration of B7-33 or H2 relaxin, after eight weeks of MI-induced injury, over a four-week treatment period, significantly reduced the build-up of MI-induced collagen confirming that B7-33 had similar anti-fibrotic activity to H2 relaxin in experimental heart failure. Furthermore, improved left ventricular (LV) function, evidenced by a significant reduction in left ventricular end-diastolic pressure (LVEDP) was also observed.

Cardiomyopathy-related cardiac fibrosis model: The efficacy of systemic B7-33 administration (throughout the onset of injury; from days 1–14) in a mouse model of cardiomyopathy-related cardiac fibrosis was investigated (**Figure 1.2D-F**)<sup>35</sup> and showed that B7-33 had similar efficacy to H2 relaxin in preventing isoproterenol (ISO)-induced cardiac fibrosis, confirming its potential again as a drug candidate for the treatment of fibrosis and related dysfunction associated with heart failure.

Mouse models with ovalbumin (OVA)-induced chronic allergic airways disease (AAD): Finally, the anti-remodelling and anti-fibrotic effects of B7-33 given by intranasal administration in a nine-week OVA-induced murine model of chronic AAD *in vivo* was assessed (**Figure 1.2G-I**)<sup>35</sup>. When administered daily from weeks 9–11, B7-33 significantly diminished airway epithelial thickening, total lung collagen concentration (a measure of fibrosis) and airway hyperresponsiveness (a measure of lung dysfunction) to a similar extent



**Figure 1.2:** *In vivo* anti-fibrotic activity of B7-33. (A–C) B7-33 significantly reduced MI-induced fibrosis and improved heart function. (A) Representative photomicrographs of collagen stained with picrosirius red in LVs of the heart of rat. (B) H2 relaxin and B7-33 significantly reduced LV fibrosis post-MI compared with saline-treated rats. (C) Animals treated with H2 relaxin or B7-33 were also associated with reduced LVEDP compared to saline-treated animals. Numbers in parentheses represent the number of animals used per group. \* $p < 0.05$ , \*\* $p < 0.01$  vs. saline group. Scale bar 100  $\mu\text{m}$ . (D–F) B7-33 prevented ISO-induced cardiac fibrosis of mice. (D) Collagen was stained with picrosirius red to determine (E) % interstitial LV collagen and assessed by hydroxyproline to determine (F) total LV collagen concentration. B7-33 ( $0.25 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) significantly prevented LV fibrosis to a similar extent as native H2 relaxin ( $0.5 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) by day 14 post-ISO-induced injury. Numbers in parentheses represent the number of animals used per group. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. saline (control) group; ### $p < 0.01$  vs. ISO group. Scale bar = 50  $\mu\text{m}$ . (G–I) B7-33 reduced OVA-induced airway/lung fibrosis and improved airway function. In (G) representative photomicrographs of Masson's trichrome-stained airway/lung sections of mice. Scale bar = 50  $\mu\text{m}$ . (G–I) B7-33 reduced OVA-induced airway/lung fibrosis and improved airway function. In (G) representative photomicrographs of Masson's trichrome-stained airway/lung sections of mice. Scale bar = 100  $\mu\text{m}$ ; In (H) subepithelial collagen thickness (mm) in the lamina reticularis as mean  $\pm$  SEM. In (I) total lung collagen (hydroxyproline) content (a measure of fibrosis) from each of the groups studied. numbers in parentheses represent number of mice analysed per group; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. saline group; # $p < 0.05$ , ### $p < 0.01$ , #### $p < 0.001$  vs. OVA group. Figure adapted from Hossain et al. Chem Sci 2016.

to native H2 relaxin, confirming that it also had the therapeutic potential for chronic inflammatory lung diseases.

The most recent study compared the rapid vascular effects of an acute bolus injection of B7-33 compared with H2 relaxin<sup>43</sup>. Male Wistar rats received a tail vein injection of placebo, B7-33 or H2 relaxin (equimolar concentration). Three hours later, vascular function in the mesenteric artery was assessed by wire myography. B7-33 and serelaxin selectively enhanced bradykinin-mediated endothelium-dependent relaxation in the rat mesenteric artery by increasing endothelium-derived hyperpolarisation.

Like H2 relaxin, B7-33 (i.p. vs. s.c. vs. i.n.) showed clear efficacy in all animal models (rat and mouse) tested to date in chronic or acute settings. However, the mechanism by which B7-33 is achieving its potent cell-specific actions via RXFP1 are still to be determined.

### **1.3.5 RXFP1 antagonist**

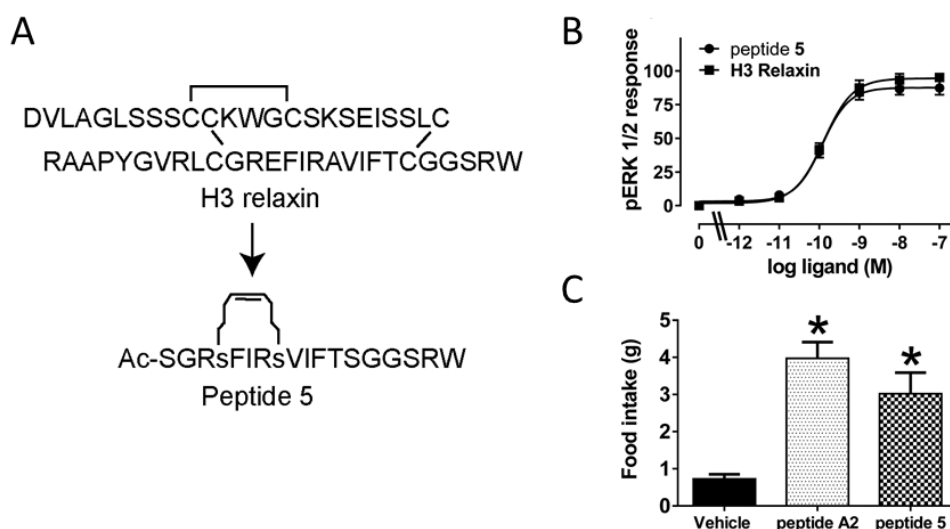
Bullesbach et al. showed the relaxin binding motif (Arg-XXX-Arg-XXX-Ile/Val) is essential for relaxin receptor binding and activity<sup>44</sup>. When arginine at 13 and 17 positions was replaced by lysine, a relaxin antagonist analogue (B-R13/17K H2) was developed which reduced the growth of prostate xenograft tumors<sup>26,45</sup>.

## **1.4 H3 relaxin and RXFP3**

H3 relaxin is predicted to have an A chain of 24 residues and a B chain of 27 residues (**Figure 1.3A**) based on the native pig relaxin-3 peptide which was extracted from brain<sup>14</sup>. It is a neuropeptide which is co-expressed in the brain with its receptor RXFP3 (also known as GPCR 135)<sup>14</sup>. H3 relaxin also strongly binds to and activates RXFP1, the native receptor for H2 relaxin, and RXFP4 (GPCR 142)<sup>46,47</sup>, the native receptor for INSL5. However, the physiological significance of these interactions is not known, especially as RXFP4 is not expressed in the brain.

### 1.4.1 Structure-activity relationship studies

SAR studies on H3 relaxin<sup>25,31,46–48</sup> and the RXFP3 receptor and modelling of the H3 relaxin/RXFP3 complex<sup>5,6,49,50</sup> have shown that the primary binding site is located within the surface of the helical domain in the B-chain. The single B-chain alone has been shown to bind to RXFP3, albeit with considerably lower affinity and potency compared to native H3 relaxin<sup>14</sup>. Although the  $\alpha$ -helix is a predominant secondary structure in the B-chain of H3



**Figure 1.3:** Design, *in vitro* and *in vivo* activity of peptide 5. (A) Design of stapled peptide 5. Hydrocarbon stapling of the truncated B-chain of H3 relaxin at positions 13 and 17 resulted in peptide 5. (B) Effect of H3 relaxin and peptide 5 on ERK1/2 kinase phosphorylation in CHO-K1-RXFP3 cells. (C) Effect of icv injection of peptide 5 (n=12), H3 relaxin agonist (A2) (n=6) and vehicle (n=12) on food intake in satiated rats. \*p<0.001 compared to vehicle treatment. Figure adapted from Hojo et al. J Med Chem 2016.

relaxin, the single B-chain alone does not adopt an  $\alpha$ -helical conformation<sup>24</sup>. Consequently, the low affinity of the B-chain was thought to be due to the lack of this secondary  $\alpha$ -helical structure that is maintained by the A-chain linked by two disulfide bridges<sup>51</sup>.

### 1.4.2 Design of a single B-chain mimetic of H3 relaxin, peptide 5

There were attempts of utilising judiciously placed salt bridges, and single and bis-cystine bridges<sup>52</sup> as potential means of stabilising the bioactive conformations of the B-chain. Unfortunately, these approaches were not successful in that the constrained analogues did not show any improvement in helical structure<sup>52</sup>. From structure-activity relationship studies on the H3 relaxin peptide, the primary binding site (R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>) is located in the same surface of the helical domain in the B-chain of H3 relaxin<sup>25</sup>. Also, the activation domain (R<sup>B26</sup> and W<sup>B27</sup>) is located at the C-terminus of the B-chain. A classical helical wheel representation reveals that two faces of the helix with the active site (R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>) is exposed on one face and the hydrophobic residues that interact with the A-chain in the native structure is exposed on the second face. Based on this analysis, Jayakody et al. carried out a ring-closing metathesis reaction to staple B18 and B22 positions and B14 and B18 positions (i, i+4)<sup>53</sup>. These stapled analogues showed poor RXFP3 activation<sup>53</sup>. Hojo et al. stapled positions B13 and B17, and B17 and B21 and deleted the N-terminal-seven residues of the B-chain which was previously found to be not crucial for RXFP3 activity<sup>52</sup>. The best position to mimic the B-chain was found to be B13 and B17. Stapling these positions (B13 and B17) together with the truncation at the N-terminus resulted in an 18 residue peptide, peptide 5 (**Figure 1.3B**), which was shown to have helical structure<sup>21</sup>. Peptide 5 was then tested *in vitro* and *in vivo* and compared its bioactivity with H3 relaxin as outlined below.

### 1.4.3 The activity of H3 relaxin and peptide 5 in cells

Upon activation, RXFP3 couples to G $\alpha_{i/o}$  proteins to inhibit the production of cAMP<sup>14,54</sup> and induce phosphorylation of ERK1/2, p38 MAPK, and JNK1/2<sup>55,56</sup>. The endogenous ligand for RXFP3, H3 relaxin, can also bind to and activate the related G $\alpha_{i/o}$ -coupled receptor RXFP4<sup>46,47</sup>, and the G $\alpha_s$ -coupled receptor RXFP1<sup>2,29</sup>.

H3 relaxin and peptide 5 were compared for their ability to activate RXFP3 in CHO-K1 cells stably expressing RXFP3 (CHO-K1-RXFP3 cells). Peptide 5 was a full agonist for inhibition of forskolin-stimulated cAMP activity and was only slightly less potent than H3 relaxin (H3



relaxin pEC<sub>50</sub>:  $9.08 \pm 0.07$ ; peptide 5 pEC<sub>50</sub>:  $8.48 \pm 0.06$ )<sup>21</sup>. Similarly, both agonists were measured for their ability to induce ERK1/2 phosphorylation, which demonstrated that peptide 5 is a full agonist for pERK1/2, and is equipotent to H3 relaxin (H3 relaxin pEC<sub>50</sub>:  $9.83 \pm 0.10$ ; peptide 5 pEC<sub>50</sub>:  $9.92 \pm 0.10$ ) (**Figure 1.3B**)<sup>21</sup>. As peptide 5 showed only slightly weaker cAMP inhibition than H3 relaxin in CHO-K1-RXFP3 cells, which was likely a result of the experimental conditions (six-hour ligand stimulation), and showed equal activity to H3 relaxin for ERK1/2 phosphorylation, peptide 5 is considered to be a true mimetic of H3 relaxin, rather than a biased agonist for RXFP3<sup>21</sup>. H3 relaxin and peptide 5 were also compared for activity at RXFP4 expressed in CHO-K1 cells (CHO-K1-RXFP4 cells), and RXFP1 expressed in HEK293T cells (HEK-RXFP1 cells). At RXFP4, peptide 5 was about 100 times less potent than H3 relaxin for cAMP inhibition, consistent with the weaker affinity of peptide 5 for RXFP4 (H3 relaxin pEC<sub>50</sub>:  $8.94 \pm 0.13$ ; peptide 5 EC<sub>50</sub>:  $6.95 \pm 0.10$ )<sup>21</sup>. Additionally, whereas H3 relaxin binds to and activates RXFP1 to increase cAMP activity, peptide 5 showed little or no cAMP activity at RXFP1<sup>21</sup>. Thus, in comparison to H3 relaxin, peptide 5 is a high-affinity agonist at RXFP3, a weaker agonist at RXFP4, and has weak or no activity at RXFP1.

#### 1.4.4 The activity of H3 relaxin and peptide 5 in animals

Effects of *icv* administration of H3 relaxin agonist (A2) and peptide 5 on food intake was measured<sup>21</sup> (**Figure 1.3C**). Peptide 5 significantly stimulated food intake, compared to control, similar to RXFP3 agonist, A2<sup>21</sup> (**Figure 1.3C**).

#### 1.4.5 RXFP3 antagonist

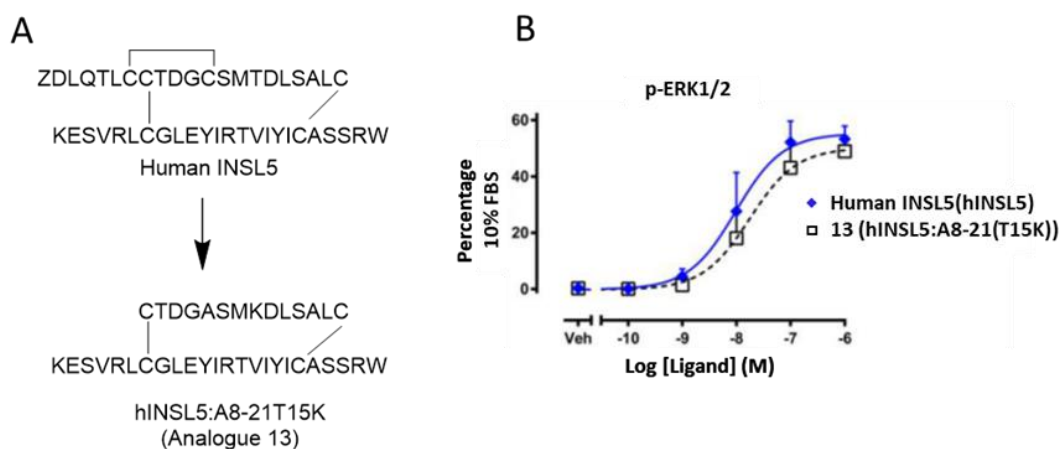
SAR studies lead to R3(BΔ23-27)R/I5 antagonist, which was selective for RXFP3 and RXFP4 over RXFP1<sup>25</sup>. Later Haugaard-Kedstrom et al. developed single-chain antagonist H3B1-22R, which is selective for RXFP3<sup>24</sup>. H3B1-22R antagonised the H3 relaxin/RXFP3 and induced increases in feeding in rats after *icv*<sup>24</sup>.

## 1.5 INSL5 and RXFP4

Insulin-like peptide 5 (INSL5) was discovered in 1999 by a search of EST databases<sup>12</sup>. This relaxin family peptide signals through the RXFP4 receptor<sup>47</sup>. INSL5 contains a 21 residue A-chain and a 24 residue B-chain<sup>57</sup> (**Figure 1.4A**). INSL5 is orexigenic hormone<sup>58</sup>, and our recent data suggest that INSL5 is also involved in colon motility<sup>59</sup>. Therefore, the INSL5 receptor RXFP4 is a promising target for developing drugs for the treatment of anorexia, obesity and constipation.

### 1.5.1 Structure-activity relationship studies

INSL5 is one of the most difficult peptides to synthesise chemically<sup>57,60</sup> or recombinantly<sup>61</sup> among its family because B-chain of human INSL5 is highly aggregating and the A-chain is susceptible to aspartimide formation due to presence of Asp-Gly in the sequence<sup>57</sup>.



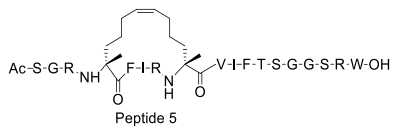
**Figure 1.4:** Design and *in vitro* activity of Analogue 13. (A) Design of analogue 13. (B) ERK1/2 phosphorylation assays on CHO-K1-RXFP4 cells of analogue 13 and human INSL5 (hINSL5). Figure adapted from Patil et al. J Med Chem 2016.

Therefore, preliminary structure-activity studies were done on mouse INSL5 (mINSL5), which has an overall higher positive charge and hence, may help in solubility<sup>60</sup>. The A-chain

of mINSL5 has Glu-Gly instead of Asp-Glu in hINSL5 which will omit aspartimide formation<sup>60</sup>. Mouse INSL5 found to be more potent than human INSL5 on human RXFP4 receptor<sup>60</sup>. Truncation and mutation studies on mouse INSL5 suggest that K<sup>B6</sup>, R<sup>B13</sup> and Y<sup>B18</sup> play an important role in binding<sup>62,63</sup>. Wang et al. showed that R<sup>B13</sup> and R<sup>B23</sup> play important roles in INSL5 binding and activity<sup>64</sup>. The free C-terminus of the B-chain was shown to be important for activation of RXFP4 receptor<sup>65</sup>. Further SAR studies led to the generation of simplified RXFP4 agonist hINSL5:8-21(T15K (Analogue 13) (**Figure 1.4A**); which can be produced in 17.4-fold higher yield than native hINSL5<sup>22</sup>. Analogue 13 showed hINSL5 like affinity and activity for RXFP4 receptor (**Figure 1.4B**)<sup>22</sup>. *In vivo* studies for analogue 13 in our lab suggested that RXFP4 agonist is a colon motility regulator<sup>59</sup>. A separate study carried out by Liu et al. demonstrated that chimeric analogue ( $\Delta$ R3/I5) acts as an antagonist for both RXFP3 and RXFP4<sup>46</sup>. This peptide consists of INSL5 A-chain and modified H3 relaxin B-chain. To date, there is no selective antagonist for RXFP4.

**Table 1.1** below summarises the simplified relaxin peptides (agonist and antagonist) we have found in the literature to date.

**Table 1.1:** Summary of native and simplified ligands of relaxin family peptides

| Peptide-Receptor pair | Native ligands                                                                                                                                                                                          | Simplified ligands                                                                                                                                                                                                                                                                                                                                                                                                            |
|-----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| H2 relaxin-RXFP1      | $\begin{array}{c} \text{Z-L-Y-S-A-L-A-N-K-C-C-H-V-G-C-T-K-R-S-L-A-R-F-C} \\ \text{D-S-W-M-E-E-V-I-K-L-C-G-R-E-L-V-R-A-Q-I-A-I-C-G-M-S-T-W-S} \end{array}$ <p style="text-align: center;">H2 relaxin</p> | <p><b>Agonist</b></p> <p>VIKLSGRELVRQAIAISGMSTWSKRSL<br/>B7-33</p> <p><b>Antagonist</b></p> $\begin{array}{c} \text{Z-L-Y-S-A-L-A-N-K-C-C-H-V-G-C-T-K-R-S-L-A-R-F-C} \\ \text{D-S-W-M-E-E-V-I-K-L-C-G-K-E-L-V-K-A-Q-I-A-I-C-G-M-S-T-W-S} \end{array}$ <p style="text-align: center;">B-R13/17K H2 relaxin</p>                                                                                                                 |
| H3 relaxin-RXFP3      | $\begin{array}{c} \text{D-V-L-A-G-L-S-S-S-C-C-K-W-G-C-S-K-S-E-I-S-S-L-C} \\ \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-G-G-S-R-W} \end{array}$ <p style="text-align: center;">H3 relaxin</p>     | <p><b>Agonist</b></p>  <p style="text-align: center;">Peptide 5</p> <p><b>Antagonist</b></p> <p>RAAPYGVRLSGREFIRAVIFTSR<br/>H3B1-22R</p>                                                                                                                                                                                                    |
| INSL5-RXFP4           | $\begin{array}{c} \text{Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C} \\ \text{K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W} \end{array}$ <p style="text-align: center;">Human INSL5</p>                | <p><b>Agonist</b></p> $\begin{array}{c} \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \\ \text{K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W} \end{array}$ <p style="text-align: center;">Analogue 13</p> <p><b>Antagonist (RXFP3 and RXFP4)</b></p> $\begin{array}{c} \text{Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C} \\ \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R} \end{array}$ <p style="text-align: center;">ΔR3/15</p> |

## 1.6 Outline and objective of this thesis

Considering the physiological importance of relaxin family peptide receptors, we need to have simplified peptide or peptidomimetic ligands, which are an invaluable tool to dissect the physiology of these peptide-receptor systems. B7-33 is a perfect template which I want to further improve as agonist and antagonist with improved therapeutic values. Considering the challenges to prepare stapled peptide 5 chemically, I want to develop a novel method to

produce high yielding RXFP3 ligands. Currently, there is no RXFP4-specific antagonist reported in the literature. Considering the vital role of INSL5 in appetite regulation and colon motility, I want to develop an RXFP4-specific antagonist.

Therefore, my thesis has the following chapters and aims:

**Chapter 1:** Introduction

**Chapter 2:** Materials and methods

**Chapter 3:** SAR studies on RXFP1 ligands: H2 relaxin, B7-33 and B-R13/17K H2

- a. Further minimisation of B7-33
- b. Improving the half-life of B7-33
- c. Developing B7-33 based potential antagonist

**Chapter 4:** SAR studies on RXFP3 ligands: H3 relaxin, A2 peptide, peptide 5, B1-22R

**Part 1:** H3 analogue A2: Effects of C-terminal B-chain modifications

**Part 2:** Development of novel RXFP3 agonist and antagonist

**Chapter 5:** SAR studies on RXFP4 ligands: INSL5, analogue 13, and  $\Delta$ R3/I5

**Chapter 6:** Conclusion and future directions

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## Chapter 2 Materials and methods

### 2.1 Materials

9-fluorenylmethoxycarbonyl (Fmoc), O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU)/ 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) were purchased from GL Biochem (Shanghai, China). Fmoc-Trp(Boc) TentaGel S PHB resin (substitution 0.24 mmol/g), Fmoc-Rink-Tentagel-resin (0.24 mmol/g) and Fmoc-Cys(Acm) TentaGel S PHB resin (substitution 0.23 mmol/g) were purchased from RAPP Polymer. Rink Amide-MBHA Resin (substitution 0.285 mmol/g and substitution 0.36 mmol/g) and chloro-2-chlorotrityl-PS-resin (0.95mmol/g) resins were purchased from GL Biochem (Shanghai, China). Trifluoroacetic acid (TFA) was obtained from Auspep (Melbourne, Australia). Acetonitrile, dichloromethane, piperidine, diethyl ether, N,N'-dimethylformamide (DMF) and methanol were from Merck (Australia). 2-pyridyl disulfide (DPDS) was purchased from Fluka (Switzerland). Fmoc- $\alpha$ -methyl-L-phenylalanine, Fmoc-L-HomoArg(Pbf)-OH and Fmoc-L-NorArg(Pbf)-OH were purchased from CHEM IMPEX INT'L INC. Fmoc-Agp(Boc)<sub>2</sub>-OH was purchased from Novabiochem. Palmitic acid, myristic acid, decanoic acid, TIPS (triisopropylsilane), DODT (3, 6-dioxa-1, 8-octanedithiol), anisol, Dimethylaminopyridine (DMAP), sinapinic acid (3,5-dimethyl-4-hydroxycinnamic acid), CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid), DHB (2,5-dihydroxybenzoic acid) and human serum were purchased from Sigma-Aldrich (Australia). Fmoc-L-Lys(Palm-L-Glu-OtBu)-OH and Fmoc-L-Lys(Palm)-OH were purchased from Iris Biotech GMBH. Fmoc-NH-PEG-COOH, Fmoc-NH-(PEG)3-COOH, Fmoc-NH-(PEG)5-COOH and Fmoc-NH-(PEG)11-COOH were purchased from Merk-millipore. 3-(Tritylthio)propionic acid was purchased from GL Biochem (Shanghai, China). All other reagents were from Sigma-Aldrich (Australia). 9-fluorenylmethoxycarbonyl (Fmoc) protected D- $\alpha$ -amino acids were purchased from GL Biochem (Shanghai, China).

## 2.2 Solid phase peptide synthesis

All the single-chain peptides were synthesised using Fmoc solid phase synthesis on microwave assisted liberty peptide synthesiser (CEM Liberty, Mathew, USA) or manually using Fmoc protected L- $\alpha$ -amino acids. Some polypeptide chains were assembled on pre-loaded resins using Fmoc solid phase synthesis, and some polypeptide chains were assembled on Rink amide resins. All side chain-protecting groups of amino acids were TFA-labile, except for tert-butyl (tBu)-protected and acetamidomethyl (Acm)-protected cysteine. The peptides were synthesised on either 0.1 or 0.2 mmol scale using instrument default protocols or manually with a 4-fold molar excess of Fmoc-protected amino acids activated by 4-fold excess of HCTU/HATU in the presence of excess (6-fold) of diisopropylethelene amine (DIEA). N $\alpha$ -Fmoc protecting groups were removed by treating the resin attached peptide with piperidine (20% v/v) in DMF. Using the microwave synthesiser, the coupling and deprotection were carried out at 75 °C using 25 W microwave power for 5 minutes and 60 W microwave power for 5 or 3 minutes, respectively. For manual synthesis, the coupling and deprotection were carried out for 60 minutes and 5 minutes three times, respectively.

## 2.3 Peptide cleavage from the solid support

After completion of solid phase synthesis, peptides were cleaved by using a cleavage cocktail of trifluoroacetic acid (TFA): TIPS (triisopropylsilane): water/DODT (3, 6-dioxa-1, 8-octanedithiol): Anisol (94: 1: 2: 3) for 2 hours. Cleavage solution was filtered using a filter and evaporated under nitrogen. The cleaved peptide was precipitated in cold ether and centrifuged for 5 minutes; this step was repeated at least four times.

## 2.4 Peptide purification and characterisation

Analytical RP-HPLC analysis of the purified peptides was performed with Waters RP-HPLC systems. Empower software was used for data collection, monitoring, and analysis. The RP-HPLC profiles were acquired using a Phenomenex Gemini C18 analytical column (4.6  $\times$  250 mm, pore size 110 Å, particle size 5  $\mu$ m), at a constant flow rate of 1.5 mL/min, in a gradient

mode with buffer A, 0.1% TFA in water, and buffer B, 0.1% TFA in acetonitrile, monitoring at a wavelength of 214 nm, which is characteristic for the amide bond. All HPLC purifications were performed using a Phenomenex C18 preparative column ( $21.2 \times 150$  mm), at a constant flow rate of 10mL/min, in a gradient mode with eluent A, 0.1% TFA in water, and eluent B, 0.1% TFA in acetonitrile.

Peptide characterisation was carried out on a Bruker Ultraflex II instrument (Bruker Daltonics, Bremen, Germany) MALDI-TOF MS (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry). Sinapinic acid (3,5-dimethyl-4-hydroxycinnamic acid), CHCA ( $\alpha$ -cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid) in 0.1% TFA in ACN/water (7:3) were used as matrix.

Some spectra were recorded using Thermo Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with high voltage electrospray ionisation source (H-ESI) for high resolution and accurate mass analysis, methods used for mass are 200 to 2000 m/z and 400 to 4000 m/z, in positive-ion mode.

## **2.5 Formation of disulfide bonds**

### **2.5.1 A-chain oxidation (formation of intramolecular disulfide bridge)**

Crude A-chain was dissolved in deionised H<sub>2</sub>O/ACN (8:2 v/v, 2 ml/ $\mu$ mol). Dropwise DIEA (base) was added to the mixture to have complete dissolution. To this solution, one equivalent of 2-pyridyl disulfide (DPDS) in MeOH (methanol) was added, and the reaction was stirred. The progress was monitored by analytical RP-HPLC analysis, which confirmed that the reaction was completed in 1-2 hours at RT or heating if required. The solution was loaded onto a preparative RP-HPLC column. The peptide was purified and freeze-dried to give pure intramolecular disulfide-bond A-chain.

### **2.5.2 A-chain conversion of Cys(tBu) to Cys(Pyr)**

The purified A-chain and DPDS (4 equivalents) were dissolved in TFA/anisole (9:1 v/v). The solution was chilled on ice before adding (TFMSA)/TFA (1:4 v/v). The reaction was allowed to continue for 45 minutes or 60 minutes at 0°C before the peptide was collected by chilled diethyl ether precipitation followed by centrifugation at least five times, and purified on a preparative RP-HPLC column. The freeze-dried purified product weighed and was identified as A-chain having one intramolecular disulfide bridge.

### **2.5.3 Combination of A-chain with B-chain (formation of second disulfide bridge)**

The A-chain was dissolved in 6M GnHCl, 0.1 M Gly/NaOH buffer (pH 8.5). The B-chain was dissolved in 6M GnHCl, buffer (pH 5) and added slowly to the A-chain solution. Analytical HPLC/MALDI monitored the reaction. The product was purified by preparative RP-HPLC and freeze dried to give combination A-B product.

### **2.5.4 Iodine oxidation (formation of third disulfide bridge)**

The A-B peptide was dissolved in a solvent mixture of acetic acid (3.15 ml/ $\mu$ M) and 60 mM HCl (0.377 ml/ $\mu$ M). Then 20 mM iodine/acetic acid (4.2 ml/ $\mu$ M) were added to the solution. Analytical HPLC monitored the reaction. After 45 minutes, the reaction was stopped by addition of ice-cold diethyl ether. The precipitate peptide was purified by preparative RP-HPLC, and freeze-dried to give pure final peptide containing three disulfide bonds.

## **2.6 Circular dichroism (CD) spectroscopy**

CD spectra data of all peptides were recorded using an Aviv Model 410 (Piscataway, NJ) spectrophotometer at 25° C using a 1 mm path length cell. The peptides were dissolved in 10 mM phosphate buffer at pH 7.5 and 20% TFE (2,2,2-Trifluoroethanol) in 10 mM phosphate buffer. The parameters used to obtain the spectra were wavelengths 190 to 260 nm with a

data pitch of 0.1 nm, continuous scanning mode at a speed of 50 nm per minute and the number of accumulations taken per peptide was 3. The concentration of peptide used was 0.2 µg/µl.

## **2.7 *In vitro* serum stability assay**

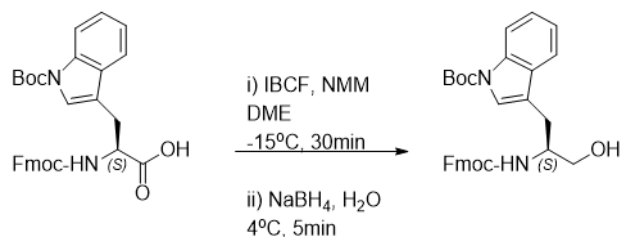
400 µl of human serum was taken, and 200 µg peptide dissolved in 100 µl of water was added to human serum at 37°C. 80 µl of samples were removed at various time points (0 min, 30 min, 60 min, 120 min, 240 min, and 480 min), and 160 µl of acetonitrile was added to precipitate plasma proteins. The solution was then centrifuged at 12000 rpm for 15 minutes at 4°C. 200 µl of supernatant was taken out and mixed with 240 µl of milli-Q water, each sample was analysed by RP-HPLC (2 x 80 µL injection), by measuring the area under the peak at appropriate retention time compared to the peak area of the peptide at time zero minute (100%). Data analysis (n = 3, duplicate) was performed using Prism Version 8.0.2 a non-linear fit one-phase decay model.

## **2.8 Amino acid analyses**

The peptide content was determined using Direct Detect® assay-free sample cards and the Direct Detect® spectrometer. Each card contains hydrophilic spots surrounded by a hydrophobic ring to retain the analysed sample within the IR beam for convenient sample application and analysis. All measurements were performed using 2 µL of the sample solution.

## 2.9 Amino acid and resin preparations (performed by Dr Julien Tailhades)

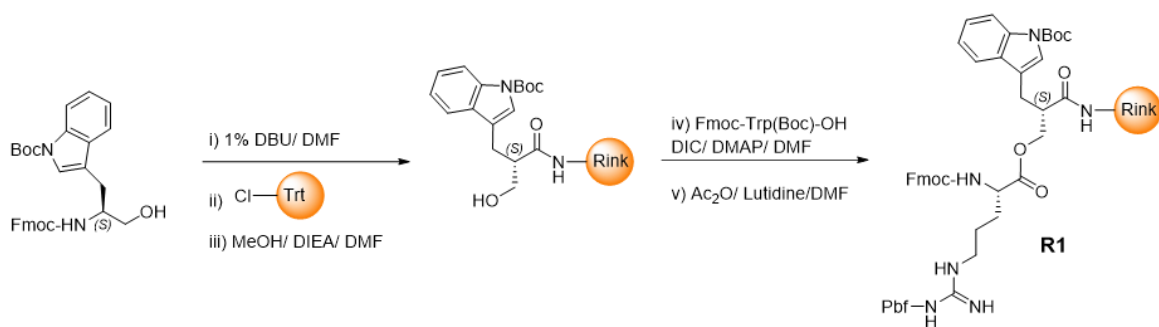
### 2.9.1 Fmoc-Trp(Boc)ol preparation



**Scheme 2.1:** Preparation of Fmoc-Trp(Boc)ol

To a cold ( $-15^{\circ}\text{C}$ ) solution of Fmoc-Trp(Boc)-OH (2.5mmol, 1.3g) in 1,2-dimethoxyethane (25ml) were successively added N-methylmorpholine (3mmol, 330 $\mu\text{l}$ ) and isobutylchloroformate (3mmol, 389 $\mu\text{l}$ ). After 30 minutes, the precipitate was filtered off, and a solution of  $\text{NaBH}_4$  (2 eq, 189mg) in water (5ml) was added at once (**Scheme 2.1**). The mixture was poured into a saturated aqueous  $\text{NaHCO}_3$  solution. Then, the Fmoc-Trp(Boc)ol was extracted three times with ethyl acetate. The combined ethyl acetate phases were washed with a 1 M solution of  $\text{KHSO}_4$ , brine and dried over anhydrous  $\text{MgSO}_4$ . After removal of the solvent under reduced pressure, a solid residue was obtained. Yield 0.92 g (78%). The compound was straightaway used for the resin preparation as outlined below.

### 2.9.2 Resin preparation for H3 B-chain bearing a C-terminal alcohol group (R1)

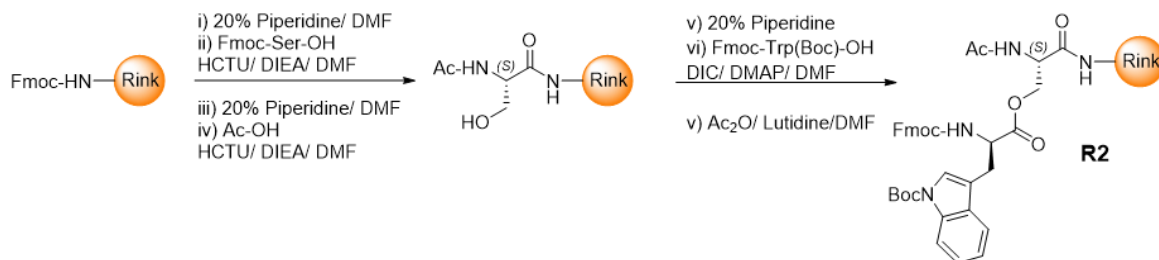


**Scheme 2.2:** Preparation of the iso-dipeptide resin for SPPS



Resin preparation: 500 mg of chloro-2-chlorotrityl resin (0.95mmol/g) was pre-activated with  $\text{SOCl}_2$  (0.5eq, 36 $\mu\text{l}$ ) in anhydrous DCM for 30 minutes. After filtration and washing with anhydrous DCM ( $5 \times 5\text{ml}$ ), a solution of Fmoc-Trp(Boc)ol (0.15mmol, 77mg) in 2% DBU in DMF (5ml) was added on the pre-activated chloro-2-chlorotrityl resin for 2 hours. After filtration and washing with DMF ( $3 \times 5\text{ml}$ ), the remaining chlorotrityl functions were capped with a solution MeOH/DIEA/DMF 5ml (5/5/90, v/v'/v'') for 15 minutes. Then, Fmoc-Arg(Pbf)-OH (0.5 mmol, 324mg) was pre-activated with DIC (0.25mmol, 37  $\mu\text{l}$ ) in 5ml of anhydrous DMF at 4°C for 30 minutes before to be added on the pre-loaded resin together with DMAP (0.025mmol, 3mg). Finally, the remaining alcohol functions were capped with a solution  $\text{Ac}_2\text{O}$ /Lutidine/DMF (5/6/89, v/v'/v'') for 15 minutes (**Scheme 2.2**)<sup>1</sup>.

### 2.9.3 Resin preparation for H3 B-chain bearing a C-terminal serinyl ester group (R2)

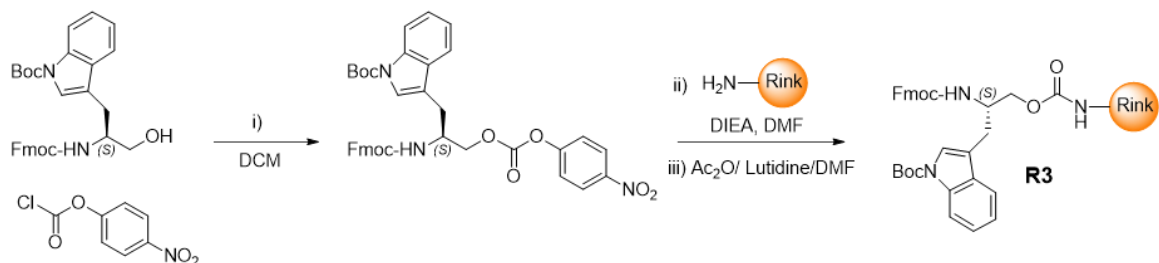


**Scheme 2.3:** Preparation of the serinyl ester resin for SPPS.

500 mg of rink amide resin (0.24mmol/g) was loaded after Fmoc removal with Fmoc-Ser-OH (0.15mmol, 49mg) pre-activated with HCTU (0.15mmol, 62mg) and DIEA (0.3mmol, 52 $\mu\text{l}$ ). After the Fmoc group removal, the amino group was capped with acetic acid (0.3mmol, 17 $\mu\text{l}$ ) pre-activated with HCTU (0.3mmol, 124mg) and DIEA (0.6mmol, 104mg). Then, Fmoc-Trp(Boc)-OH (0.5 mmol, 263mg) was pre-activated with DIC (0.25mmol, 37  $\mu\text{l}$ ) in 5ml of anhydrous DMF at 4°C for 30 minutes before to be added on the pre-loaded

resin together with DMAP (0.025mmol, 3mg). Finally, the remaining alcohol functions were capped with a solution  $\text{Ac}_2\text{O}$ /Lutidine/DMF (5/6/89, v/v'/v'') for 15 minutes (**Scheme 2.3**).

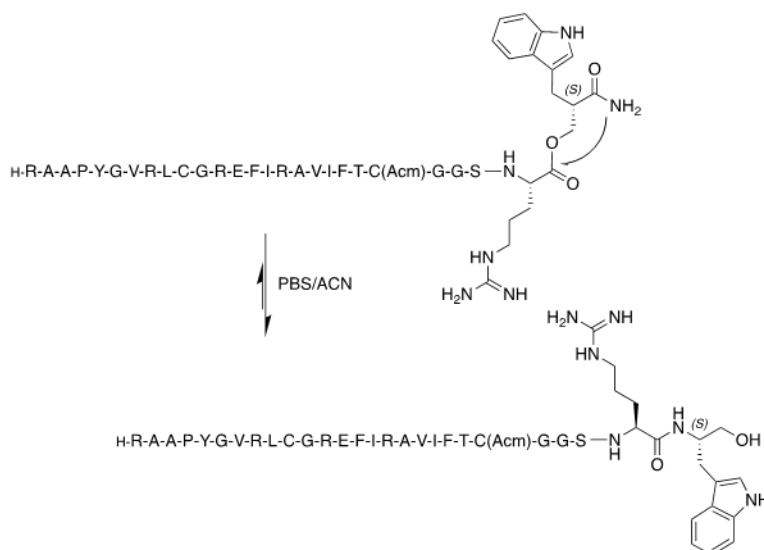
#### 2.9.4 Resin preparation for H3 B-chain bearing a C-terminal carbamate group (R3)



**Scheme 2.4:** Preparation of the carbamate resin for SPPS.

Fmoc-Trp(Boc)ol (0.3mmol, 154mg) and 4-nitrophenyl chloroformate (0.29mmol, 58mg) were solubilised in DCM (3ml). After 1 hour at room temperature, the resulting carbonate compound was added to 500 mg of rink amide resin (0.24mmol/g) in DMF (3ml) and DIEA (0.6mmol, 104 $\mu$ l). Finally, the remaining alcohol functions were capped with a solution  $\text{Ac}_2\text{O}$ /Lutidine/DMF (5/6/89, v/v'/v'') for 15 minutes (**Scheme 2.4**)<sup>2,3</sup>.

## 2.10 Preparation for H3 B-chain bearing a C-terminal alcohol



**Scheme 2.5:** Preparation of the H3 B-chain bearing C-terminal alcohol.

The H3 B-chain bearing a C-terminal iso-dipeptide (2.7  $\mu\text{mol}$ , 10mg) was dissolved in phosphate buffer ACN (80:20) at pH 7.4 (**Scheme 2.5**)<sup>1,4</sup>. ACN was used to avoid the precipitation of the final peptide<sup>4</sup>. The monitoring of the O-to-N acyl transfer<sup>5</sup> was achieved by RP-HPLC. After 2 hours at room temperature, the resulting H3 B-chain bearing C-terminal alcohol was purified by RP-HPLC preparative to lead to 8.2 mg of pure material for chain combination (reaction Y = 80%).

## 2.11 Solution NMR spectroscopy (performed by Dr Ashish Sethi)

All NMR experiments were routinely collected at 25 °C on a 700 MHz Bruker Advance HDIII spectrometer equipped with triple resonance cryoprobe. 2 mg of the peptide was dissolved in 0.5 mL of 90% H<sub>2</sub>O–10% D<sub>2</sub>O at pH ~ 4. Two-dimensional data sets including a homonuclear proton TOCSY (1024 x 256 complex points) with a mixing time of 80 ms, 1H-1H NOESY (1024 x 256 complex points) with a mixing time of 200 ms and a 1H–15N HSQC (1024 x 256 complex points) at natural abundance were recorded. Spectra were

processed using NMRPipe<sup>6</sup> and typically Fourier-transformed after applying Lorentz-to-Gauss window functions in the direct dimension and cosine bells in the indirect dimensions. NMR data were analysed using SPARKY<sup>7</sup>.

## **2.12 Bioassays (performed by Sharon Layfield and Tania Ferraro)**

### **2.12.1 HEK-7BP binding assays**

HEK-293T cells stably expressing the 7BP protein (HEK-7BP; RXFP1 extracellular domain attached with a single TM domain instead of the native 7 TM domain<sup>8</sup>) were plated on 96 well plates. The medium was aspirated off, and the cells were washed with phosphate-buffered saline (PBS) before competition binding assays were performed with Eu-H2 Relaxin (RXFP1 ligand) as previously described<sup>9</sup>. Competition binding curves for each peptide were performed in triplicate, and each experiment was performed independently at least three times. Fluorescent measurements were carried out on a BMG POLARstar plate reader (BMG Labtech, Melbourne, Australia). Pooled data are presented as mean  $\pm$  SE of specific binding and are fitted using one-site binding curve in GraphPad Prism version 8 (GraphPad Inc., San Diego, USA). Statistical analyses were conducted using one-way analysis of variance with uncorrected Fisher's least significant difference (LSD) post-hoc analysis in GraphPad Prism version 8.

### **2.12.2 RXFP3 and RXFP4 binding assays**

Chinese hamster ovary CHO-K1 cells stably transfected with RXFP3 (CHO-K1-RXFP3)<sup>10</sup> or RXFP4 (CHO-K1-RXFP4)<sup>11</sup> were plated out onto precoated poly-L-lysine 96-well view plates at a density of 50000 cells per well. The medium was aspirated off, and the cells were washed with phosphate-buffered saline (PBS) before competition binding assays were performed with 5 nM Eu-H3B1-22R<sup>12</sup> (RXFP3 ligand) or 5 nM Eu-DTPA-mINSL5<sup>11</sup> (RXFP4 ligand) as previously described. Competition binding curves for each peptide were performed in triplicate, and each experiment was performed independently at least three

times. Fluorescent measurements were carried out at excitation of 340 nm and emission of 614 nm on a BMG POLARstar plate reader (BMG Labtech, Melbourne, Australia). Pooled data are presented as mean  $\pm$  SE of specific binding and are fitted using one-site binding curve in GraphPad Prism version 8 (GraphPad Inc., San Diego, USA). Statistical analyses were conducted using one-way analysis of variance with uncorrected Fisher's least significant difference (LSD) post-hoc analysis in GraphPad Prism version 8.

### **2.12.3 RXFP3 and RXFP4 cAMP (agonism or antagonism) assays**

The peptides were tested for their ability to inhibit forskolin induced cAMP activity in CHO-K1-RXFP3 or CHO-K1-RXFP4 cells transfected with a pCRE (cAMP Response Element)  $\beta$ -galactosidase reporter plasmid as previously described<sup>11,12</sup>. For agonist assays, cells were stimulated with forskolin (5  $\mu$ M for RXFP3, 1  $\mu$ M for RXFP4) plus or minus increasing concentrations of each peptide for 6 hours. For antagonist assays cells were stimulated with forskolin as in agonist assays plus increasing concentrations of each peptide for 10 minutes followed by stimulation with agonist at an  $\sim$ EC<sub>80</sub> concentration for a further 6 hours (10 nM Analogue 2 for RXFP3 and 16.6 nM Analogue 13 for RXFP4). Each peptide was tested in triplicate, and each experiment was performed independently at least three times. Agonist data was expressed as the % Forskolin activity whereby 100% was defined as Forskolin alone and 0% as maximum agonist stimulation (1  $\mu$ M Analogue 2 for RXFP3 and 1  $\mu$ M Analogue 13 for RXFP4). Antagonist data was expressed as % inhibition whereby 0% was defined as agonist alone and 100% Forskolin alone. Data were analysed and plotted using GraphPad PRISM 8 and are expressed as the mean  $\pm$  SEM of the pooled data. Statistical analysis was conducted using one-way ANOVA with Uncorrected Fisher's LSD post-hoc analysis in GraphPad Prism version 8.

### **2.12.4 RXFP1 antagonist assay (performed by A/Prof Nasir Uddin)**

Cell Culture and Treatment with B7-33:

The human extravillous CTB cell line Sw.71 utilised in these studies was derived from first-trimester chorionic villus tissue. It was kindly provided by Dr Gil G. Mor at Yale University School of Medicine, New Haven, CT, USA. These cells are well-characterised and share many characteristics with isolated primary cells, including the expression of cytokeratin-7, HLA class I antigen, HLA-G, BC-1, CD9, human chorionic gonadotropin, and human placental lactogen. Sw.71 cells were cultured in DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum, 10 mM Hepes, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 100 U/mL penicillin/streptomycin. Cells were incubated at 37°C, 5% CO<sub>2</sub>, and 99% humidity (Fisher, Isotemp CO<sub>2</sub> Incubator), with no exposure to hypoxic conditions.

Treatment with B7-33 and RXFP1 Receptor Antagonist:

CTBs were seeded on 6 well plates and treated with DMSO (vehicle) or 5, 10, 25 and 50 nM B7-33, while some cells are pre-treated with RXFP1 receptor antagonist (1.0 µM) either single or two chain analogues prior to treating with B7-33.

ELISA for VEGF: After the treatment, the media removed from cells placed in tubes. Levels of angiogenic factor (VEGF was measured in the culture media by the commercially available ELISA kits from Boster (Pleasanton, CA).

## 2.13 References

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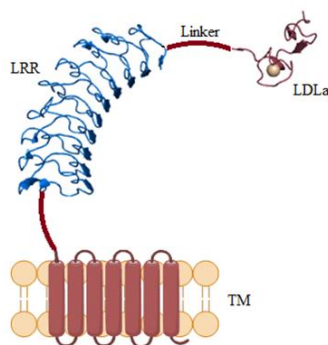
## **Chapter 3 SAR studies on RXFP1 ligands: H2 relaxin, B7-33 and B-R13/17K H2**

### **3.1 Abstract**

Human relaxin-2 (H2 relaxin) is therapeutically very important due to its strong anti-fibrotic, vasoprotective, and vasodilatory effects. Relaxin's receptor, RXFP1, is a potential target for the treatment of heart failure, fibrosis and related disorders, including liver cirrhosis, and preeclampsia. Our laboratory has designed and synthesised a B-chain-only analogue, B7-33, which was shown to be potent in activating pERK1/2, MMPs, and cGMP in fibroblast cells and in reducing fibrosis in four animal models. However, the peptide B7-33 is 27 residues long and has short circulation time in serum ( $t_{1/2}$  = ~6 min). Our laboratory also has reported an RXFP1 antagonist, B-R13/17K H2, which is a mutated analogue of H2 relaxin. I have carried out structure-activity relationship studies on B7-33. The results suggest that I can further truncate the peptide without altering its activity and can significantly improve its half-life. I also have developed B7-33 based single-chain RXFP1 antagonist for the first time.

## 3.2 Introduction

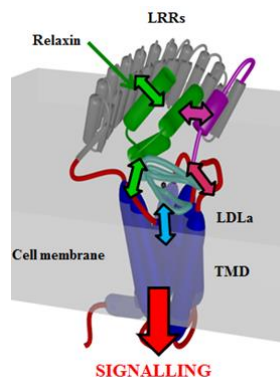
Human relaxin 2 (H2 relaxin) was discovered by Hisaw et al. in 1926<sup>1</sup>. Initially, it was considered as a hormone of reproduction, but recent studies show that H2 relaxin is a pleiotropic hormone with strong anti-fibrotic<sup>2</sup> and vasodilatory effects<sup>3</sup>. While the native receptor for H2 relaxin is Relaxin Family Peptide Receptor 1 (RXFP1)<sup>4</sup>, it can also bind to and activate the related Relaxin Family Peptide Receptor 2 (RXFP2, a cognate receptor for INSL3<sup>5</sup>). The discovery of RXFP1 as the receptor for relaxin was made 18 years ago<sup>4</sup>. RXFP1 has a 7-transmembrane domain region (TM), a large extracellular domain containing ten leucine-rich repeats (LRRs) and a unique *N*-terminal low-density lipoprotein type A (LDLa) module (**Figure 3.1**)<sup>4</sup>.



**Figure 3.1:** Schematic representation of RXFP1. RXFP1 receptor consists of seven transmembrane domain; a large extracellular domain consists of 10 Leucine-Rich Repeats (LRR), linker and LDLa (low-density lipoprotein class A) modules.

A cascade of signalling pathways is activated downstream of RXFP1, including generation of cAMP, cGMP, ERK 1/2, and MAPKs<sup>6</sup>. However, not all of these pathways are activated in every cell type. Binding of H2 relaxin to RXFP1 requires several interactions involving the LRR domain<sup>7</sup> and the extracellular TM loops<sup>8-9</sup>, whereas the *N*-terminal LDLa module is essential for its activation<sup>10-13</sup>. A recent paper demonstrated that H2 relaxin binding stabilises

the  $\alpha$ -helical conformation of the LDLa-LRR linker that positions both residues of the linker and the LDLa module to bind the TM domain to activate RXFP1 (**Figure 3.2**)<sup>14</sup>.



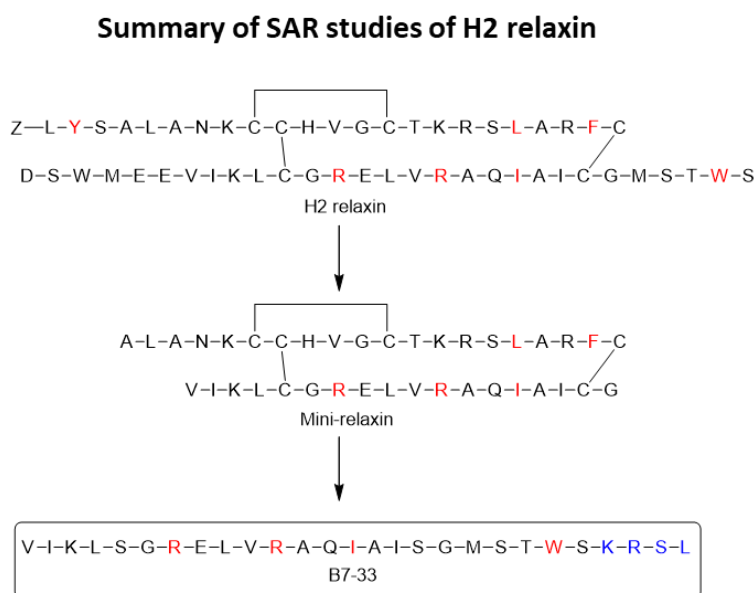
**Figure 3.2:** Schematic diagram that shows RXFP1 activation by H2 relaxin. Green arrows show interactions between the B-chain of H2 relaxin and the LRR region of RXFP1. Pink arrows indicate the interaction between the A-chain and the linker region of RXFP1. Figure adapted from Sethi et al. Nat Commun, 2016, 7, 11344.

H2 relaxin has insulin-like two-chain structure, A- and B-chain (**Figure 3.3**). Three disulfide bridges connect these two chains. The binding cassette ( $R^{B13}XXXXR^{B17}XXI^{B20}$ ) within the mid-region of the B-chain (**Figure 3.3**) is responsible for the primary interaction with the LRR of RXFP1<sup>15</sup>. Extensive structure-activity studies (SAR)<sup>8,9,14,16,17</sup> suggest that the A-chain of H2 relaxin is involved in additional interactions necessary for RXFP1 activation. Such a complex mechanism of H2 relaxin-RXFP1 interaction suggests considerable challenges for modifying and minimising the size of the peptide without altering its biological activity. In a recent attempt to identify the secondary interaction site in the A-chain, our laboratory carried out extensive SAR studies in the A-chain<sup>16</sup>. Unexpectedly, no single amino acid residue in the A-chain of H2 relaxin acts as a primary driver for RXFP1 binding and activation<sup>18</sup>. Two residues (L20 and F23) in the A-chain were found to be important for RXFP2 interaction<sup>19</sup>. This finding suggested that it might be possible to develop a B-chain-only agonist and/ antagonist. Preliminary single-chain analogues were insoluble and thus inactive<sup>20</sup>. However, by producing a B-chain with an *N*-terminal truncation and *C*-terminal elongation, a soluble peptide B7-33 was developed in our laboratory (**Figure 3.3**)<sup>21</sup>. B7-33 was tested in rat renal myofibroblasts (from injured kidneys) and human cardiac fibroblasts

(endogenously expressing RXFP1 cells) for its ability to promote MMP-2, a collagen-degrading enzyme. B7-33 exhibited similar potency to H2 relaxin.<sup>21</sup> Most importantly, like H2 relaxin, B7-33 reversed or cured fibrosis in three animal models (rat vs. mouse; heart fibrosis vs. lung fibrosis) (more details in chapter 1 section 1.3.4). Based on these exciting results, I have utilised B7-33 as a template to further develop this peptide into promising drug lead.

This chapter has three main aims:

- Further minimisation of B7-33
- Improving the half-life of B7-33
- Developing B7-33 based potential antagonist



**Figure 3.3:** Summary of SAR studies on H2 relaxin. Red colour shows important residues that interact with RXFP1/and or RXFP2. Blue colour residues KRSL are derived from isomeric isoform of B1-33 at the C-terminus, and six residues truncation from N-terminal resulted in B7-33. Z is pyroglutamate, which is a cyclic form of glutamine that is produced by the loss of a water molecule.

### 3.3 Further minimisation of B7-33

B7-33 is a 27 amino acid long peptide with an overall positive charge of five (+5). This study was undertaken to investigate further truncation of B7-33 without compromising its binding affinity. To determine the minimised structure of B7-33, a series of analogues were prepared. They were sequentially truncated from both *C*-terminus and *N*-terminus. The peptides were synthesised (**Figure 3.4**) by using Fmoc solid-phase peptide synthesis method (please see section 2.2 in chapter 2 for description). The overall yield of all analogues was similar to B7-33 (supplementary information, **Table S3.1**). The correct synthesis of all the analogues was confirmed by HPLC and MALDI-TOF mass spectrometric (supplementary information).

#### Primary structure of truncated analogues

##### a) Truncation from *C*-terminus

V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R-S-L

A) B7-33

V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R

B) B7-31

Ac-V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S

C) AcB7-29

##### b) Truncation from *N*-terminus

Ac-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R

D) AcB9-31

S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R

E) B11-31

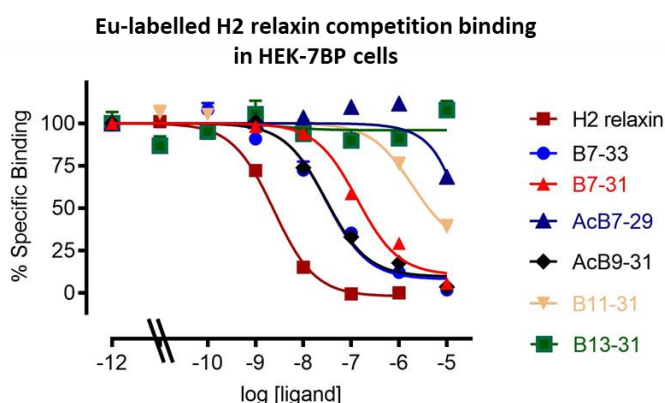
R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R

F) B13-31

**Figure 3.4:** Primary structure of truncated analogues, a) truncation from the *C*-terminus and b) truncation from *N*-terminus.

The initial competition binding assays in HEK-7BP cells (please see section 2.12.1 in chapter 2 for description) suggest that truncation of two residues from *C*-terminus and *N*-terminus is

tolerated, whereas further truncation leads to reduced binding affinity (**Figure 3.5, Table 3.1**). Therefore, B9-31 is the shortest analogue possible, which is four residues smaller than B7-33 and has similar B7-33-like binding affinity for its receptor. Binding affinity for RXFP1 decreases with truncating the C-terminal positive charged residues (KR) (**Table 3.1**).



**Figure 3.5:** Eu-H2 relaxin competition binding of truncated analogues in HEK-7BP cells. Truncation of two residues at the C-terminus and N-terminus respectively gives minimised B7-33 analogue B9-31. Further truncation results in reduced binding affinity compared to B7-33. The data are the result of n=3-4 independent experiments and expressed as mean  $\pm$  SEM.

**Table 3.1:** List of truncated analogues and their binding (pKi) in HEK-7BP cells

| Analogues | HEK-7BP binding Eu-H2 pKi (n) |
|-----------|-------------------------------|
| B7-33     | 7.56 $\pm$ 0.02 (3)           |
| B7-31     | 7.30 $\pm$ 0.14 (3)           |
| AcB7-29   | <5 (3) <sup>#</sup>           |
| AcB9-31   | 7.85 $\pm$ 0.16 (3)           |
| B11-31    | 5.70 $\pm$ 0.20 (3)***        |
| B13-31    | No binding (3)                |

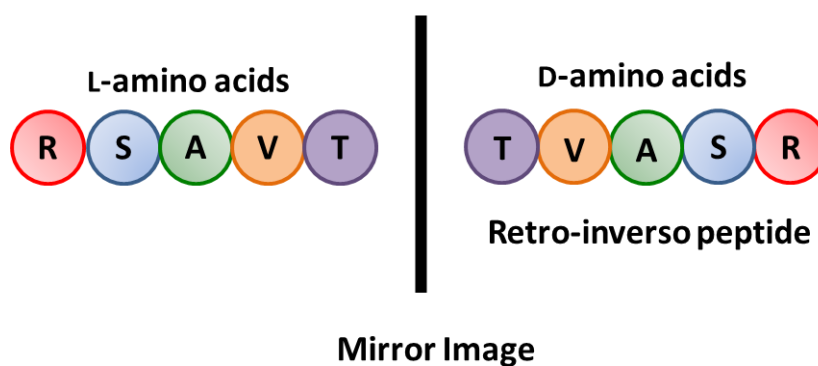
<sup>#</sup> Bought from mimotope, \*\*\*p<0.001 vs B7-33

### 3.4 Improving the *in vivo* half-life

A significant limitation of peptides as drug candidates is their short half-life<sup>22,23</sup> as they are rapidly degraded *in vivo* by enzymes and excreted by the kidneys<sup>22,24</sup>. The *in vivo* half-life of H2 relaxin is approximately ten minutes<sup>25,26</sup>. Therefore, it requires continuous intravenous infusion into patients over 48 hours for demonstrating efficacy<sup>27</sup>. B7-33 is an unstructured single-chain peptide<sup>21</sup>. Consequently, its *in vivo* half-life is expected to be even less than H2 relaxin. To improve its therapeutic value, we need to enhance its half-life. This study will focus on enhancing the half-life of B7-33 by using the following strategies:

#### 3.4.1 Retro-inverso peptide

Retro-inverso peptide is an isomer of a linear peptide in which the direction of the amino acid sequence is reversed, and the chirality of each amino acid is inverted (**Figure 3.6**). Enzymes cannot recognise D-amino acids. Therefore, this strategy provides stability against enzymatic degradation<sup>28-31</sup>.



**Figure 3.6:** Retro-inverso peptide is an isomer of a linear peptide in which direction of amino acid sequence is reversed, and chirality of each amino acid is inverted.

Two peptides, retro-inverso-B7-33 and retro-inverso-B15-29 (only the helical region of B-chain), were synthesised (**Figure 3.7**) and evaluated for its binding in HEK-7BP cells. Unfortunately, neither of the retro-inverso analogues showed binding to HEK-7BP cells

(**Figure 3.8, Table 3.2**). Our result is consistent with many studies in the literature that shows that retro-inverso strategy works poorly in mimicking biologically active helical peptides. This can be due to inherent differences at the secondary and tertiary structure levels between an L-peptide and its retro-inverso isomer despite their similar side chain topologies at the primary structure level<sup>32,33</sup>. This is supported by circular dichroism (CD) spectra (please see section 2.6 in chapter 2 for description), where CD graphs of retro-inverso analogues are opposite to B7-33 in structure (**Figure 3.9**).

### Primary structure of retro-inverso analogues

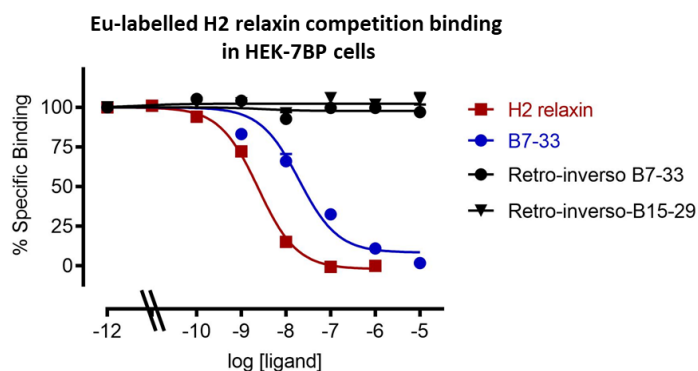
I-s-r-k-w-t-s-m-g-s-i-a-i-q-a-r-v-l-e-r-g-s-l-k-i-v

A) Retro-inverso-B7-33

m-g-s-i-a-i-q-a-r-v-l-e-r-g-s

B) Retro-inverso-B15-29

**Figure 3.7:** Primary structure of retro-inverso analogues, A) retro-inverso-B7-33 and B) retro-inverso-B15-29. Small letters indicate D-amino acid.



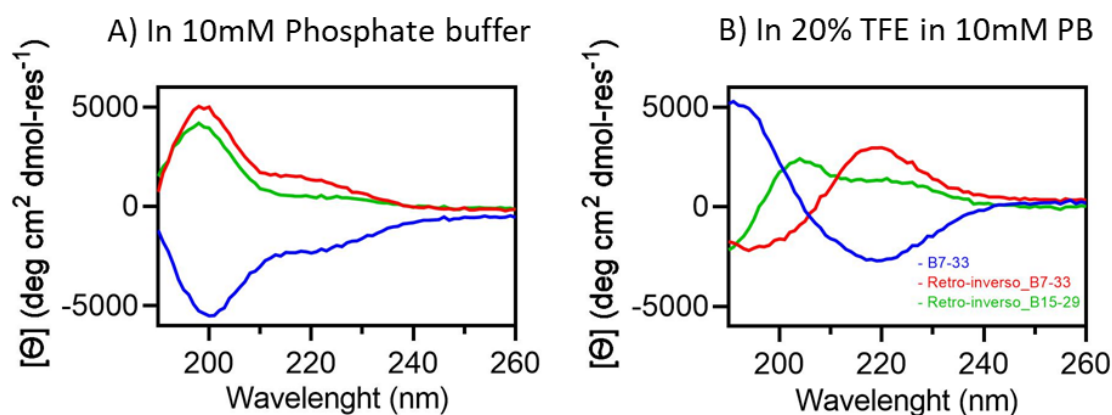
**Figure 3.8:** Eu-H2 relaxin competition binding of retro-inverso analogues in HEK-7BP cells. Retro-inverso analogues exhibited no binding affinity. The data are the result of n=3-4 independent experiments and expressed as mean  $\pm$  SEM.



**Table 3.2:** List of retro-inverso analogues and their binding (pKi) in HEK-7BP cells

| Analogues            | HEK-7BP binding Eu-H2 pKi (n) |
|----------------------|-------------------------------|
| B7-33                | 7.56 ± 0.02 (3)               |
| Retro-inverso-B7-33  | No binding (3)                |
| Retro-inverso-B15-29 | No binding (3)                |

### CD spectra of B7-33 and retro-inverso analogues

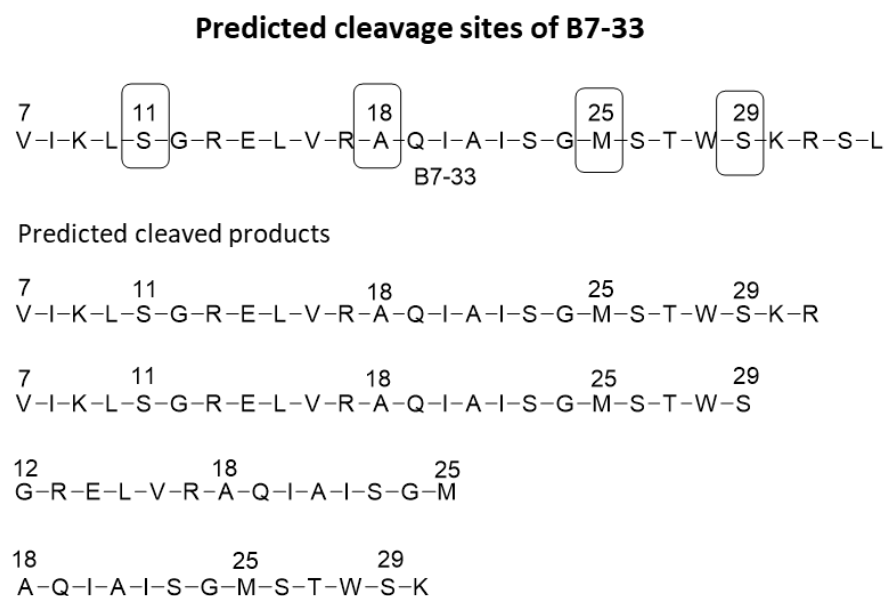


**Figure 3.9:** CD spectra of retro-inverso analogues, A) in 10 mM phosphate buffer (PB) and B) in 20% TFE in 10 mM PB.

### 3.4.2 Strengthening the cleavage site

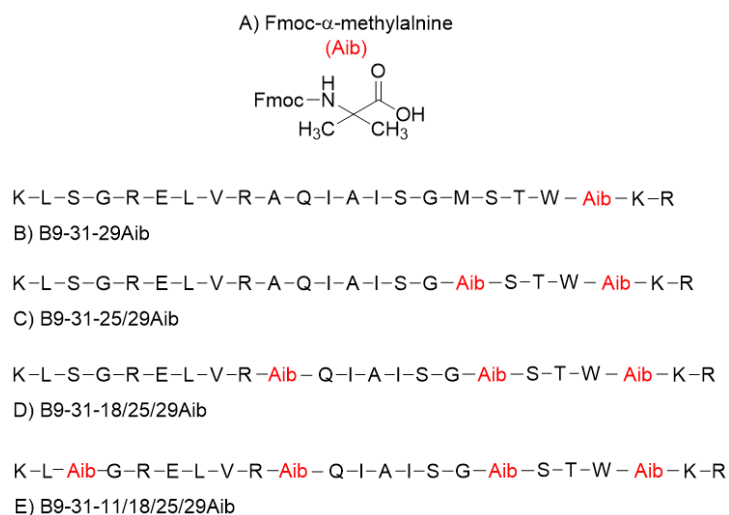
Another way to stabilise peptide against proteolytic degradation is to identify the cleavage sites of peptide and replace those amino acids with unnatural amino acid so that enzyme cannot recognise those unnatural amino acids, thus, gives stability against proteolytic degradation. Therefore, I have hydrolysed B7-33 and predicted four labile sites, as shown in **Figure 3.10**. Subsequently, I used Aib (amino iso-butyric acid) (**Figure 3.11A**), an unnatural amino acid, to replace those labile sites in the minimised analogue B9-31. Aib not only helps to prevent proteolytic degradation but also allows peptides to adopt a helical structure<sup>34</sup>. I made a series of Aib analogues (**Figure 3.11B-E**); preliminary HEK-7BP binding data

(**Figure 3.12, Table 3.3**) showed Aib analogues had exhibited reduced binding affinity compared with B7-33. Therefore, this strategy needs to be revised. N-Methylation may be the way to go that may preserve the binding affinity of the peptides.

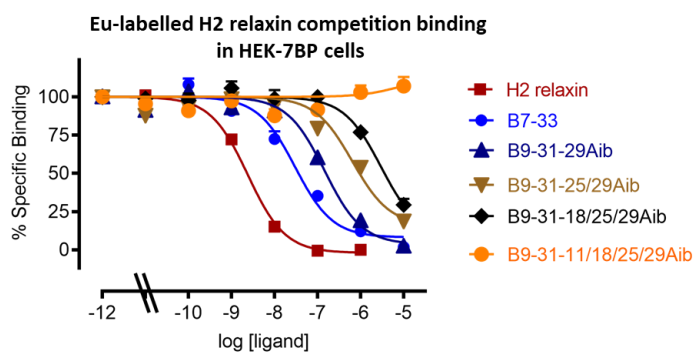


**Figure 3.10:** Predicted cleavage sites of B7-33. Boxed residues show cleavage sites and predicted cleavage products.

### Primary structure of Aib and Aib mutated analogues



**Figure 3.11:** Primary structure of A) Aib and B-E) Aib analogues. B) S is replaced by Aib at 29 position, C) S and M are replaced with Aib at 29 and 25 positions respectively, D) Aib replaces A, M and S at 18, 25 and 29 positions respectively and E) S, A, M, and S are replaced with Aib at 11, 18, 25 and 29 respectively.



**Figure 3.12:** Eu-H2 relaxin competition binding of Aib analogues in HEK-7BP cells. Aib mutated B9-31 analogues showed reduced binding affinity. The data are the result of n=3-4 independent experiments and expressed as mean  $\pm$  SEM.

**Table 3.3:** List of Aib analogues and their binding (pKi) in HEK-7BP cells

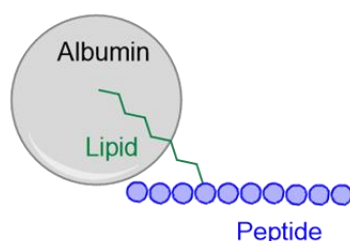
| Analogues            | HEK-7BP binding Eu-H2 pKi (n) |
|----------------------|-------------------------------|
| B7-33                | $7.56 \pm 0.02$ (3)           |
| B9-31-29Aib          | $7.26 \pm 0.01$ (3)*          |
| B9-31-25/29Aib       | $6.62 \pm 0.01$ (3)***        |
| B9-31-18/25/29Aib    | $5.89 \pm 0.15$ (3)***        |
| B9-31-11/18/25/29Aib | No binding (3)                |

\*p<0.05; \*\*\*p<0.001 vs B7-33

### 3.4.3 Lipidation

A lipidation strategy was developed in the mid-1990s<sup>35</sup>. Lipid conjugated peptides interact with albumin at three high-affinity interaction sites, between domains IIA and IIB, and within the domain III<sup>36</sup>. Therefore, as a consequence of their bulky size, they get excreted slower (**Figure 3.13**). Albumin is the most abundant protein in plasma, and it has a half-life of 19 days in humans and, its size is above the renal filtration size. Today, the most well-known lipidated peptide drugs on the market are detemir (Levemir®)<sup>37</sup>, degludec (Tresiba®)<sup>38</sup>, liraglutide (Victoza®)<sup>39</sup> and semaglutide<sup>40</sup>. I have used the following three strategies to lipidate B7-33:

- 1) The different chain length of fatty acid
- 2) Solubilising tag
- 3) Different length of spacers between the fatty acid and B7-33

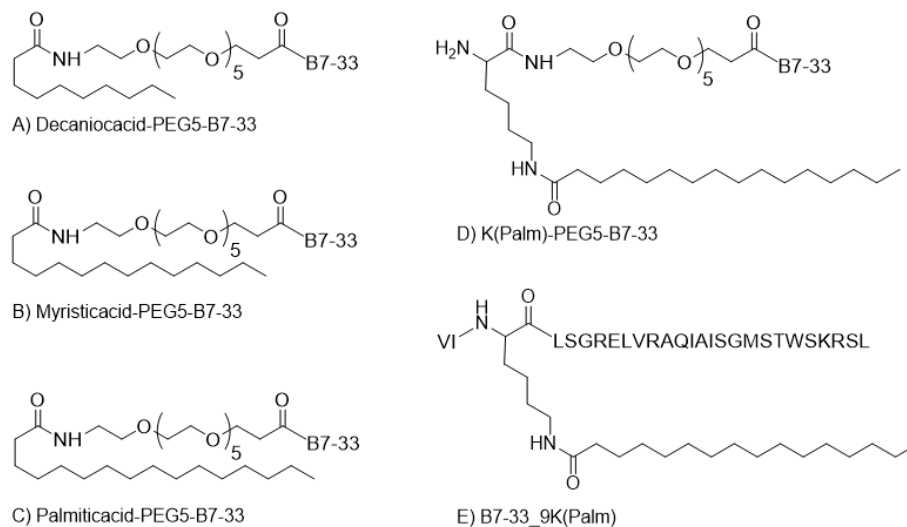


**Figure 3.13:** Schematic representation of fatty acid interacting with albumin. Lipid interacts non-covalently to albumin.

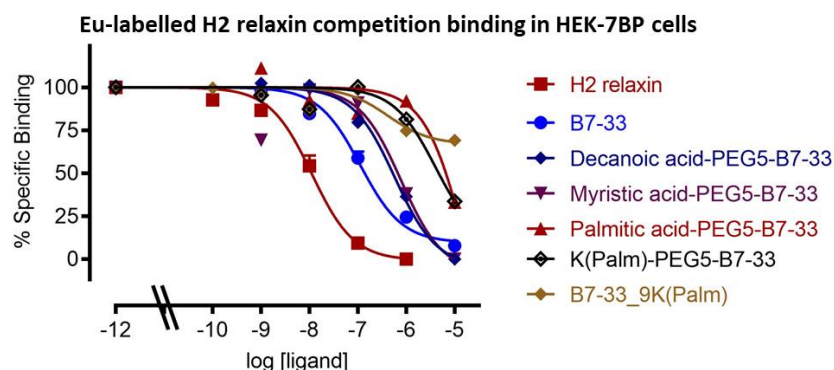
### 3.4.3.1 The different chain length of fatty acid

I used different types and lengths of fatty acids to synthesise a series of lipidated-B7-33 analogues (**Figure 3.14A-D**). I conjugated these fatty acids at the *N*-terminus of B7-33 with PEG5 as a spacer so that the fatty acid will not interfere with the binding sites of B7-33. I have synthesised one analogue where palmitic acid was conjugated at the side chain of lysine 9 of B7-33 (**Figure 3.14E**). We tested these analogues for binding in HEK-7BP cells in bovine serum albumin (BSA) free condition because we do not want lipid to interact with albumin in cell assay. Binding data suggest (**Figure 3.15, Table 3.4**) that increasing the carbon length of fatty acids decreases their binding affinity. As a result, ten-carbon decanoic acid (C10) derivative of B7-33 has a stronger binding affinity compared with fourteen-carbon myristic acid (C14) and sixteen-carbon palmitic acid (C16). This is possibly more due to the hydrophobic nature of palmitic acid, or we need a longer spacer than PEG5 that can separate the fatty acids from B7-33.

### Primary structure of different length of fatty acid analogues



**Figure 3.14:** Primary structure and sequence of lipidated analogues. Different length of fatty acid, A) decanoic acid (C10), B) myristic acid (C14), C) palmitic acid (C16) D) lysine palmitic acid (C16), E) lysine replaced with lysine palmitic acid at 9<sup>th</sup> position of B7-33.



**Figure 3.15:** Eu-H2 relaxin competition binding of lipidated analogues in HEK-7BP cells. The binding data suggest that increasing the carbon length of fatty acids causes decreasing the binding affinity. Decanoic acid (C10) derivative of B7-33 has a stronger binding affinity compared to myristic acid (C14) and palmitic acid (C16). The data are the result of n=3-4 independent experiments and expressed as mean  $\pm$  SEM.

**Table 3.4:** List of lipidated analogues and their binding (pKi) in HEK-7BP cells, BSA free assay

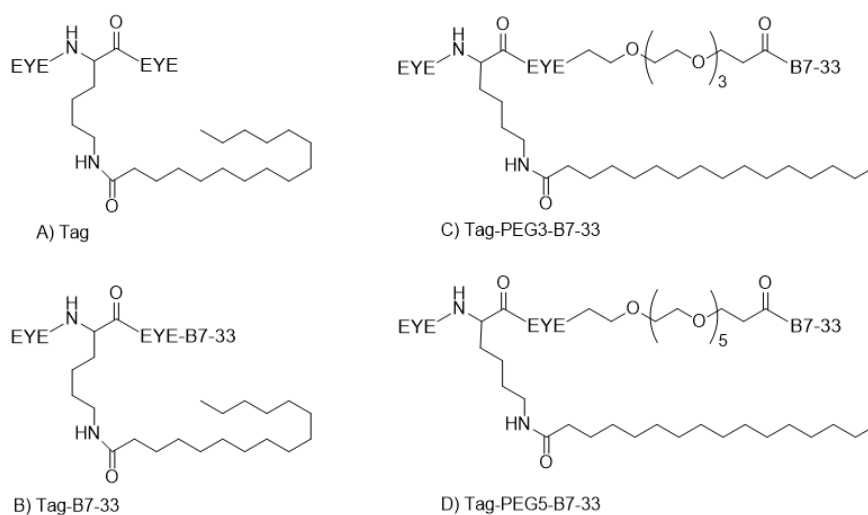
| Analogues                | HEK-7BP binding BSA free Eu-H2 pKi (n) |
|--------------------------|----------------------------------------|
| B7-33                    | 7.28 ± 0.11 (7)                        |
| Decanoic acid-PEG5-B7-33 | 6.63 ± 0.14 (3)                        |
| Myristic acid-PEG5-B7-33 | 6.0 ± 0.11 (3)**                       |
| Palmitic acid-PEG5-B7-33 | <5 (3)                                 |
| K(Palm)-PEG5-B7-33       | 5.71 ± 0.14 (3) ***                    |
| B7-33_9K(Palm)           | <5 (3)                                 |

\*\*p<0.01; \*\*\*p<0.001 vs B7-33

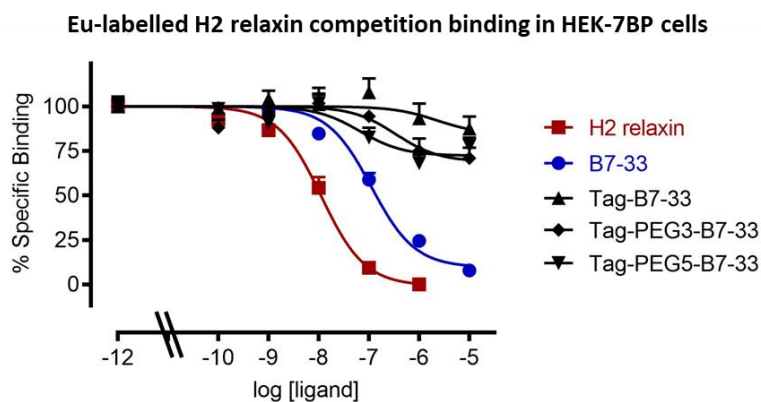
### 3.4.3.2 Solubilising tag

As fatty acid analogues were challenging to solubilise, therefore I used a tag heptapeptide (**Figure 3.16A**) developed by Zorzi et al. to increase the solubility and half-life of my lipidated analogues<sup>41</sup>. In a recently published study, it was shown that this tag peptide was able to improve the proteolytic stability of a series of peptides<sup>41</sup>. It prolonged the circulation time of the peptides in rats and rabbits around 24-fold, increasing their half-lives for 5 to 7 hours<sup>41</sup>. I made three tag-B7-33 analogues (**Figure 3.16B-D**). While the tag peptide (tag-B7-33) alone didn't show improved solubility, tag peptide (tag-PEG3-B7-33 and tag-PEG5-B7-33) with PEG3 and PEG5 spacer were soluble in water. However, all tag-B7-33 analogues showed reduced binding affinity (**Figure 3.17, Table 3.5**). Therefore, I decided to increase the length of the spacer.

### Primary structure of Tag analogues



**Figure 3.16:** Primary structure of tag B7-33 analogues. A) Tag structure, B) Tag-B7-33 with no spacer, C) Tag-PEG3-B7-33 with PEG3 spacer and D) Tag-PEG5-B7-33 with PEG5 spacer.



**Figure 3.17:** Eu-H2 relaxin competition binding of Tag-B7-33 analogues in HEK-7BP cells. The binding data suggest that this tag is not useful for B7-33 to improve solubility and affinity. The data are the result of  $n=3-4$  independent experiments and expressed as mean  $\pm$  SEM.



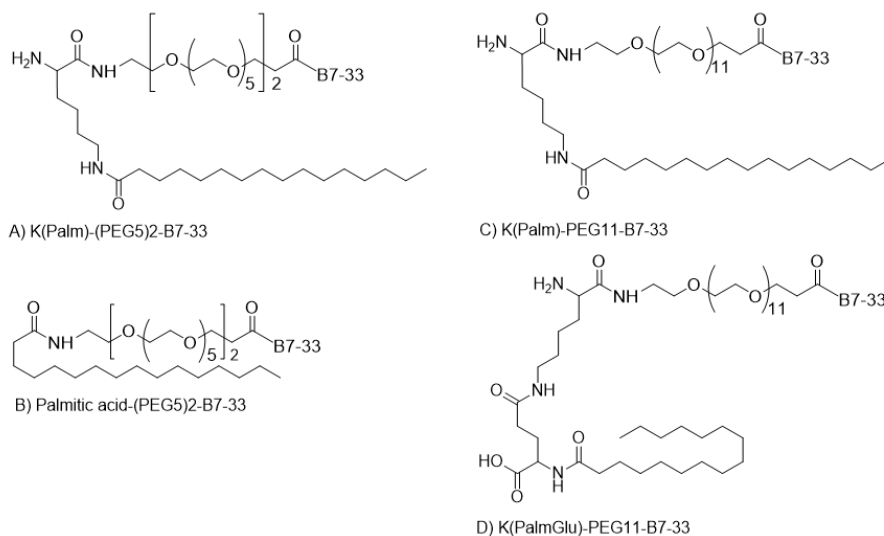
**Table 3.5:** List of Tag-B7-33 analogues and their binding (pKi) in HEK-7BP cells, BSA free assay

| <b>Analogues</b> | <b>HEK-7BP binding BSA free Eu-H2 pKi (n)</b> |
|------------------|-----------------------------------------------|
| B7-33            | 7.28 ± 0.11 (7)                               |
| Tag-B7-33        | <5 (3)                                        |
| Tag-PEG3-B7-33   | <5 (3)                                        |
| Tag-PEG5-B7-33   | <5 (3)                                        |

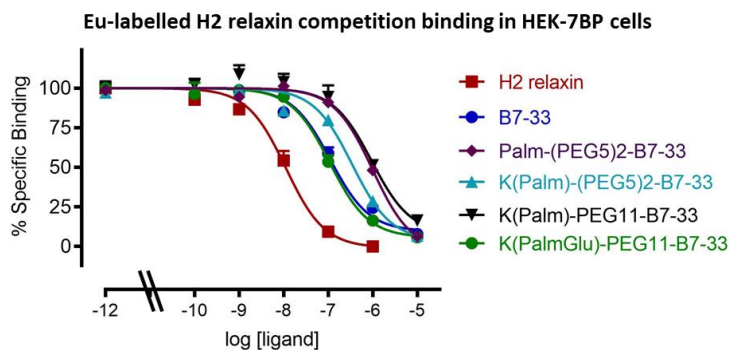
### **3.4.3.3 Different length of spacers between the fatty acid and B7-33**

I tried a longer PEG spacer between the fatty acid and B7-33 (**Figure 3.18**). I used two PEG5 spacers in K(Palm)-(PEG5)<sub>2</sub>-B7-33 and Palmitic acid-(PEG5)<sub>2</sub>-B7-33 (**Figure 3.18 A and B**). PEG11 in K(Palm)-PEG11-B7-33 (**Figure 3.18C**) and PEG11 and glutamic acid as a spacer in K(PalmGlu)-PEG11-B7-33 (**Figure 3.18D**). Binding studies showed that K(PalmGlu)-PEG11-B7-33 is the best-lipidated analogue that has similar binding to B7-33 (**Figure 3.19, Table 3.6**). I acetylated this K(PalmGlu)-PEG11-B7-33 analogue so that it will give resistance to proteolytic degradation.

### Primary structure of fatty acid analogues with different length of spacer



**Figure 3.18:** Primary structure and sequence of lipidated analogues with different length of spacer used between the fatty acid and B7-33.



**Figure 3.19:** Eu-H2 relaxin competition binding of lipidated analogues with different length of the spacer in HEK-7BP cells. The binding data suggest that spacer length between fatty acid and B7-33 is important. The data are the result of n=3-4 independent experiments and expressed as mean  $\pm$  SEM.

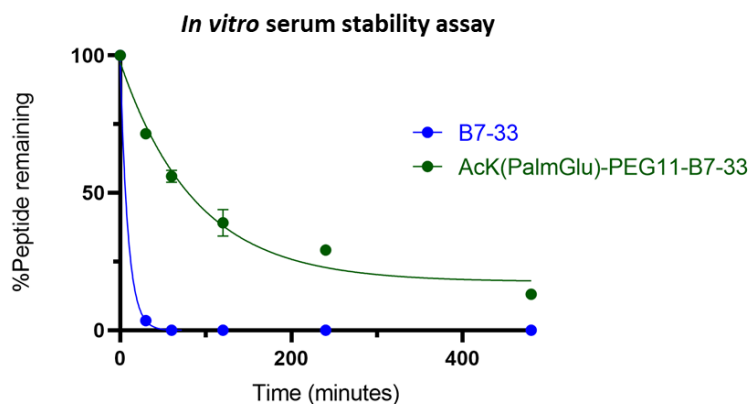
**Table 3.6:** List of lipidated analogues with increased spacer length and their binding (pKi) in HEK-7BP cells, BSA free assay

| Analogues                | HEK-7BP binding Eu-H2 pKi BSA free (n) |
|--------------------------|----------------------------------------|
| B7-33                    | 7.28 ± 0.11 (7)                        |
| K(Palm)-(PEG5)2-B7-33    | 6.80 ± 0.16 (3)                        |
| PA-(PEG5)2-B7-33         | 6.40 ± 0.10 (3) **                     |
| K(Palm)-PEG11-B7-33      | 6.56 ± 0.52 (3)**                      |
| K(PalmGlu)-PEG11-B7-33   | 7.37 ± 0.10 (3)                        |
| AcK(PalmGlu)-PEG11-B7-33 | 7.52 ± 0.13 (3)                        |

\*\*p<0.01 vs B7-33

#### 3.4.3.4 *In vitro* serum stability

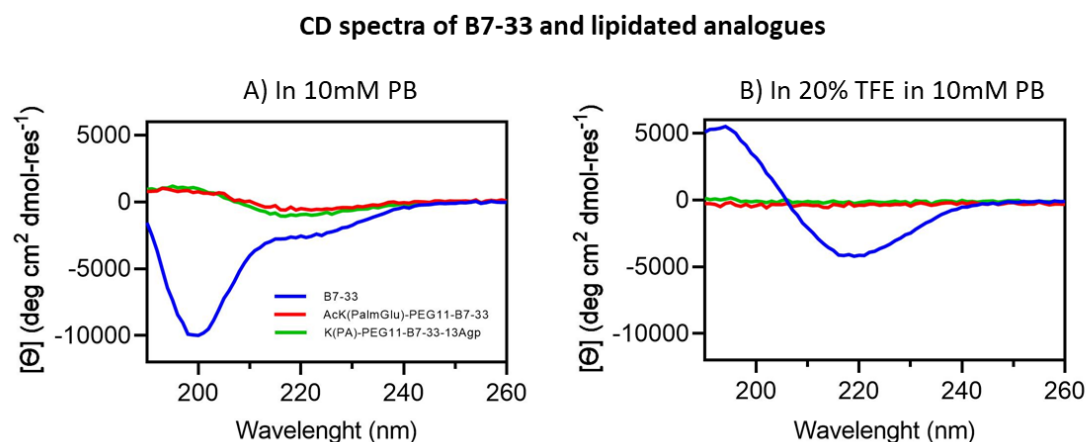
*In vitro* serum stability studies (n=3) (please see section 2.7 for the *in vitro* serum stability method in chapter 2) showed that B7-33 linear peptide degrades quickly (within minutes) whereas AcK(PalmGlu)-PEG11-B7-33 peptide resides in serum for a longer time (**Figure 3.20**). *In vitro* half-life of B7-33 and AcK(PalmGlu)-PEG11-B7-33 is 6 and 60 minutes, respectively (**Figure 3.20**). The presence of fatty acid increased the *in vitro* half-life of B7-33. It is likely that proteases have less access to albumin-bound AcK(PalmGlu)-PEG11-B7-33 compared with B7-33. The *in vivo* half-life of AcK(PalmGlu)-PEG11-B7-33 will be evaluated in animal models by using fluorescent tags (such as FITC) in the future.



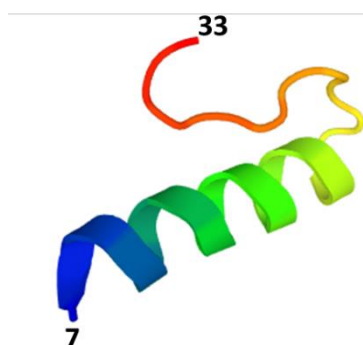
**Figure 3.20:** *In vitro* serum stability assay. B7-33 degrades within minutes, whereas AcK(PalmGlu)-PEG11-B7-33 is stable in serum over time. There were significant differences between the peptides at any time point (P-value = 0.0222, t-test). One-phase exponential decay and paired t-test analyses were performed with GraphPad Prism 8.4.3. The data are the results of n=3 independent experiments.

#### 3.4.3.5 Structural studies

B7-33 has a random coil structure shown by NMR studies<sup>21</sup>. CD spectroscopy data showed no double minima at 208 and 222 nm wavelength, therefore lipidated analogues and B7-33 have no structure (**Figure 3.21**). Even in 20% of TFE peptides are unstructured (**Figure 3.21B**). I also tried PEP-FOLD3 software to make the model structure of B7-33 (**Figure 3.22**), however it contradicts with the structure of B7-33 which is random coil in nature<sup>21</sup>.



**Figure 3.21:** CD spectra of B7-33 and lipidated analogues. A) in 10 mM PB and B) in 20% TFE in 10 mM PB.



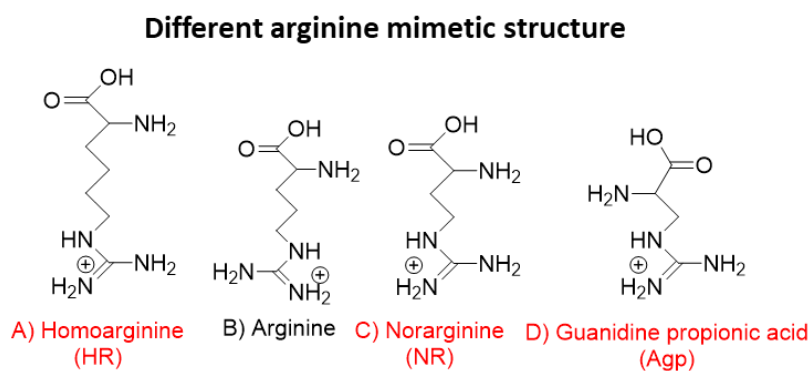
**Figure 3.22:** Modal structure of B7-33, by using PEP-FOLD3 software. <https://bioserv.rpbs.univ-parisdiderot.fr/services/PEP-FOLD3/>

### 3.5 Developing B7-33 based novel antagonist

While relaxin is well known for its reproductive and antifibrotic roles, recent studies suggest that it is produced by cancer cells and acts on RXFP1 to induce growth and metastasis<sup>42</sup>. Recently, a relaxin analogue (B-R13/17K H2) was developed that was shown to reduce the growth of prostate xenograft tumors<sup>15</sup>. However, this analogue has two-chain, and three disulfides bonded structure, which is low yielding and expensive to manufacture<sup>15</sup>. Our laboratory carried out extensive structure-function studies to determine the residues involved in agonism and antagonism functions. These findings were further investigated in our

laboratory where R<sup>B13/17</sup> were both or individually replaced by arginine mimetic, homoarginine (HR) (unpublished results).

Interestingly, the analogue which had a single HR substitution at the 13<sup>th</sup> position was found to be an antagonist in fibroblast cells. However, the analogue having HR substitution at the 17<sup>th</sup> position resulted in a very high-affinity agonist. To investigate the effects of incorporating arginine mimetic (**Figure 3.23**) residues in B7-33, a series of arginine mimetic analogues were synthesised (**Figure 3.24**) by replacing R<sup>B13</sup> and R<sup>B17</sup> by HR, norarginine (NR) and guanidine propionic acid (Agp). While arginine residue has three carbons, HR, NR, and Agp have four, two and one carbons, respectively in the side-chains together with a guanidine group (**Figure 3.23**).



**Figure 3.23:** Arginine mimetics structure, A) homoarginine one carbon extra in the side chain, B) arginine, C) norarginine one carbon less in the side chain and D) guanidine propionic acid two carbon less in the side chain.

### Primary structure of arginine mimetic analogues

#### a) Mutation at 17 position

V-I-K-L-S-G-R-E-L-V-**HR**-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

A) B7-33-17HomoArg

V-I-K-L-S-G-R-E-L-V-**NR**-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

B) B7-33-17NorArg

V-I-K-L-S-G-R-E-L-V-**Agp**-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

C) B7-33-17Agp

#### b) Mutation at 13 position

V-I-K-L-S-G-**HR**-E-L-V-R-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

D) B7-33-13HomoArg

V-I-K-L-S-G-**NR**-E-L-V-R-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

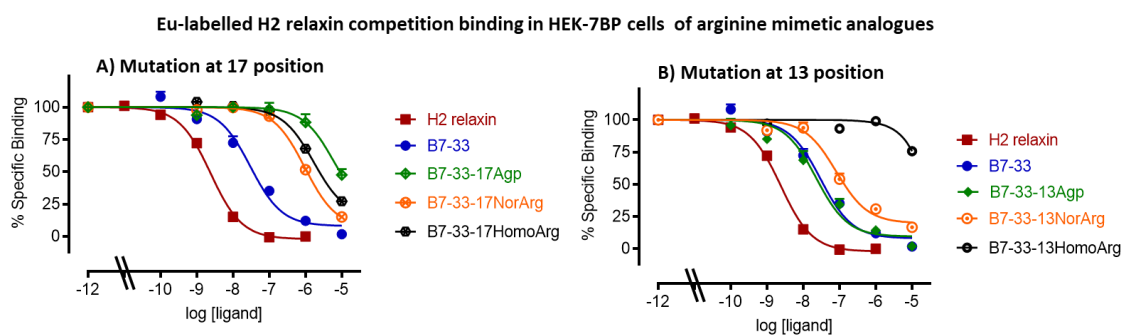
E) B7-33-13NorArg

V-I-K-L-S-G-**Agp**-E-L-V-R-A-Q-I-A-I-S-G-M-S-W-S-K-R-S-L

F) B7-33-13Agp

**Figure 3.24:** Primary structure of B7-33 analogues modified at a) 17 position and b) 13 position.

Binding studies in HEK-7BP cells show that analogues modified at 17 position have a weak binding affinity (**Figure 3.25, Table 3.7**) compared with B7-33 suggesting that binding mode of B7-33 and H2 relaxin might be different. However, one of the analogues, B7-33-13Agp, modified at 13 position with an arginine mimetic showed B7-33-like strong binding affinity (**Figure 3.25B**).



**Figure 3.25:** Eu-H2 relaxin competition binding of arginine mimetic analogues in HEK-7BP cells. A) The binding data suggest that arginine at 17 position is not replaceable. B) The binding data indicate that arginine at 13 is replaceable, and B7-33-13Agp has similar binding as B7-33. The data are the result of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

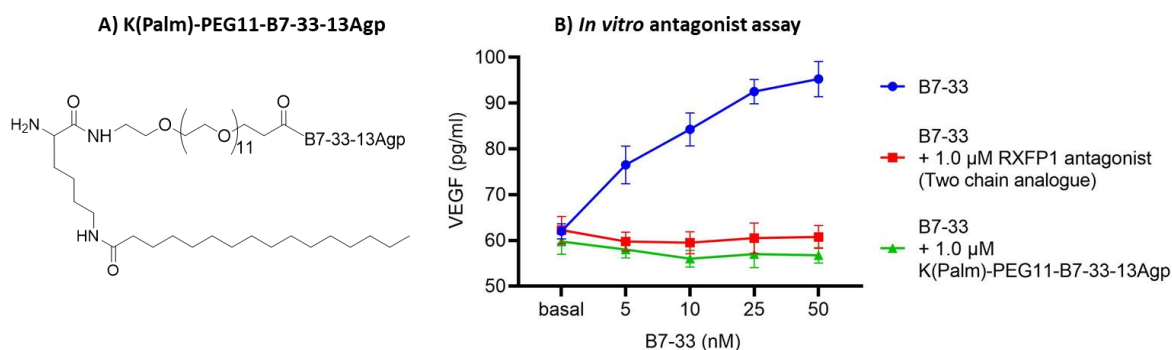
**Table 3.7:** List of arginine mimetic analogues and their binding (pKi) in HEK-7BP cells

| <b>Analogues</b>          | <b>HEK-7BP binding Eu-H2 pKi<br/>(n)</b>          |
|---------------------------|---------------------------------------------------|
| B7-33                     | 7.56 ± 0.02 (3)                                   |
| B7-33-17HomoArg           | 6.18 ± 0.13 (3)***                                |
| B7-33-17NorArg            | 6.45 ± 0.08 (3)**                                 |
| B7-33-17Agp               | 5.53 ± 0.19 (3)***                                |
| B7-33-13HomoArg           | <5 (3)                                            |
| B7-33-13NorArg            | 6.45 ± 0.10 (3)**                                 |
| B7-33-13Agp               | 8.03 ± 0.38 (3)                                   |
| <b>Analogues</b>          | <b>HEK-7BP binding Eu-H2 pKi BSA free<br/>(n)</b> |
| B7-33                     | 7.28 ± 0.11 (7)                                   |
| K(Palm)-PEG11-B7-33-13Agp | 7.39 ± 0.12 (4)                                   |

\*\*p<0.01; \*\*\*p<0.001 vs B7-33

For B7-33-13Agp analogue, I conjugated fatty acid at the *N*-terminus with a PEG11 spacer to improve its half-life (**Figure 3.26A**). The resulting analogue, K(Palm)-PEG11-B7-33-13Agp, was evaluated in VEGF (Vascular Endothelial Growth Factor) model to measure VEGF secretion by CTBs (cytotrophoblast) cells. The analogue K(Palm)-PEG11-B7-33-13Agp shown to antagonises the VEGF secretion by CTBs cells consistent with previous results that relaxin stimulates RXFP1-mediated VEGF production leading to angiogenesis<sup>6</sup>. (**Figure 3.26B**) (please see section 2.12.4 for antagonist assay method in chapter 2).



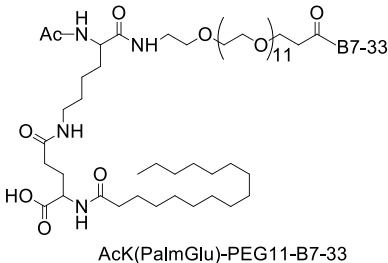
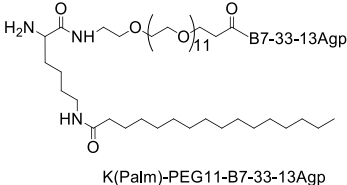


**Figure 3.26:** A) K(Palm)-PEG11-B7-33 primary structure and B) *in vitro* antagonist assay VEGF model to measure VEGF secretion by CTBs cells. K(Palm)-PEG11-B7-33-13Agp analogue antagonises the VEGF secretion by CTBs cells.

### 3.6 Conclusion

In conclusion, I have shown that B7-33 can be further truncated up to 4 residues, and by fatty acid conjugation, it's *in vitro* half-life can be increased from 6 minutes to 60 minutes. I have also developed a high yielding single-chain relaxin analogue for the first time that acts as an antagonist for RXFP1. These analogues will be used as tools to understand the physiology of RXFP1. They will also serve as drug leads for the treatment of fibrosis-related diseases, prostate, and other cancers. **Table 3.8** below summarises the new analogues I developed in this chapter.

**Table 3.8:** Summary of RXFP1 analogues

| Previously we had                                                                                                                                                     | New                                                                                                                                                                         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>RXFP1 single-chain agonist</p> <p>V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R-S-L<br/>B7-33</p>                                                              | <p>RXFP1 minimised single-chain agonist</p> <p>K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R<br/>B9-31</p>                                                                  |
| <p>B7-33 short <i>in vitro</i> half-life</p> <p>V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R-S-L<br/>B7-33</p>                                                   | <p>Single chain long-acting analogue <i>in vitro</i></p>  <p>AcK(PalmGlu)-PEG11-B7-33</p> |
| <p>RXFP1 Antagonist</p> <p>Z-L-Y-S-A-L-A-N-K-C-C-H-V-G-C-T-K-R-S-L-A-R-F-C<br/>D-S-W-M-E-E-V-I-K-L-C-G-K-E-L-V-K-A-Q-I-A-I-C-G-M-S-T-W-S<br/>B-R13/17K H2 relaxin</p> | <p>RXFP1 single chain antagonist</p>  <p>K(Palm)-PEG11-B7-33-13Agp</p>                  |

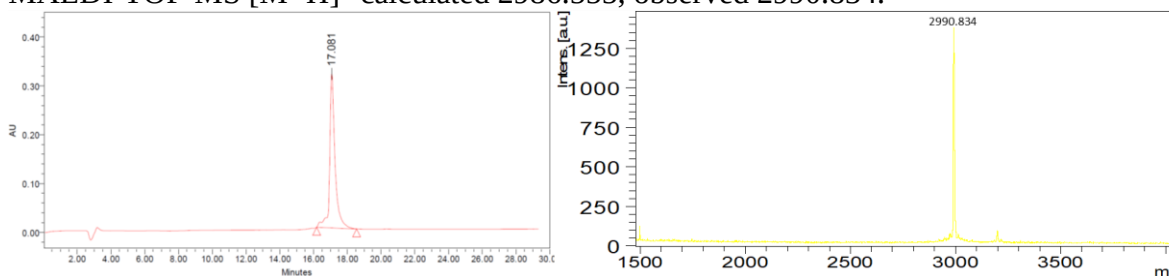
## 3.7 Supporting information

### 3.7.1 Analytical RP-HPLC and MALDI-TOF-MS of all compounds

#### 1. B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 17.081$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2986.555, observed 2990.834.

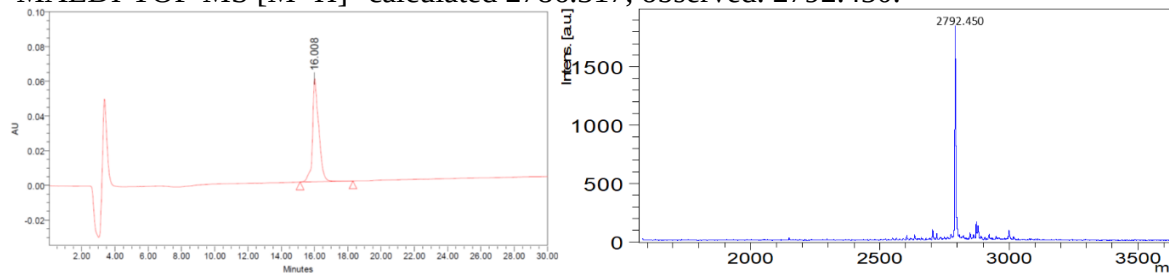


**Figure S3.1:** RP-HPLC profile of B7-33 (left) and MALDI-TOF-MS (right).

#### 2. B7-31

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 16.008$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2786.317, observed. 2792.450.

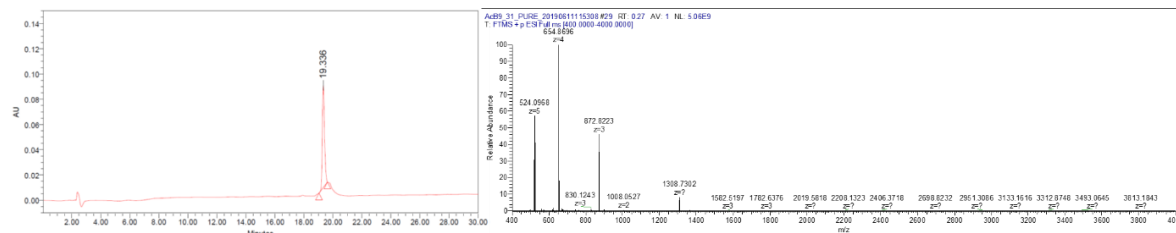


**Figure S3.2:** RP-HPLC profile of B7-31 (left) and MALDI-TOF-MS (right).

### 3. AcB9-31

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-55% in 30 minutes,  $t = 19.336$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2614.438, observed. 2615.466.

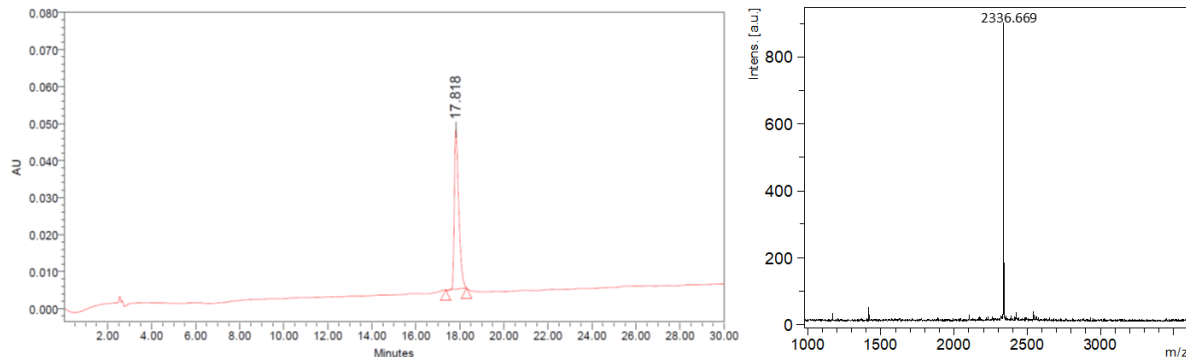


**Figure S3.3:** RP-HPLC profile of AcB9-31 (left) and MALDI-TOF-MS (right).

### 4. B11-31

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t = 17.818$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2332.693, observed 2336.669.

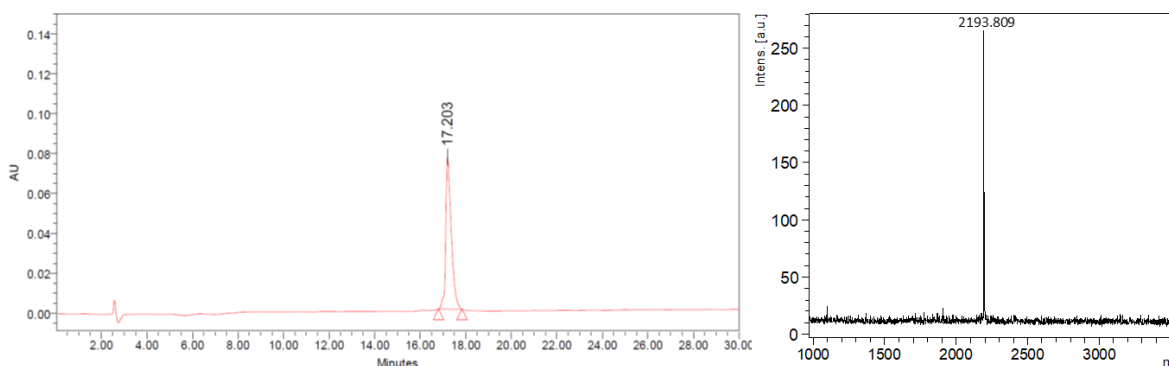


**Figure S3.4:** RP-HPLC profile of B11-31 (left) and MALDI-TOF-MS (right).

### 5. B13-31

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t = 17.203$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2188.563, observed 2193.809.

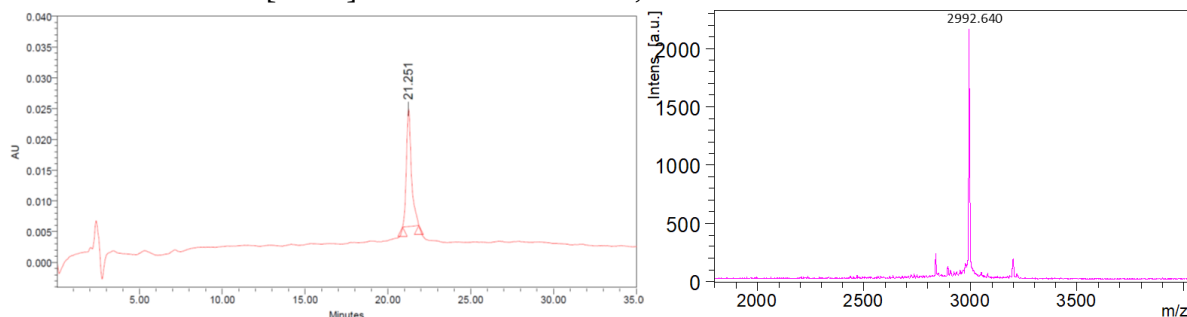


**Figure S3.5:** RP-HPLC profile of B13-31 (left) and MALDI-TOF-MS (right).

### 6. Retro-inverso-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t = 21.251$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2986.555, observed 2992.640.

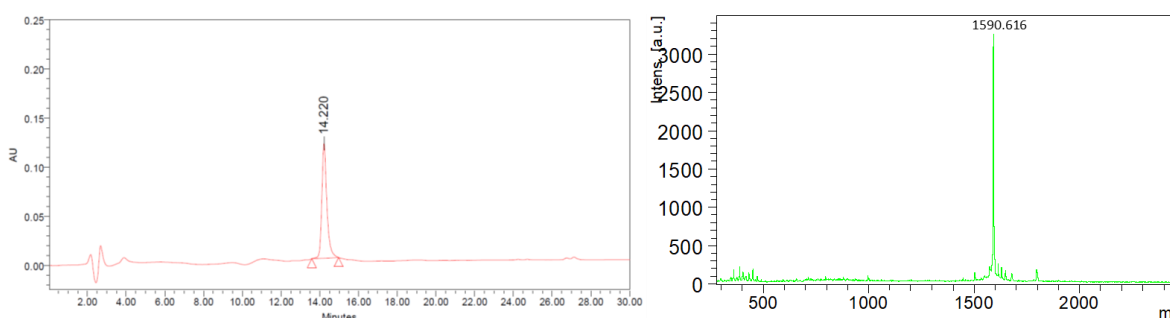


**Figure S3.6:** RP-HPLC profile of Retro-inverso-B7-33 (left) and MALDI-TOF-MS (right).

### 7. Retro-inverso-B15-29

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t = 14.22$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 1586.858, observed 1590.616.

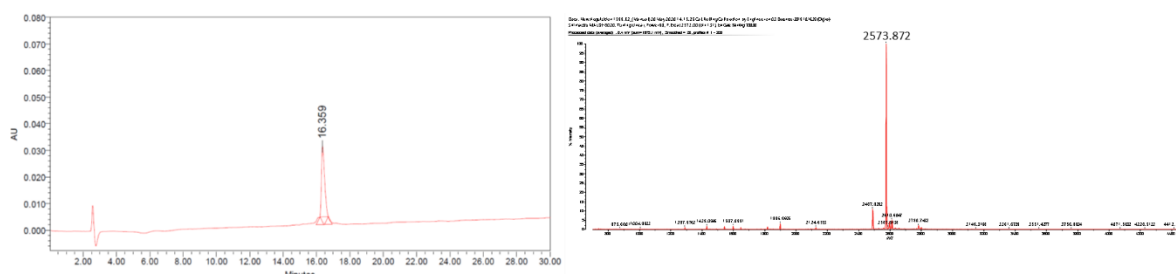


**Figure S3.7:** RP-HPLC profile of Retro-inverso-B15-29 (left) and MALDI-TOF-MS (right).

### 8. B9-31-29Aib

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 15.359$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2572.318, observed 2573.872.

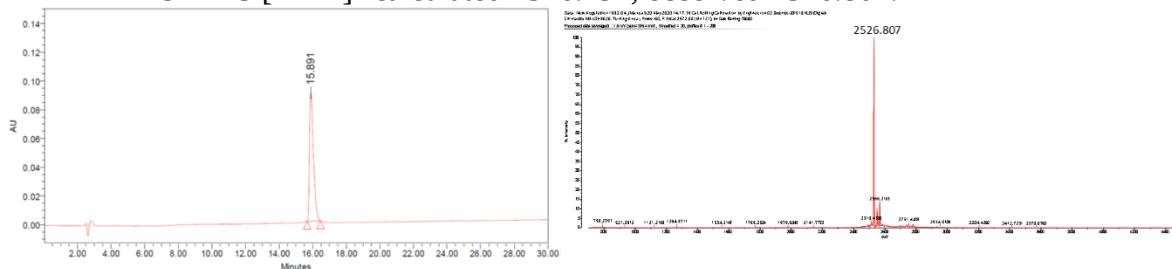


**Figure S3.8:** RP-HPLC profile of B9-31-29Aib (left) and MALDI-TOF-MS (right).

### 9. B9-31-25/29Aib

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 15.891$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2526.492, observed 2526.807.

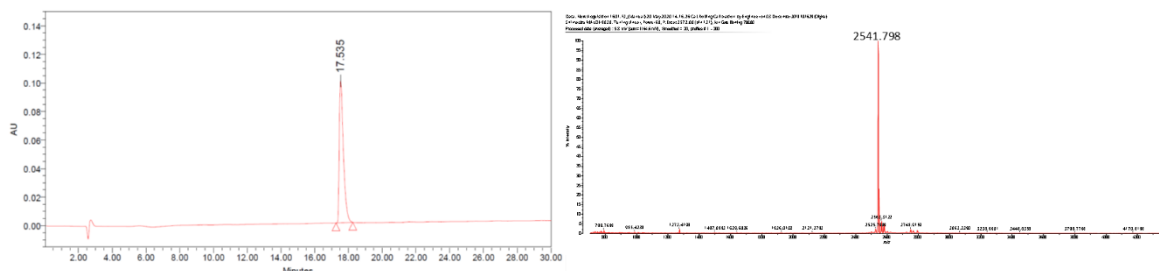


**Figure S3.9:** RP-HPLC profile of B9-31-25/29Aib (left) and MALDI-TOF-MS (right).

### 10. B9-31-18/25/29Aib

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 17.535$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2540.783, observed 2541.798.

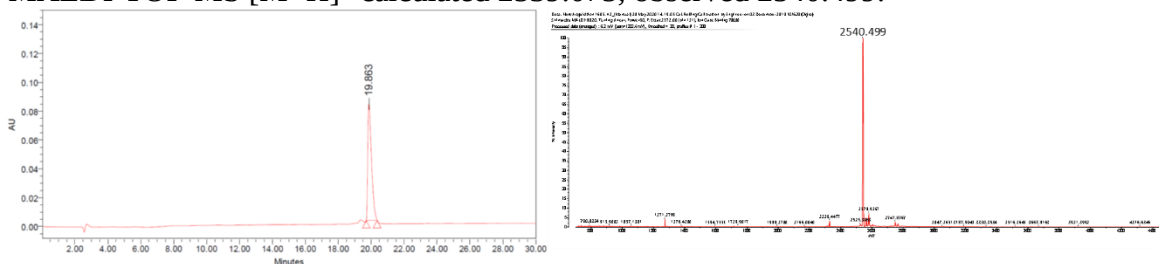


**Figure S3.10:** RP-HPLC profile of B9-31-18/25/29Aib (left) and MALDI-TOF-MS (right).

### 11. B9-31-11/18/25/29Aib

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t = 19.863$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2539.075, observed 2540.499.



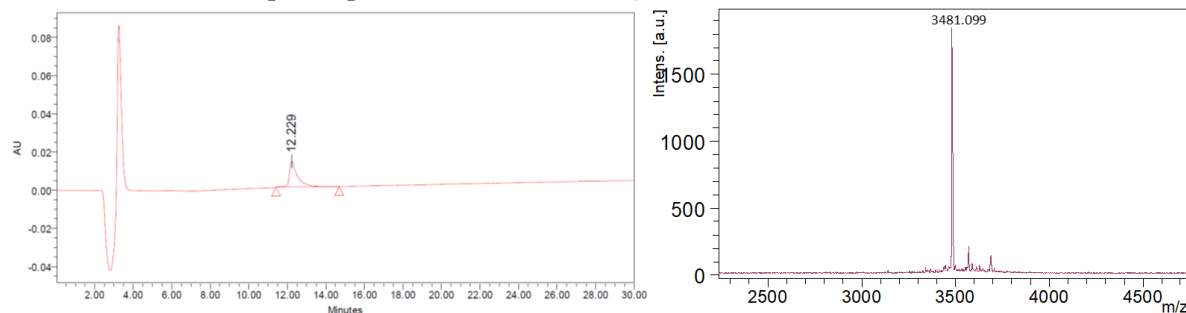
**Figure S3.11:** RP-HPLC profile of B9-31-11/18/25/29Aib (left) and MALDI-TOF-MS (right).

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### 12. Decanoic acid-PEG5-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t_r = 12.229$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3475.425, observed 3481.099.



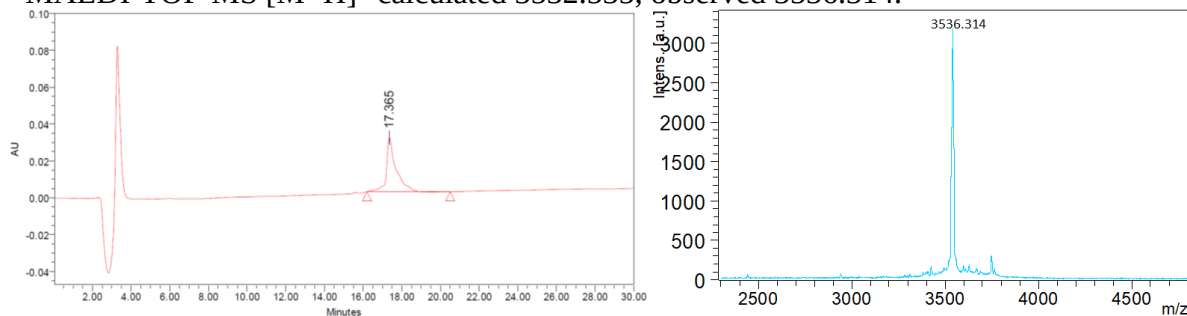
**Figure S3.12:** RP-HPLC profile of Decanoic acid-PEG5-B7-33 (left) and MALDI-TOF-MS (right).

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### 13. Myristic acid-PEG5-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t_r = 17.365$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3532.535, observed 3536.314.



**Figure S3.13:** RP-HPLC profile of Myristic acid-PEG5-B7-33 (left) and MALDI-TOF-MS (right).

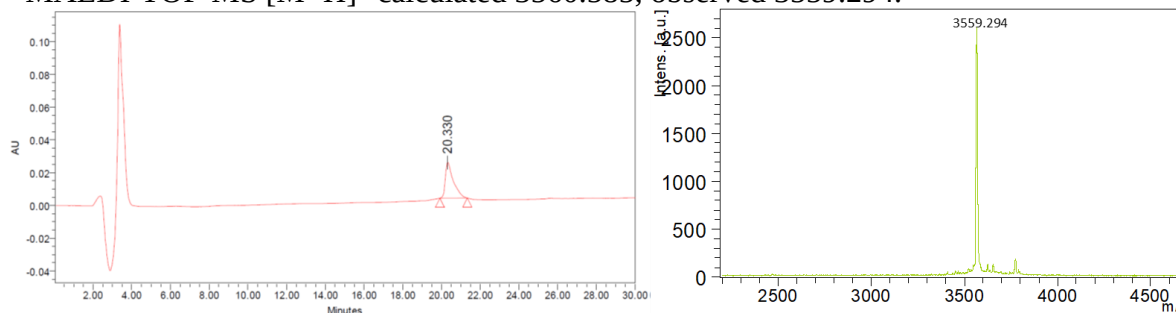
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#### 14. Palmitic acid-PEG5-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 20.330$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3560.585, observed 3559.294.



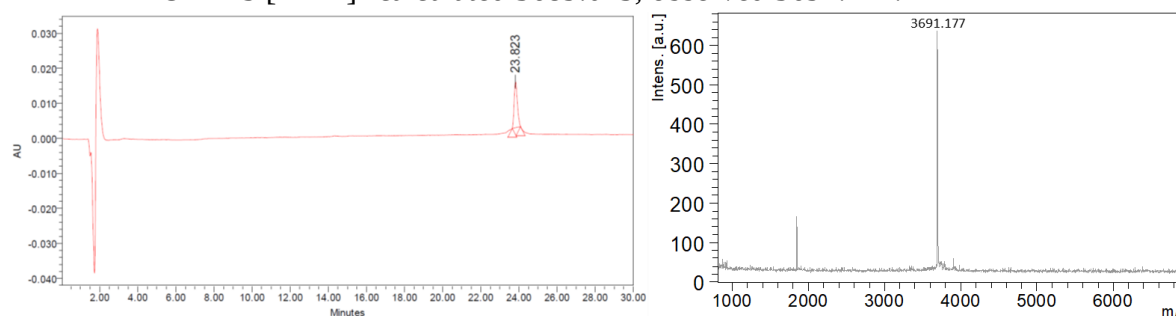
**Figure S3.14:** RP-HPLC profile of Palmitic acid-PEG5-B7-33 (left) and MALDI-TOF-MS (right).

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#### 15. K(Palm)-PEG5-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t = 23.823$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3689.015, observed 3691.177.



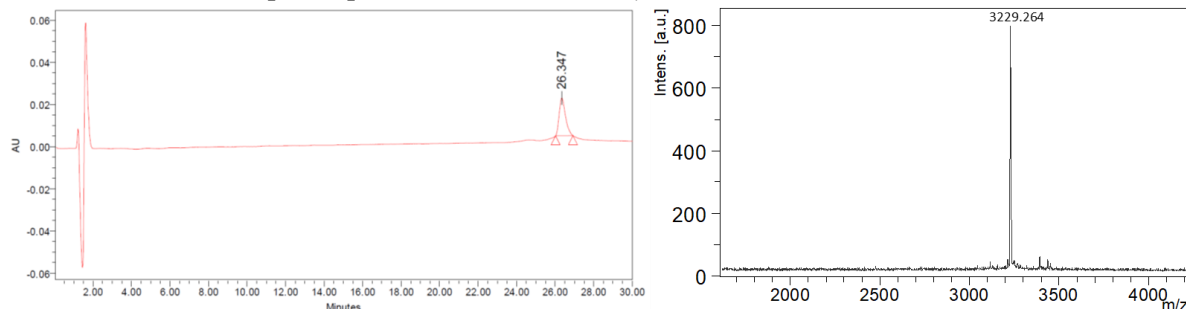
**Figure S3.15:** RP-HPLC profile of K(Palm)-PEG5-B7-33 (left) and MALDI-TOF-MS (right).



### 16. B7-33\_9K(Palm)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t = 26.347$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3225.231, observed 3229.264.

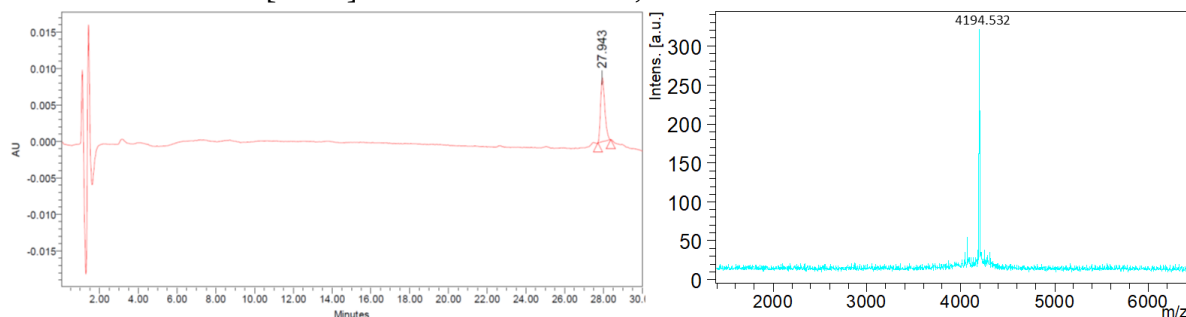


**Figure S16:** RP-HPLC profile of B7-33\_9K(Palm) (left) and MALDI-TOF-MS (right).

### 17. Tag-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t = 27.943$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4196.216, observed 4194.532.

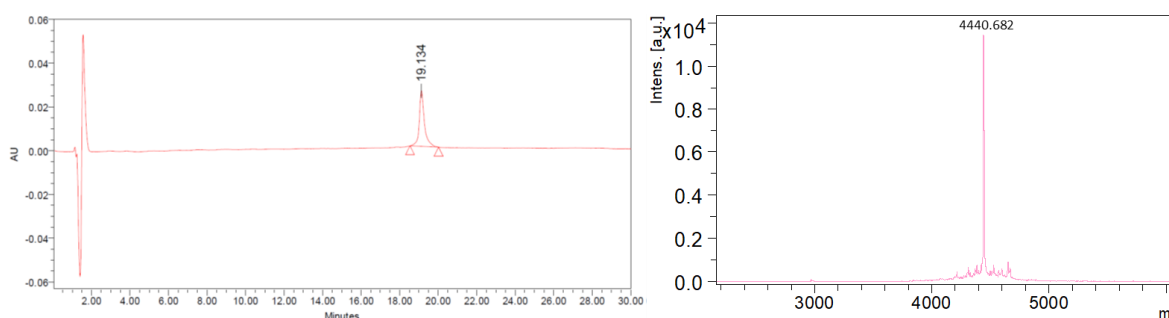


**Figure S3.17:** RP-HPLC profile of Tag-B7-33 (left) and MALDI-TOF-MS (right).

### 18. Tag-PEG3-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 19.134$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4443.766, observed 4440.682.



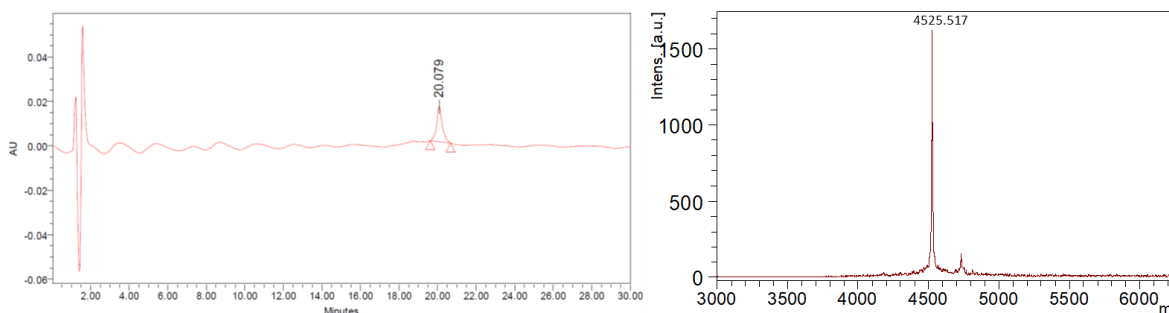
**Figure S3.18:** RP-HPLC profile of Tag-PEG3-B7-33 (left) and MALDI-TOF-MS (right).

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### 19. Tag-PEG5-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 20.079$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4531.826, observed 4525.517.



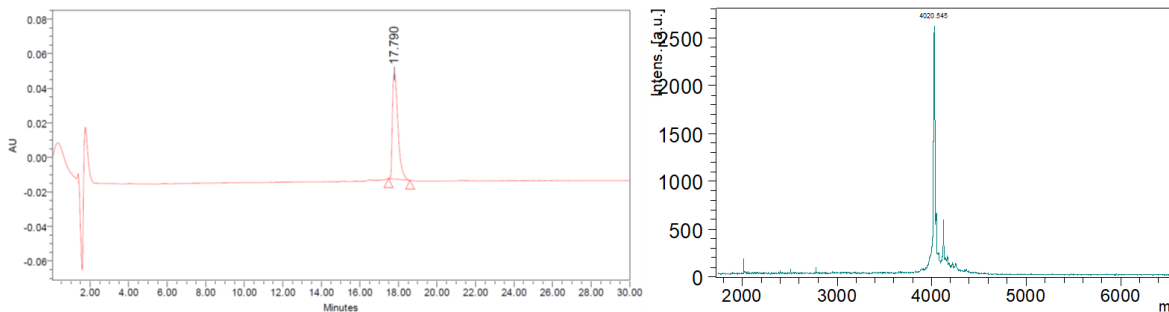
**Figure S3.19:** RP-HPLC profile of Tag-PEG5-B7-33 (left) and MALDI-TOF-MS (right).

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### 20. K(Palm)-(PEG5)2-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 17.790$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4024.625, observed 4020.545.



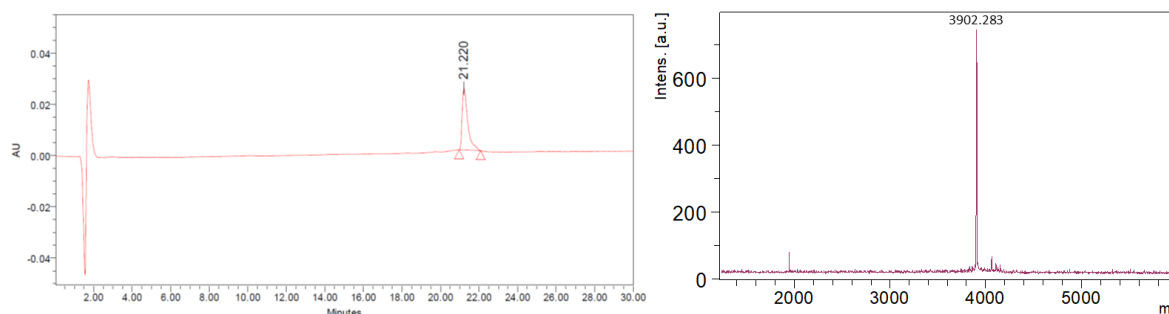
**Figure S3.20:** RP-HPLC profile of K(Palm)-(PEG5)2-B7-33 (left) and MALDI-TOF-MS (right).

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### 21. PA-(PEG5)2-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 21.220$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3896.195, observed 3902.283.



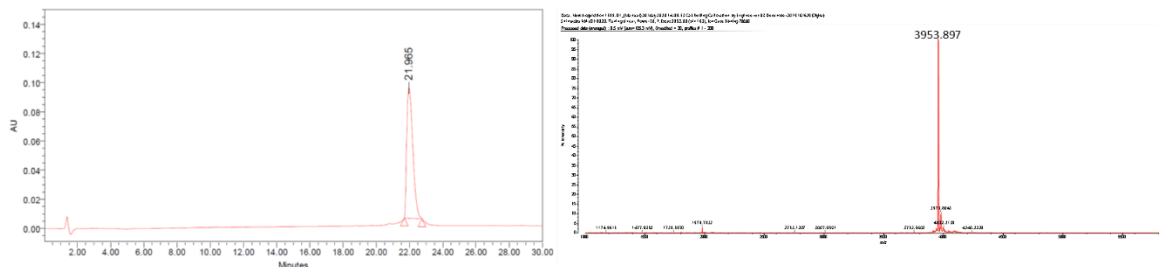
**Figure S3.21:** RP-HPLC profile of PA-(PEG5)2-B7-33 (left) and MALDI-TOF-MS (right).

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## 22. K(Palm)-PEG11-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t = 21.965$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3953.365, observed 3953.897.

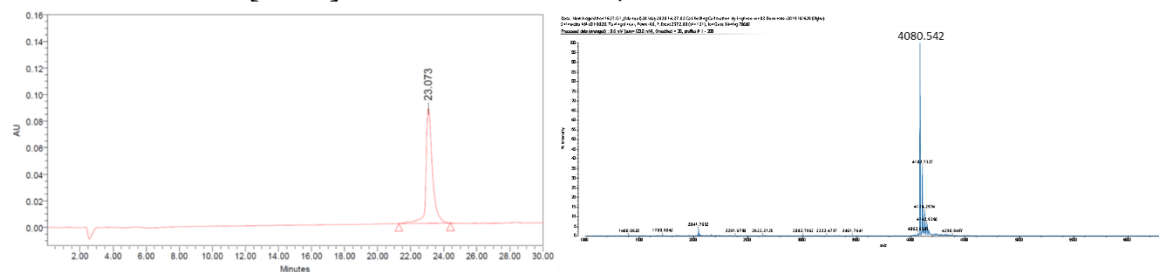


**Figure S3.22:** RP-HPLC profile of K(Palm)-PEG11-B7-33 (left) and MALDI-TOF-MS (right).

## 23. K(PalmGlu)-PEG11-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t = 23.073$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4082.506, observed 4080.542.

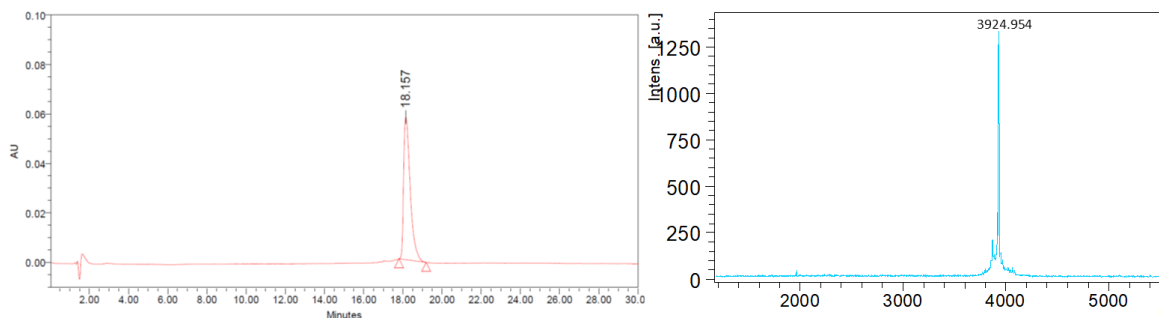


**Figure S3.23:** RP-HPLC profile of K(PalmGlu)-PEG11-B7-33 (left) and MALDI-TOF-MS (right).

## 24. K(Palm)-PEG11-B7-33-13Agp

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t = 18.157$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3925.365, observed 3924.954.



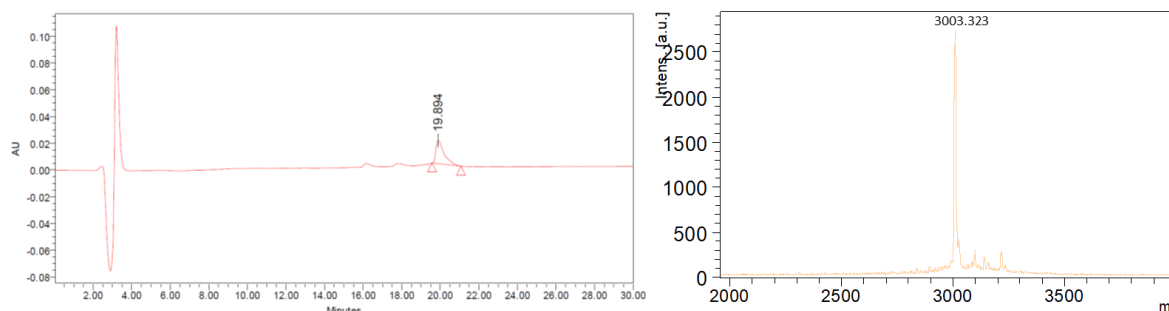
**Figure S3.24:** RP-HPLC profile of K(Palm)-PEG11-B7-33-13Agp (left) and MALDI-TOF-MS (right).

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### 25. B7-33-17HomoArg

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 19.894$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3000.555, observed 3003.323.



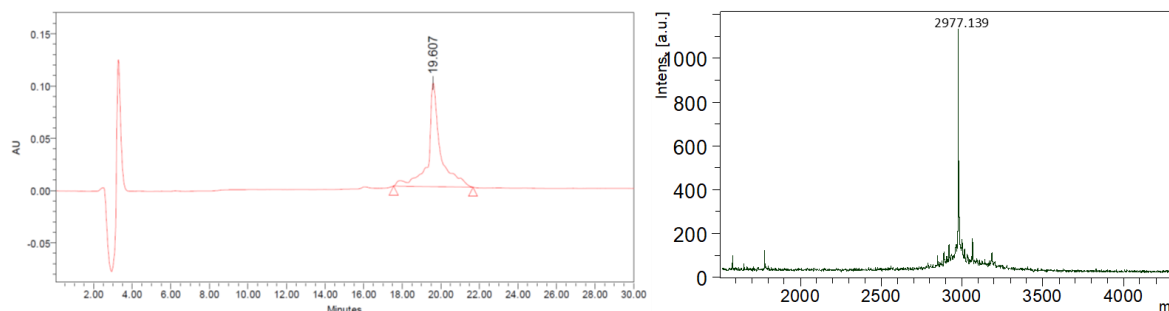
**Figure S3.25:** RP-HPLC profile of B7-33-17HomoArg (left) and MALDI-TOF-MS (right).

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### 26. B7-33-17NorArg

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 19.607$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2972.555, observed 2977.139.



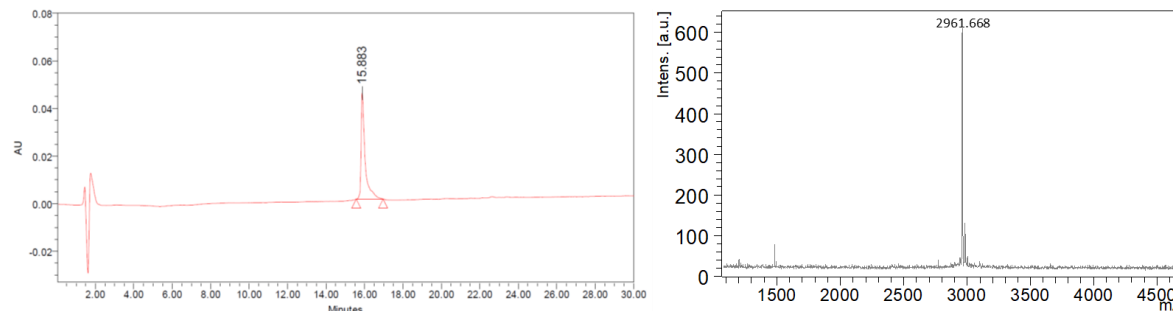
**Figure S3.26:** RP-HPLC profile of B7-33-17NorArg (left) and MALDI-TOF-MS (right).

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### 27. B7-33-17Agp

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 15.883$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2958.555, observed 2961.668.

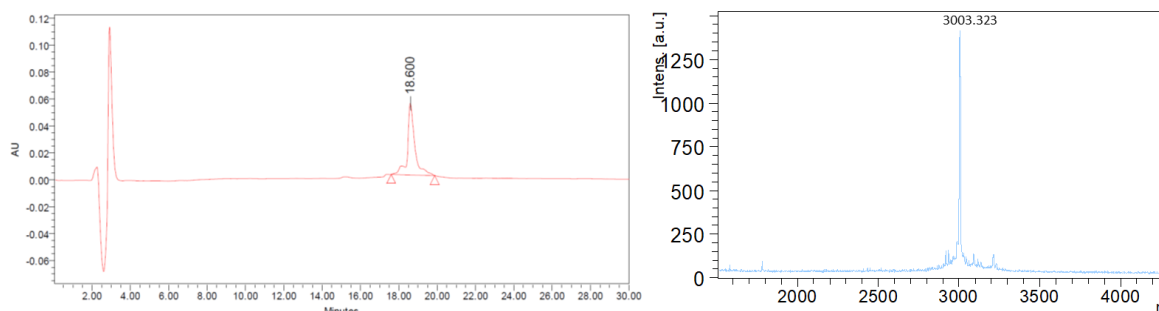


**Figure S3.27:** RP-HPLC profile of B7-33-17Agp (left) and MALDI-TOF-MS (right).

### 28. B7-33-13HomoArg

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 18.600$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 3000.555, observed 3003.323.

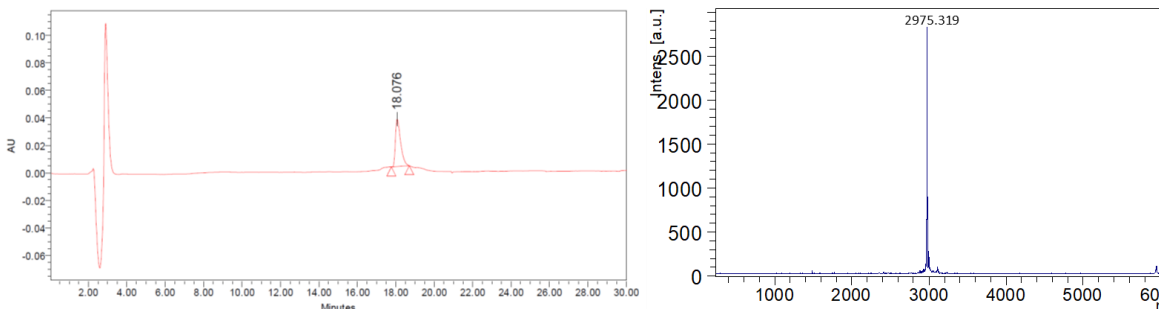


**Figure S3.28:** RP-HPLC profile of B7-33-13HomoArg (left) and MALDI-TOF-MS (right).

### 29. B7-33-13NorArg

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 18.076$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2972.555, observed 2975.319.

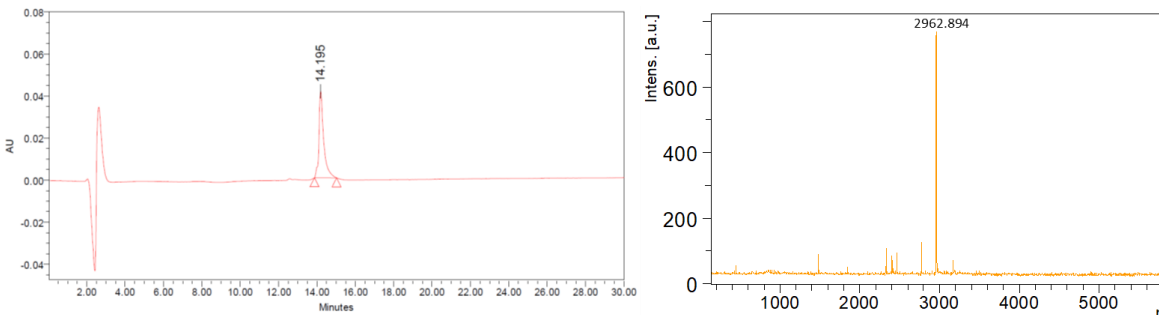


**Figure S3.29:** RP-HPLC profile of B7-33-13NorArg (left) and MALDI-TOF-MS (right).

### 30. B7-33-13Agp

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t = 14.195$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2958.555, observed 2962.894.

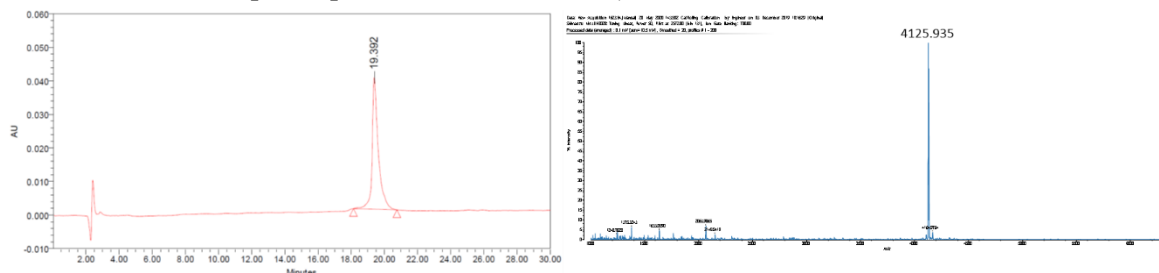


**Figure S3.30:** RP-HPLC profile of B7-33-13Agp (left) and MALDI-TOF-MS (right).

### 31. AcK(PalmGlu)-PEG11-B7-33

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 35-65% in 30 minutes,  $t_r = 19.392$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4124.517, observed 4125.935.



**Figure S3.31:** RP-HPLC profile of AcK(PalmGlu)-PEG11-B7-33 (left) and MALDI-TOF-MS (right).

**Table S3.1:** Summary of peptide characterization by MALDI-TOF MS and HPLC

| Analogues            | MALDI TOF MS |            | HPLC        | Purity (%) | Yield (%) |
|----------------------|--------------|------------|-------------|------------|-----------|
|                      | Observed*    | Calculated | $t_r$ (min) |            |           |
| B7-33                | 2990.834     | 2986.555   | 17.081      | 99.09      | 55        |
| B7-31                | 2792.450     | 2786.317   | 16.008      | 99.74      | 30        |
| AcB9-31              | 2615.466     | 2614.438   | 19.336      | 99         | 35        |
| B11-31               | 2336.669     | 2332.693   | 17.818      | 99.47      | 40        |
| B13-31               | 2193.809     | 2188.563   | 17.203      | 99.85      | 45        |
| Retro-inverso-B7-33  | 2992.640     | 2986.555   | 21.251      | 99.84      | 18        |
| Retro-inverso-B15-29 | 1590.616     | 1586.858   | 14.22       | 99.93      | 22        |
| B9-31-29Aib          | 2573.872     | 2572.318   | 15.359      | 99.34      | 43        |
| B9-31-25/29Aib       | 2526.807     | 2526.492   | 15.891      | 99.9       | 50        |
| B9-31-18/25/29Aib    | 2541.798     | 2540.783   | 17.535      | 99.99      | 35        |
| B9-31-11/18/25/29Aib | 2540.499     | 2539.075   | 19.863      | 97.84      | 40        |

|                          |          |          |        |       |    |
|--------------------------|----------|----------|--------|-------|----|
| Decanoic acid-PEG5-B7-33 | 3481.099 | 3475.425 | 12.229 | 100   | 50 |
| Myristic acid-PEG5-B7-33 | 3536.314 | 3532.535 | 17.365 | 99.57 | 45 |
| Palmitic acid-PEG5-B7-33 | 3559.294 | 3560.585 | 20.33  | 99.92 | 40 |
| K(Palm)-PEG5-B7-33       | 3691.177 | 3689.015 | 23.823 | 100   | 45 |
| B7-33_9K(Palm)           | 3229.264 | 3225.231 | 26.347 | 97.34 | 25 |
| Tag-B7-33                | 4194.532 | 4196.216 | 27.943 | 97.68 | 25 |
| Tag-PEG3-B7-33           | 4440.682 | 4443.766 | 19.134 | 99.7  | 33 |
| Tag-PEG5-B7-33           | 4525.517 | 4531.826 | 20.079 | 99.69 | 30 |
| K(Palm)-(PEG5)2-B7-33    | 4020.545 | 4024.625 | 17.790 | 99.68 | 55 |
| PA-(PEG5)2-B7-33         | 3902.283 | 3896.195 | 21.22  | 99.69 | 35 |
| K(Palm)-PEG11-B7-33      | 3953.897 | 3953.365 | 21.965 | 99.94 | 42 |
| K(PalmGlu)-PEG11-B7-33   | 4080.542 | 4082.506 | 23.073 | 99.72 | 45 |
| AcK(PalmGlu)-PEG11-B7-33 | 4125.935 | 4124.517 | 19.392 | 100   | 45 |
| B7-33-17HomoArg          | 3003.323 | 3000.555 | 19.894 | 100   | 44 |
| B7-33-17NorArg           | 2977.139 | 2972.555 | 19.607 | 94.18 | 40 |
| B7-33-17Agp              | 2961.668 | 2958.555 | 15.883 | 99.15 | 43 |
| B7-33-13HomoArg          | 3003.323 | 3000.555 | 18.6   | 96.6  | 35 |
| B7-33-13NorArg           | 2975.319 | 2972.555 | 18.076 | 100   | 45 |
| B7-33-13Agp              | 2962.894 | 2958.555 | 14.195 | 99.69 | 35 |

*This chapter contains confidential information*

|                               |          |          |        |       |    |
|-------------------------------|----------|----------|--------|-------|----|
| K(Palm)-PEG11-B7-<br>33-13Agp | 3924.954 | 3925.365 | 17.831 | 99.77 | 45 |
|-------------------------------|----------|----------|--------|-------|----|

\* Observed molecular weights were determined by MALDI-TOF MS of the purified peptides



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## **Chapter 4 SAR studies on RXFP3 ligands: H3 relaxin, A2 peptide, peptide 5, B1-22R**

### **4.1.1 Part 1: H3 analogue A2: Effects of C-terminal B-chain modifications**

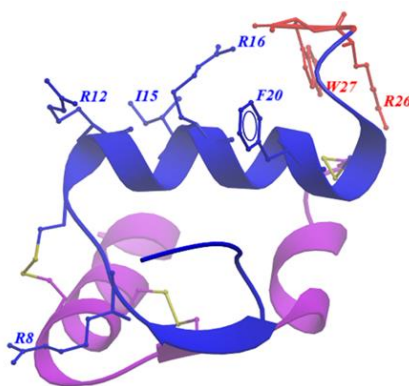
#### **4.1.2 Abstract**

The receptor for the neuropeptide H3 relaxin, RXFP3, is an attractive pharmacological target for the control of eating, addictive and psychiatric behaviours. Several structure-activity relationship studies on both H3 relaxin (containing three 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.

### 4.1.3 Introduction

H3 relaxin is a member of the insulin/ relaxin hormone superfamily<sup>1-4</sup>. While H3 relaxin interacts with the endogenous relaxin family peptide receptor 3 (RXFP3). It can also bind to and activate related peptide receptors, RXFP1 and RXFP4<sup>3</sup>. Current literature suggests that RXFP3 is a potential therapeutic target for psychiatric disorders such as anxiety and depression<sup>5</sup>. Central administration of RXFP3 agonists into rats increases feeding behaviours and that of antagonists inhibits food consumption and addictive behaviours<sup>6</sup>. These findings suggest that RXFP3 is also a potential target for pharmacological control of eating and addictive disorders.

H3 relaxin is structurally related to insulin with two chains (A and B) and three disulfide bonds<sup>1,7</sup>. Extensive SAR data on H3 relaxin suggests that the B-chain is the sole determinant of RXFP3 binding and activation<sup>8,9</sup>. Briefly, R<sup>B8</sup>, R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup> are involved in RXFP3 binding, and R<sup>B26</sup> and W<sup>B27</sup> are involved in RXFP3 activation (Figure 4.1.1)<sup>10,11</sup>.

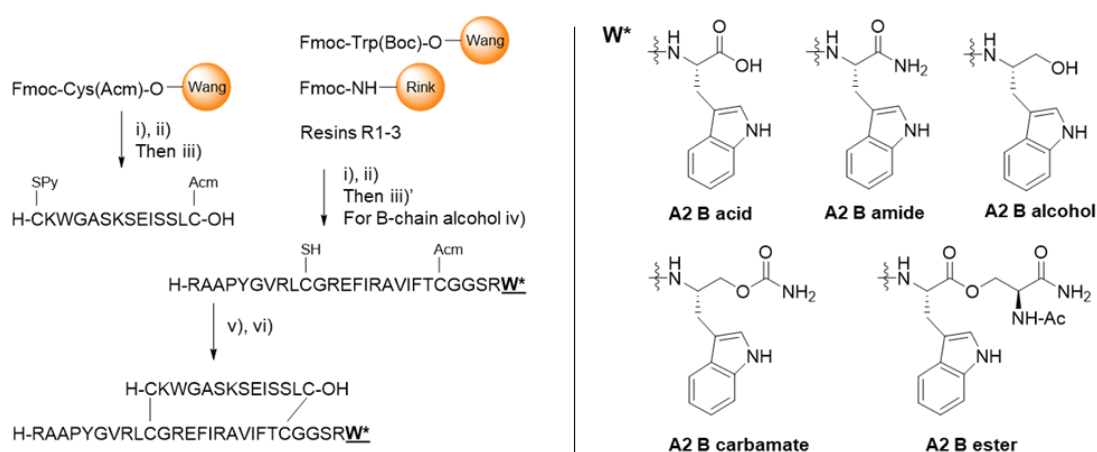


**Figure 4.1.1:** NMR structure of H3 relaxin (PDB: 2FHW), highlighting all the key residues Magenta: A-chain; Blue: B-chain, highlighting the amino acids involved in RXFP3 binding; Red: The C-terminal dipeptide important for RXFP3 activation; Yellow: disulfide bridges. The image was generated by Molsoft LLC software (ICM-browser Pro(x64); ActiveICM (version 1.2-4).

As H3 relaxin also interacts with RXFP1 and RXFP4 receptors, and it has a complex three disulfide-bonded structure which is difficult to produce in large scale, we removed the intra-A-chain disulfide bond and truncated ten residues from the N-terminus of A-chain,

resulting in a minimised analogue, A2<sup>12</sup>. This high yielding analogue exhibits H3 relaxin-like activity both *in vitro* and *in vivo*<sup>12</sup>. Our SAR studies on H3 relaxin and the related INSL5 peptides revealed that a free C-terminus (COOH) of the B-chain, not that of the A-chain, is favoured for RXFP3<sup>13</sup> and RXFP4<sup>14,15</sup> 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<sup>16</sup>. Thus, the effects of C-terminal modifications are peptide and receptor-dependent.

The aim of this study was to investigate the effects of the C-terminal modifications on the high yielding A2 analogue<sup>12</sup>. I have designed and synthesised four novel A2 analogues for this study (**Figure 4.1.2**). Some of the modifications at the carboxylic acid level were intended to improve the stability of the A2 peptides in regard to carboxypeptidases while others were designed to reversibly hide the C-terminal to explore the possibility of a prodrug strategy (**Figure 4.1.2**).



**Figure 4.1.2:** Solid phase peptide synthesis and regioselective disulfide bridge formation to generate A2 analogues. Synthetic conditions: i) 20% piperidine in DMF, 20 min, RT; ii) Fmoc-AA-OH, HCTU, DIEA, DMF, 1 hour, RT; iii) TFA/ DODT/ TIS/ H<sub>2</sub>O, 2 hour, RT; iii) TFA/ DODT/ TIS/ H<sub>2</sub>O with 10eq of DPDS, 2 hour, RT; iv) PBS/ ACN (80:20) at pH 7.4, 2 hour, RT; v) Addition of A-chain in 6M GnHCl (pH 8.5) to B-chain in 6M GnHCl (pH 5.5), 15 min, RT; vi) I<sub>2</sub>, AcOH, 1 hour, RT.

#### 4.1.4 Result and discussion

To study the effects of the *C*-terminal modifications of the A2 peptide, I chemically synthesised four novel analogues (A2 B alcohol, A2 B ester, A2 B carbamate, and A2 B amide) together with the parent A2 B carboxylic acid (A2 B acid) as control (**Figure 4.1.2**). For this study, two orthogonal cysteine S-protecting groups (Trt and Acn) were used for each A and B chains. 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.

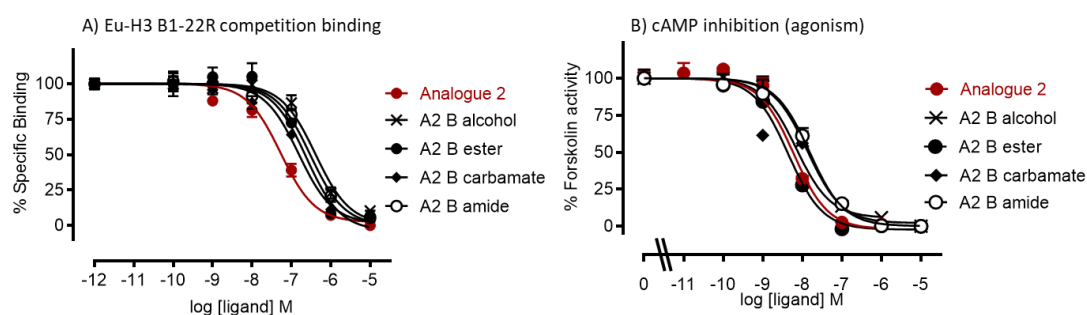
Briefly, the A-chain was generated from the preloaded Wang resin after solid phase peptide synthesis (SPPS) and TFA cleavage, in which 2,2-dipyridyl disulfide (DPDS) was supplemented to generate the *N*-terminal Cys(SPy). B-chains with *C*-terminal amide and carboxylic acid were obtained from commercially 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** (please see section 2.9 for methods in chapter 2). Briefly, the B-chain with a *C*-terminus alcohol was achieved 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**, please see section 2.9.2 for methods in chapter 2) and the SPPS afforded after TFA cleavage the iso- or depsi- peptide<sup>17</sup>. Then, an intramolecular O-to-N acyl transfer<sup>18</sup> 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<sup>19</sup>. The *C*-terminus with a serinyl ester was obtained after acylation of a supported acetyl-serine by Fmoc-L-Trp(Boc)-OH (**R2**, please see section 2.9.3 for methods in chapter 2). After SPPS and TFA cleavage, the presence of the acyl group on the serine blocks the intramolecular O-to-N acyl transfer<sup>18</sup> which consequently leads to a stable ester bond in this regard. Lastly, the *C*-terminus 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**, please see section 2.9.4 for methods in chapter 2)<sup>20,21</sup>. Solid phase peptide synthesis of the separate, selectively S-protected A- and B-chains followed by reversed phase chromatographic purification. Subsequent



stepwise formation of each of the two disulfide bonds was performed by thiololysis and iodolysis leading to the successful preparation of A2 analogues. Their analytical RP-HPLC profiles and MALDI TOF MS spectrometric analysis revealed highly purified synthetic products (Supporting Information; **Table S4.1.1**). The synthesis of parent peptide A2 peptides was found to be higher-yielding compared with modified analogues (Supporting Information; **Table S4.1.1**).

The peptides were tested for their ability to bind to their native receptor, RXFP3, in cell-based assays (**Figure 4.1.3**). The parent A2 analogue (A2 B acid) with free C-terminus of the B-chain showed higher affinity compared to A2 B amide (**Figure 4.1.3A, Table 4.1.1**) 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 alcohol and A2 B ester also showed lower binding affinity, the affinity of A2 B carbamate was not significantly different from A2 B acid. The peptides were then tested for their ability to activate RXFP3. As RXFP3 coupled to the inhibitory G proteins  $G_{i/o}$  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<sup>13</sup>, while some analogues appeared to show lower potency the difference to the activity of A2 B chain acid was not statistically significant (**Figure 4.1.3B, Table 4.1.1**). 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-hour stimulation during which time it is highly probable that the CHO-K1 cells are liberating esterase into the media that will generate the native A2 B acid as a metabolite from the A2 B ester (**Figure 4.1.3B**). This prodrug strategy can have a useful application for generating long-lasting molecules, in which the parent drug or at least one of its metabolites is activating RXFP3<sup>22</sup>. 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 six-hour activation time of the reporter assay. 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 the C-terminal amide substitution resulted in 10 times lower binding and activity at RXFP3<sup>13</sup> the modification at the C-terminal of the B-chain is less detrimental for A2. It is possible that the less restrained structure of the truncated

peptide, which lacks a large portion of the A-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<sup>22</sup>. RXFP3 mutagenesis and modelling data 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<sup>23</sup>. 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 free carboxylic acid.



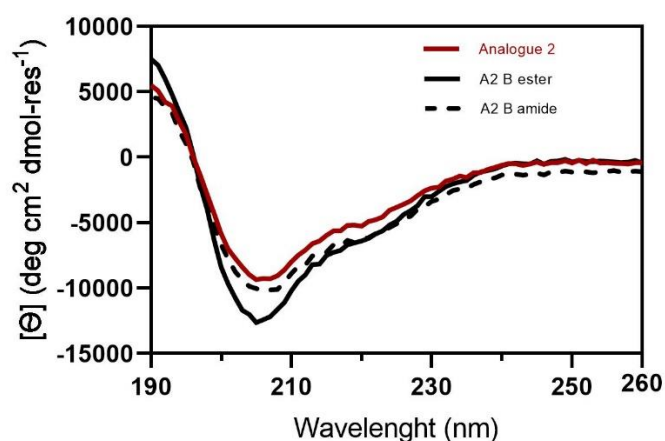
**Figure 4.1.3:** Competition binding (A) and cAMP activity assays (B) of A2 and its analogues in CHO-K1-RXFP3 cells. Europium-labelled H3 B1-22R was used as the labelled ligand in the competition binding assays. The data are the result of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

**Table 4.1.1:** Pooled binding affinity (pKi), (pEC<sub>50</sub>) data. RXFP3 *in vitro* assays were carried out on A2 and the four analogues.

| Analogues      | Eu-H3 B1-22R pKi (n) | cAMP pEC <sub>50</sub> (n) |
|----------------|----------------------|----------------------------|
| Analogue 2     | 7.34 ± 0.14 (3)      | 8.21 ± 0.08 (3)            |
| A2 B ester     | 6.73 ± 0.03 (3)**    | 8.36 ± 0.16 (3)            |
| A2 B carbamate | 6.96 ± 0.15 (3)      | 8.21 ± 0.17 (3)            |
| A2 B alcohol   | 6.48 ± 0.16 (3)***   | 7.88 ± 0.10 (3)            |
| A2 B amide     | 6.72 ± 0.09 (4)**    | 7.80 ± 0.13 (3)            |

\*\*\*p<0.001, \*\*p<0.01 vs Analogue 2.

Finally, we carried out circular dichroism (CD) studies to understand whether the peptide's structure is altered due to C-terminal modifications. We thus analysed the secondary structure of two analogues with reduced binding affinity A2 B ester and A2 B amide (**Figure 4.1.4**). The other analogues were not tested due to lack of sufficient amount of peptides. The CD spectra showed a typical  $\alpha$ -helical pattern with double minima at 208 and 222 nm. There was no observable perturbation in the overall secondary structure of A2 B ester or A2 B amide compared to A2 B acid (**Figure 4.1.4**).



**Figure 4.1.4:** CD spectra of A2 analogues modified at C-terminal. The overall secondary structure is maintained despite the C-terminal B-chain modification.

#### 4.1.5 Conclusion

In conclusion, we have chemically prepared four novel two chained, two disulfide-bonded 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 being 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 the serum of this modification, could potentially be utilised for developing A2 analogues with longer-lasting effects for animal studies.

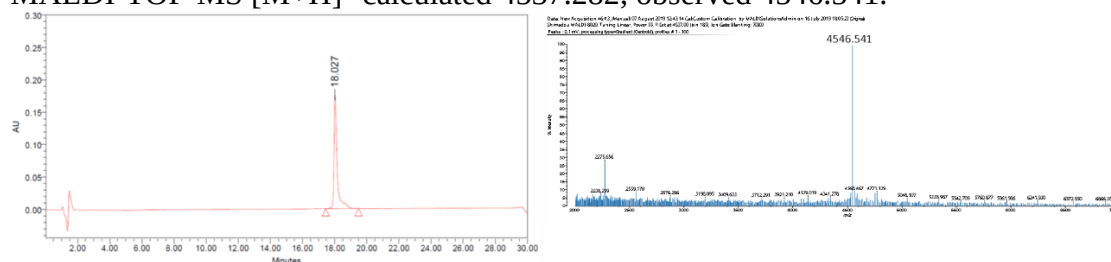
## 4.1.6 Supplementary information

### 4.1.6.1 Analytical RP-HPLC and MALDI-TOF-MS of all compounds

#### 1. Analogue 2

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 18.027 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4537.282, observed 4546.541.

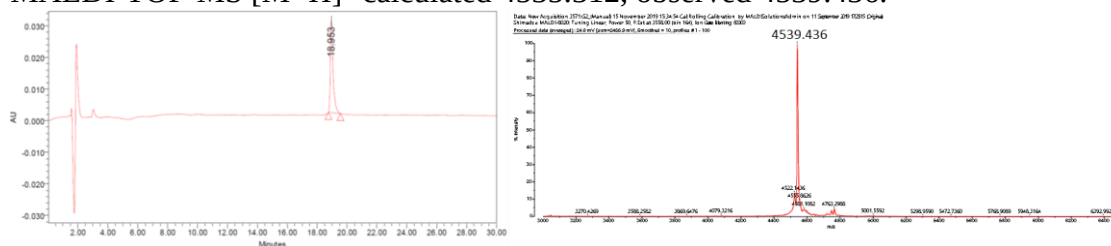


**Figure S4.1.1:** RP-HPLC profile of Analogue 2 (left) and MALDI-TOF-MS (right).

#### 2. A2 B amide

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 18.953 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4535.312, observed 4539.436.

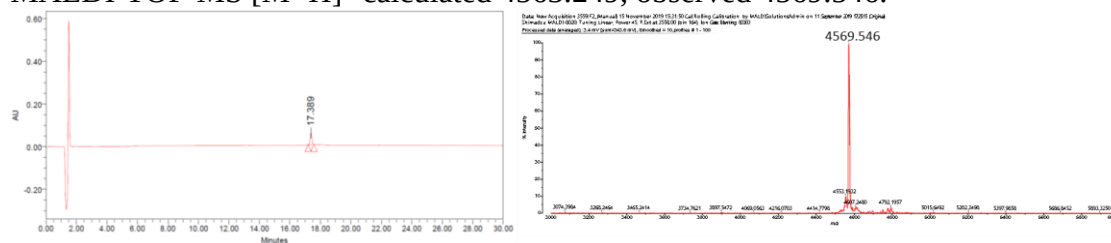


**Figure S4.1.2:** RP-HPLC profile of A2 B amide (left) and MALDI-TOF-MS (right).

#### 3. A2 B carbamate

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 17.389 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4563.249, observed 4569.546.

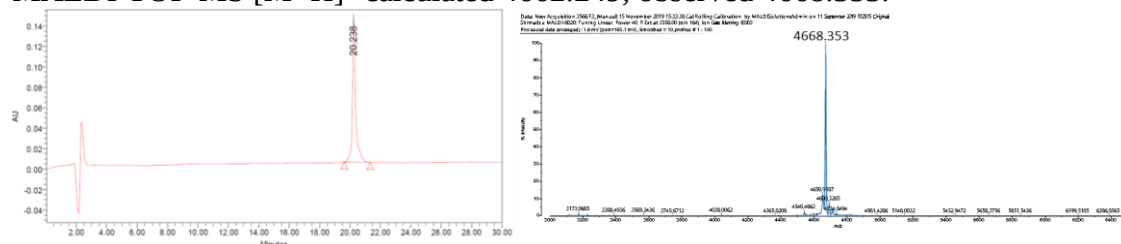


**Figure S4.1.3:** RP-HPLC profile of A2 B carbamate (left) and MALDI-TOF-MS (right).

#### 4. A2 B ester

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r = 20.238$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4662.249, observed 4668.353.



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## **4.2 Chapter 4: SAR studies on RXFP3 ligands: H3 relaxin, A2 peptide, peptide 5, B1-22R**

### **4.2.1 Part 2: Development of novel RXFP3 agonist and antagonist**

#### **4.2.2 Abstract**

Previously reported two-chain minimised RXFP3 agonist (A2 analogue), single-chain RXFP3 agonist (known as peptide 5), and antagonist (B1-22R; 23 residues) still suffer from low yield. Peptide 5 was shown to mimic the  $\alpha$ -helical structure of H3 relaxin B-chain. While this peptide is small, however, this peptide was generated by all-hydrocarbon stapling, which involves the use of Grubb's catalyst, which is difficult to separate. The yield of the hydrocarbon-stapled peptide is also low. I have developed a new method by utilising non-native amino acid in *i, i+4* manner at positions 13 and 17 that generated high yielding RXFP3 agonist and antagonist (14 residues) peptides.

### 4.2.3 Introduction

In the previous chapter 4 part 1, I have elaborated on two-chain H3 relaxin analogue, A2 peptide, as an RXFP3 agonist<sup>1</sup>. A2 is simplified as it has two disulfide bonds and truncated A chain compared with H3 relaxin which has three disulfide bonds. The B-chain alone was previously shown to bind to and activate the RXFP3 receptor with low affinity and potency<sup>2,3</sup>. This was because the B-chain alone contains all amino acids necessary for RXFP3 binding and activation. However, it does not have the correct  $\alpha$ -helical conformation, which is essential for high-affinity binding<sup>4</sup>. Therefore, several attempts were made to make B-chain-specific helical peptides. Shabanpoor et al. utilised judiciously placed salt bridges and single and bis-cystine bridges; unfortunately, these B-chain analogues showed no  $\alpha$ -helical structure<sup>1</sup>. Later Jayakody et al.<sup>5</sup> used a ring-closing metathesis reaction (RCM), a popular method to produce  $\alpha$ -helical peptide<sup>6-8</sup>. They stapled i, i+4 positions of B chain: B14 and B18 positions and B18 and B22 positions. These peptides were reported to be helical, but they showed poor RXFP3 binding and activation<sup>5</sup>. Hojo et al. in 2016 stapled the 13<sup>th</sup> and 17<sup>th</sup> positions of B-chain and truncated seven residues from the *N*-terminal which were not critical for RXFP3 binding and activity<sup>1</sup>. This method produced an all-hydrocarbon stapled analogue called peptide 5<sup>9</sup>. This peptide mimicked the helical structure of the B-chain of H3 relaxin and exhibited full agonistic activity both *in vitro* and *in vivo*<sup>9</sup>. Like R3/I5 and A2 analogues, peptide 5 was also selective for RXFP3/RXFP4 over RXFP1<sup>9</sup>. While peptide 5 has the simplest structure to date, however, this peptide was generated by hydrocarbon stapling, which involves the use of Grubb's catalyst, which is difficult to separate by HPLC. The yield of the hydrocarbon stapled product is low, and the separation of Grubb's catalyst from the product is difficult<sup>10-13</sup>. It is also challenging to control *E/Z* isomerism that arises from ring-closing metathesis reaction and distinguish between the isomers by solution NMR spectroscopy<sup>13</sup>. Other  $\alpha$ -helix-stabilising methods (e.g. lactam bridge, triazole bond)<sup>14</sup> also have limitations including low yield. Therefore, a novel, a high-yielding stapling method is required to contribute to peptide drug design and development.

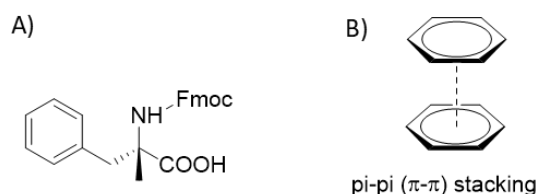
I hypothesised that I could generate  $\alpha$ -helical peptide by utilising non-native amino acids and without ring-closing metathesis reaction that requires Grubb's catalyst. Therefore, this chapter has two major main aims:

- 1) Developing novel RXFP3 agonist
- 2) Developing novel RXFP3 antagonist

#### **4.2.4 Developing novel RXFP3 agonist**

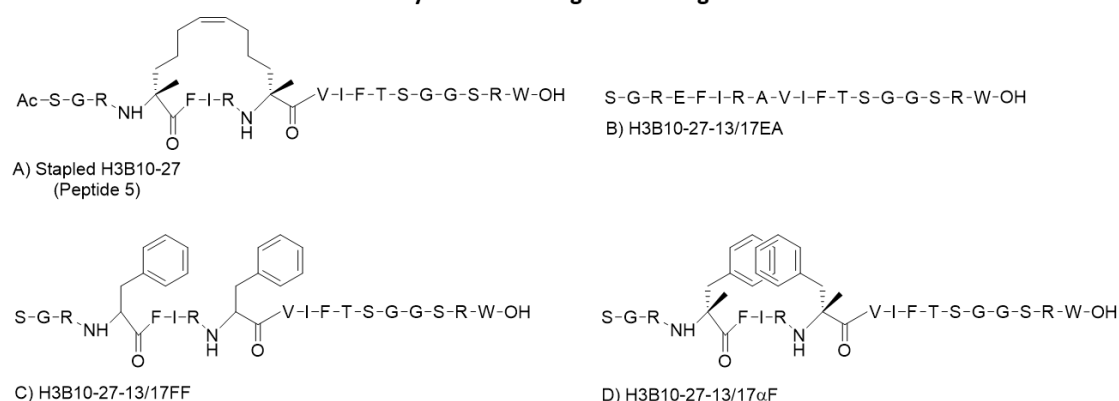
I aimed to use a non-covalent strategy to generate  $\alpha$ -helical peptides. Stabilising helical structure using non-covalent strategies such as  $\pi$ - $\pi$  stacking and/or hydrophobic interactions<sup>15</sup> has been studied. However, these methods never became popular as they did not work for a wide range of peptides. The  $\alpha,\alpha$ -disubstituted amino acids are also known to stabilise helical peptides which are also sequence-specific<sup>16</sup>. My idea is to use  $\pi$ - $\pi$  stacking interaction in combination with  $\alpha,\alpha$ -disubstituted amino acids. In this respect, I used two  $\alpha$ -methyl phenylalanine residues (**Figure 4.2.1A**) at positions 13 and 17, instead of (*S*)-2-(4-pentenylalanine) in stapled peptide 5. In addition to the  $\alpha$ -methyl group that puts steric constraints on the amino acid backbone that favours helical  $\phi$  and  $\psi$  angles, there will be  $\pi$ - $\pi$  interaction (**Figure 4.2.1B**) between two phenyl rings on the same side of the peptide leading to a non-covalent stapled peptide. This method will not require a catalyst and will also lead one single product in high yield. Four analogues, including three controls (**Figure 4.2.2A-C**) and one  $\alpha$ -methyl phenylalanine analogue (**Figure 4.2.2D**) were designed and synthesised with free C-termini by using Fmoc solid-phase peptide synthesis. The synthesis of all the analogues was confirmed using HPLC and MALDI-TOF mass spectroscopy (Supporting information).

### Structure of alpha-methyl phenylalanine and pi-pi ( $\pi$ - $\pi$ ) interaction



**Figure 4.2.1:** Structure of A) Fmoc- $\alpha$ -methyl phenylalanine and B) example of pi-pi ( $\pi$ - $\pi$ ) stacking between two benzene rings.

### Primary structure of agonist analogues

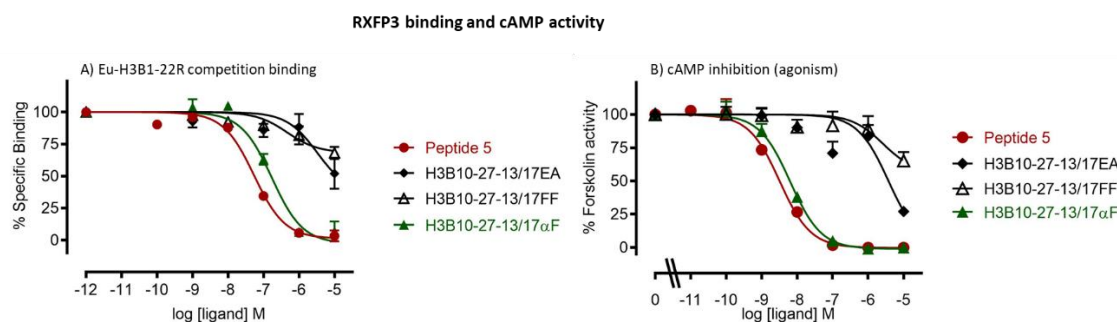


**Figure 4.2.2:** Primary structure of RXFP3 ligands. A) Hydrocarbon stapled peptide 5, B) control peptide H3B10-27-13/17EA, C) phenylalanine control H3B10-27-13/17FF and D)  $\alpha$ -methyl phenylalanine analogue H3B10-27-13/17 $\alpha$ F.

#### 4.2.4.1 RXFP3 binding and cAMP activity of agonist analogues

The incorporation of  $\alpha$ -methyl phenylalanine in i, i+4 manner at positions 13 and 17 resulted in an analogue, H3B10-17-13/17 $\alpha$ F (**Figure 4.2.2D**). Eu-H3B1-22R competition binding assay in CHO-K1-RXFP3 cells suggests that H3B10-27-13/17 $\alpha$ F has a similar RXFP3 binding affinity to peptide 5, whereas control peptides (H3B10-27-13/17FF and H3B10-27-13/17EA) have a weak binding affinity (**Figure 4.2.3A**, **Table 4.2.1**). Since RXFP3 is coupled to  $G_{\alpha i}$ , these analogues were then tested for their ability to activate the RXFP3 receptor as measured via their ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP3 cells. As expected, unlike control peptides (H3B10-27-13/17FF and H3B10-27-13/17EA), H3B10-27-13/17 $\alpha$ F was able to activate RXFP3 with

similar potency to peptide 5 (**Figure 4.2.3B, Table 4.2.1**). Please see section 2.12.2 and 2.12.3 for the competition binding and cAMP inhibition methods in chapter 2.



**Figure 4.2.3:** Binding and agonist activity of RXFP3 ligands in CHO-K1-RXFP3 cells. A) Competition binding curve of the agonist with 5 nM of Eu-B1-22R; B) cAMP activity as measured by cAMP changes. The data are the results of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

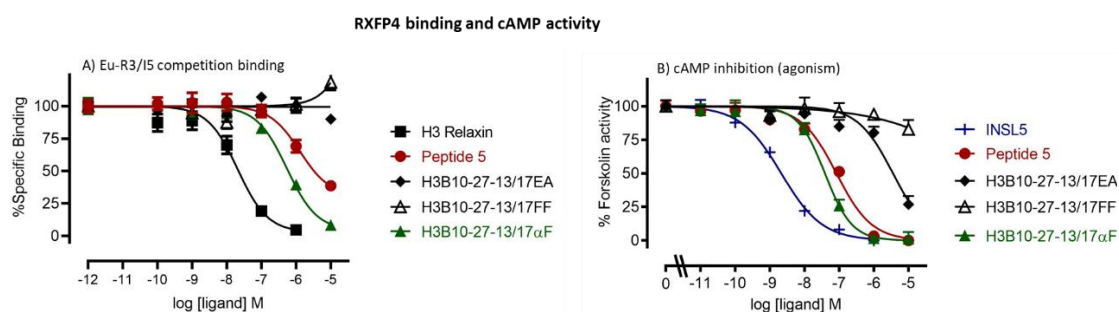
**Table 4.2.1:** RXFP3 and RXFP4 binding affinity (pKi) and cAMP activation (pEC<sub>50</sub>) of RXFP3 agonist

| Analogues        | RXFP3<br>Eu-H3B1-<br>22R pKi<br>(n) | RXFP3<br>cAMP pEC <sub>50</sub><br>(n) | RXFP4<br>Eu-R3/I5 pKi<br>(n)        | RXFP4<br>cAMP<br>pEC <sub>50</sub><br>(n) |
|------------------|-------------------------------------|----------------------------------------|-------------------------------------|-------------------------------------------|
| Peptide 5        | 7.38 $\pm$ 0.03<br>(3)              | 8.48 $\pm$ 0.06<br>(5)                 | 6.08 $\pm$ 0.17<br>(4)              | 6.95 $\pm$ 0.10<br>(4)                    |
| H3B10-27-13/17EA | 5.09 $\pm$ 0.29<br>(3) <sup>#</sup> | <5<br>(3)                              | No binding<br>(3)                   | 5.43 $\pm$ 0.22<br>(3) <sup>#</sup>       |
| H3B10-27-13/17FF | <5<br>(3)                           | <5<br>(3)                              | No binding<br>(3)                   | No activity<br>(4)                        |
| H3B10-27-13/17αF | 6.83 $\pm$ 0.04<br>(3) <sup>#</sup> | 8.16 $\pm$ 0.08<br>(6) <sup>^</sup>    | 6.81 $\pm$ 0.11<br>(3) <sup>θ</sup> | 7.41 $\pm$ 0.13<br>(6) <sup>θ</sup>       |

<sup>#</sup>p<0.001, <sup>^</sup>p<0.01, <sup>θ</sup>p<0.05 vs Peptide 5

#### 4.2.4.2 RXFP4 binding and cAMP activity of agonist analogues

Since H3 relaxin also binds to and activates the RXFP4 receptor, we tested these analogues for RXFP4 binding and activity. Eu-R3/I5 competition binding assay in CHO-K1-RXFP4 cells suggests that the incorporation of  $\alpha$ -methyl phenylalanine in i, i+4 manner at positions 13 and 17 results in a higher binding affinity to RXFP4 as peptide 5, whereas control peptides (H3B10-27-13/17FF and H3B10-27-13/17EA) exhibited no binding affinity (**Figure 4.2.4A, Table 4.2.1**). Since RXFP4 is coupled to  $G_{\alpha i}$ , these analogues were then tested for their ability to activate the RXFP4 receptor as measured by their ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP4 cells. As expected, H3B10-27-13/17 $\alpha$ F demonstrated higher potency at RXFP4 than peptide 5, whereas control peptides exhibited no or poor activity (**Figure 4.2.4B, Table 4.2.1**).

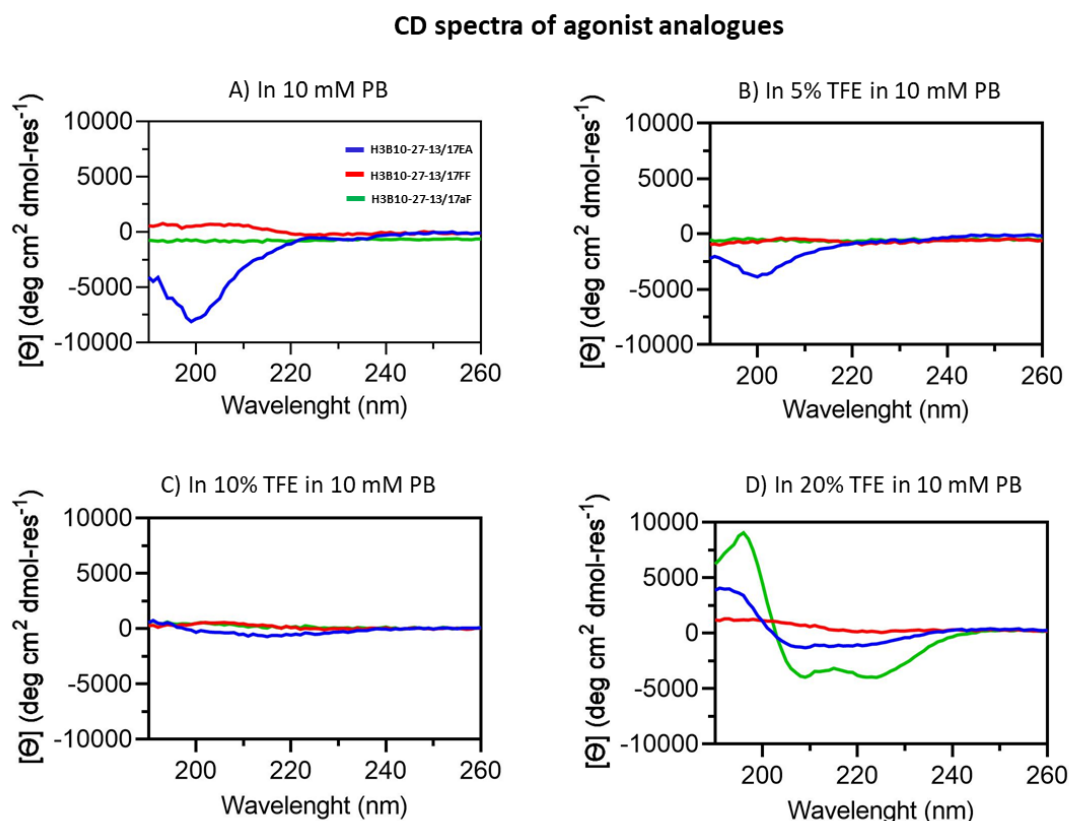


**Figure 4.2.4:** RXFP4 binding and agonist activity. A) Competition binding curve of the antagonist with 5nM of Eu-R3/I5 in CHO-K1-RXFP4 cells; B) cAMP activity as measured by cAMP changes in CHO-K1-RXFP4 cells. The data are the results of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

#### 4.2.4.3 Structural studies of agonist analogues

All-hydrocarbon stapled peptide 5 was shown to be helical by CD and NMR studies<sup>9</sup>. To confirm whether H3B10-27-13/17 $\alpha$ F peptide also has  $\alpha$ -helical structure, I conducted CD studies (**Figure 4.2.5**, please see section 2.6 for methods in chapter 2). The NMR studies were conducted by Dr Ashish Sethi (**Figure 4.2.6**, please see section 2.11 for methods in chapter 2). CD spectra of H3B10-27-13/17 $\alpha$ F analogue showed a typical  $\alpha$ -helical pattern with double minima at 208 and 222 nm wavelength in 20% TFE, whereas the control

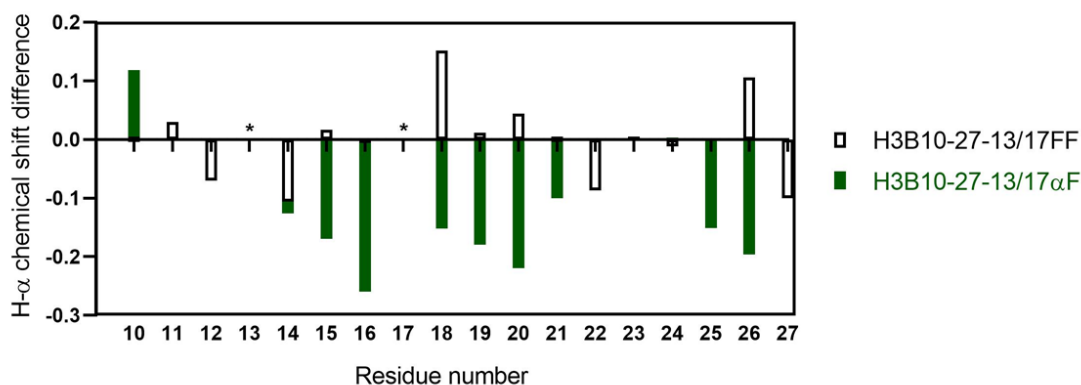
peptides (H3B10-27-13/17FF and H3B10-27-13/17EA) were not helical in the same condition (20% TFE) (**Figure 4.2.5**). The peptide H3B10-27-13/17 $\alpha$ F, however, did not show  $\alpha$ -helical structure in water or phosphate buffer. The data suggest that H3B10-27-13/17 $\alpha$ F has the propensity to form  $\alpha$ -helical structure in an appropriate solvent or environment (e.g. in the presence of RXFP3/4 receptor).



**Figure 4.2.5:** CD spectra of  $\alpha$ -agonist analogues. A) In phosphate buffer (PB) 10 mM none of the peptides is helical, B) in 5% TFE in 10 mM PB no helicity is observed, C) in 10% TFE in 10 mM PB no helicity is observed and D) in 20% TFE in 10 mM PB, H3B10-27-13/17 $\alpha$ F is helical in 20% TFE in 10 mM PB.

Secondary H- $\alpha$  chemical shifts, that is, the difference between shifts from random coil peptides and observed H- $\alpha$  chemical shifts are good indicators of the presence of secondary structure. Negative secondary H- $\alpha$  shift in NMR suggests that the H3B10-27-13/17 $\alpha$ F has the propensity to adopt  $\alpha$ -helical structure, which is consistent with CD data (**Figure 4.2.6**). On the other hand, the control peptide lacked negative secondary H- $\alpha$

shifts suggesting that it has a random coil structure (**Figure 4.2.6**). I hypothesise the peptide H3B10-27-13/17 $\alpha$ F forms induced-fit structure upon binding to the RXFP3 and RXFP4 receptors.



**Figure 4.2.6:** Secondary H- $\alpha$  chemical shift comparison for H3B10-27-13/17 $\alpha$ F and control peptide (H3B10-27-13/17FF). Stretches of negative values indicate the  $\alpha$ -helix structure. H3B10-27-13/17 $\alpha$ F shows negative values suggest it has the propensity to form a helical structure. \*The secondary shifts of residues alpha-methyl phenylalanine at 13 and 17 positions could not be determined as the random coil shift for this amino acid type is not known.

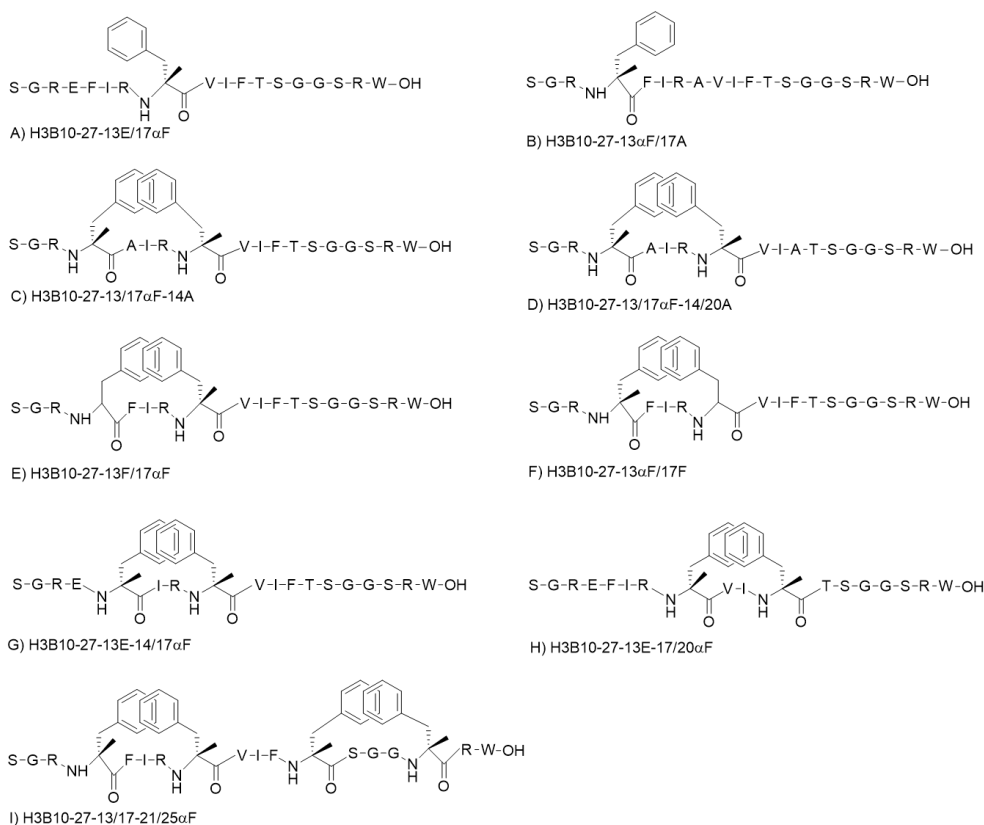
#### 4.2.4.4 Different combination of $\alpha$ -methyl phenylalanine analogues

There are two other phenylalanine residues in the H3B10-27-13/17 $\alpha$ F sequence (**Figure 4.2.2D**). I synthesised a series of different combinations of  $\alpha$ -methyl phenylalanine analogues (**Figure 4.2.7A-I**) to investigate if other phenyl rings have any interactions with the RXFP3 and RXFP4 receptors. **H3B10-27-13E/17 $\alpha$ F** analogue has only one  $\alpha$ -methyl phenylalanine at the 17<sup>th</sup> position and native residue Glu at the 13<sup>th</sup> position. **H3B10-27-13 $\alpha$ F/17A** analogue has only one  $\alpha$ -methyl phenylalanine at 13<sup>th</sup> position and native residue alanine at the 17<sup>th</sup> position. **H3B10-27-13/17 $\alpha$ F-14A** analogue has  $\alpha$ -methyl phenylalanine at the 13<sup>th</sup>, and 17<sup>th</sup> positions and phenylalanine is replaced with alanine at the 14<sup>th</sup> position. **H3B10-27-13/17 $\alpha$ F-14/20A** analogue has  $\alpha$ -methyl phenylalanine at the 13<sup>th</sup>, and 17<sup>th</sup> positions and phenylalanine is replaced with alanine at the 14<sup>th</sup> and 20<sup>th</sup> positions. **H3B10-27-13 $\alpha$ F/17F** analogue has one  $\alpha$ -methyl phenylalanine at the 13<sup>th</sup> position and phenylalanine at the 17<sup>th</sup> position. **H3B10-27-**



**13F/17 $\alpha$ F** analogue has phenylalanine at the 13<sup>th</sup> position and  $\alpha$ -methyl phenylalanine at the 17<sup>th</sup> position. **H3B10-27-13E-14/17 $\alpha$ F** analogue has glutamic acid at the 13<sup>th</sup> position and  $\alpha$ -methyl phenylalanine at the 14<sup>th</sup> and 17<sup>th</sup> position. **H3B10-27-13E-17/20 $\alpha$ F** analogue has glutamic acid at the 13<sup>th</sup> and two  $\alpha$ -methyl phenylalanine at the 17<sup>th</sup> and 20<sup>th</sup> positions. **H3B10-27-13/17-21/25 $\alpha$ F** analogue has two pairs of  $\alpha$ -methyl phenylalanine at the 13/17 and 21/25 positions.

Primary structure of different combination of  $\alpha$ -methyl phenylalanine analogues

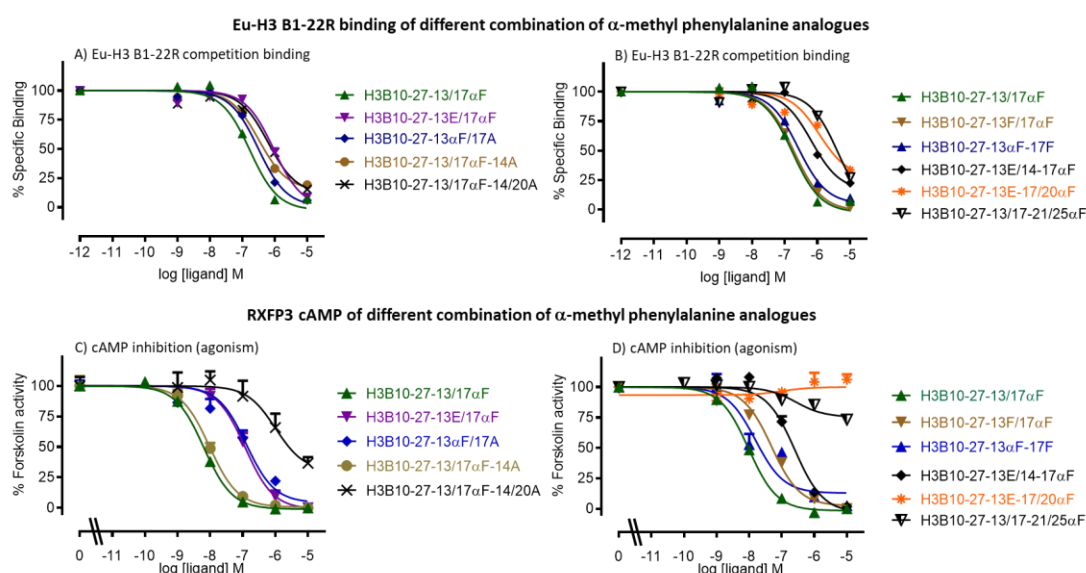


**Figure 4.2.7:** Primary structure of the different combinations of  $\alpha$ -methyl phenylalanine analogues.

#### 4.2.4.5 RXFP3 binding and cAMP activity of the different combination of $\alpha$ -methyl phenylalanine analogues

Eu-H3B1-22R competition binding assay in CHO-K1-RXFP3 cells suggests that the incorporation of  $\alpha$ -methyl phenylalanine only at one position (13<sup>th</sup> or 17<sup>th</sup>) and phenylalanine at other position reduces binding and activity compared to H3B10-27-

13/17 $\alpha$ F (**Figure 4.2.8A and C, Table 4.2.2**). Replacing phenylalanine at the 14<sup>th</sup> position with alanine does not affect binding and activity (**Figure 4.2.8A and C, Table 4.2.2**). Replacing Phe at the 20<sup>th</sup> position with Ala reduces the binding and activity drastically (**Figure 4.2.8A and C, Table 4.2.2**). This result confirms the previous finding that phenylalanine at the 20<sup>th</sup> position is important for binding<sup>9</sup>. Placing  $\alpha$ -methyl phenylalanine only at one position 13<sup>th</sup> or 17<sup>th</sup> and putting phenylalanine at the other position reduces binding and activity compared to H3B10-27-13/17 $\alpha$ F (**Figure 4.2.8B and D, Table 4.2.2**). Placing  $\alpha$ -methyl phenylalanine at 14/17 and 17/20 positions also reduces binding and activity compared to H3B10-27-13/17 $\alpha$ F (**Figure 4.2.8B and D, Table 4.2.2**). Putting two pairs of  $\alpha$ -methyl phenylalanine,  $\pi$ - $\pi$  interaction at 13/17 and 21/25 positions reduces binding and activity compared to H3B10-27-13/17 $\alpha$ F (**Figure 4.2.8B and D, Table 4.2.2**). Collectively, these different combinations of  $\alpha$ -methyl phenylalanine analogues show that presence of  $\alpha$ -methyl phenylalanine at the 13 and 17 positions is essential for binding and activity. It is likely that the side chains stabilise the  $\alpha$ -helical conformation rather than directly interacting with the receptors.



**Figure 4.2.8:** RXFP3 binding and activity of the different combination of  $\alpha$ -methyl phenylalanine analogues. A and B) Competition binding curve with 5nM of Eu-B1-22R in CHO-K1-RXFP3 cells. C and D) cAMP activity as measured by cAMP changes in CHO-K1-RXFP3 cells. The data are the results of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

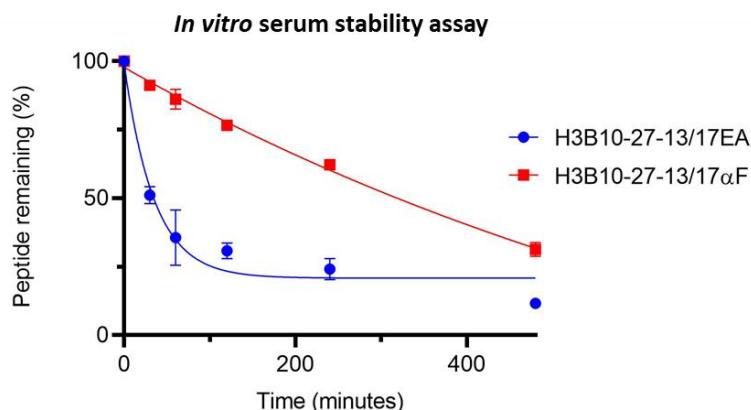
**Table 4.2.2:** List of a different combination of  $\alpha$ -methyl phenylalanine analogues and their RXFP3 binding (pKi) and cAMP activity (pEC<sub>50</sub>)

| Analogues                        | RXFP3 Eu-H3B1-22R<br>pKi (n) | RXFP3 cAMP pEC <sub>50</sub><br>(n) |
|----------------------------------|------------------------------|-------------------------------------|
| H3B10-27-13/17 $\alpha$ F        | 6.83 $\pm$ 0.04 (3)          | 8.16 $\pm$ 0.08 (6)                 |
| H3B10-27-13E/17 $\alpha$ F       | 6.07 $\pm$ 0.16 (3)**        | 6.98 $\pm$ 0.10 (3)***              |
| H3B10-27-13 $\alpha$ F/17A       | 6.35 $\pm$ 0.18 (3)*         | 6.84 $\pm$ 0.13 (3)***              |
| H3B10-27-13/17 $\alpha$ F-14A    | 6.83 $\pm$ 0.09 (3)          | 8.02 $\pm$ 0.08 (3)                 |
| H3B10-27-13/17 $\alpha$ F-14/20A | 5.88 $\pm$ 0.15 (3)***       | <5 (3)                              |
| H3B10-27-13F/17 $\alpha$ F       | 6.80 $\pm$ 0.11 (3)          | 7.27 $\pm$ 0.06 (3)***              |
| H3B10-27-13 $\alpha$ F/17F       | 6.70 $\pm$ 0.06 (3)          | 7.34 $\pm$ 0.23 (3)***              |
| H3B10-27-13E-14/17 $\alpha$ F    | 6.22 $\pm$ 0.06 (3)**        | 6.67 $\pm$ 0.07 (3)***              |
| H3B10-27-13E-17/20 $\alpha$ F    | 5.70 $\pm$ 0.26 (3)***       | No activity (3)                     |
| H3B10-27-13/17-21/25 $\alpha$ F  | 5.41 $\pm$ 0.13 (3)***       | <5 (4)                              |

\*p<0.05, \*\*<0.01, \*\*\*p<0.001 vs H3B10-27-13/17 $\alpha$ F

#### 4.2.4.6 *In vitro* serum stability of agonist analogues

To determine the stability of H3B10-27-13/17 $\alpha$ F in serum, I conducted *in vitro* serum stability assays (please see section 2.7 for the *in vitro* serum stability assay method in chapter 2). The results show that H3B10-27-13/17EA, which is control peptide, degrades quickly in serum (half-life 20 minutes), whereas H3B10-27-13/17 $\alpha$ F analogue is 20-fold more stable in serum (half-life 400 minutes) (**Figure 4.2.9**).



**Figure 4.2.9:** Serum stability assay of H3B10-27-13/17αF and H3B10-27-13/17EA at 37°C. There were significant differences between the peptides at any time point (P-value = 0.0088, t-test). One-phase exponential decay and paired t-test analyses were performed with GraphPad Prism 8.4.3. The data are the results of n=3 independent experiments.

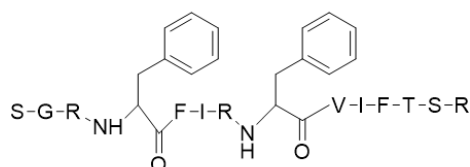
#### 4.2.5 Developing novel RXFP3 antagonist

In this study, I also aim to develop a high yielding RXFP3 antagonist. Kuei et al. truncated five residues from C-terminus of B-chain of a chimeric peptide (R3/I5; A-chain of INSL5 and B-chain of H3 relaxin), and added arginine at the C-terminus of B-chain resulted in R3(BΔ23-27)R/I5. The peptide acts as an RXFP3/4 antagonist<sup>17</sup>. Haugaard-Kedstrom et al. replaced two cysteine residues of the B-chain of R3(BΔ23-27)R/I5 that resulted in H3B1-22R, a single-chain antagonist of RXFP3<sup>18</sup>. I used the same strategy that I used for agonist development (earlier in section 4.2.4) to create a new antagonist. I have used two α-methyl phenylalanine at 13 and 17 positions of H3B1-22R but truncated nine residues from the N-terminus of the B-chain which are not important for binding<sup>18</sup>. Four analogues, including three controls (**Figure 4.2.10A-C**) and one target analogue containing two α-methyl phenylalanine residues (**Figure 4.2.10D**) were synthesised.

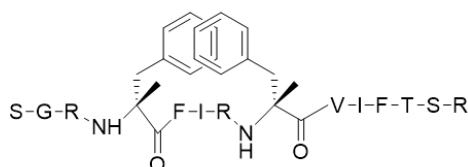
### Primary structure of antagonist analogues

R-A-A-P-Y-G-V-R-L-S-G-R-E-F-I-R-A-V-I-F-T-S-R  
A) H3B1-22R

S-G-R-E-F-I-R-A-V-I-F-T-S-R  
B) H3B10-22R-13/17EA



C) H3B10-22R-13/17FF

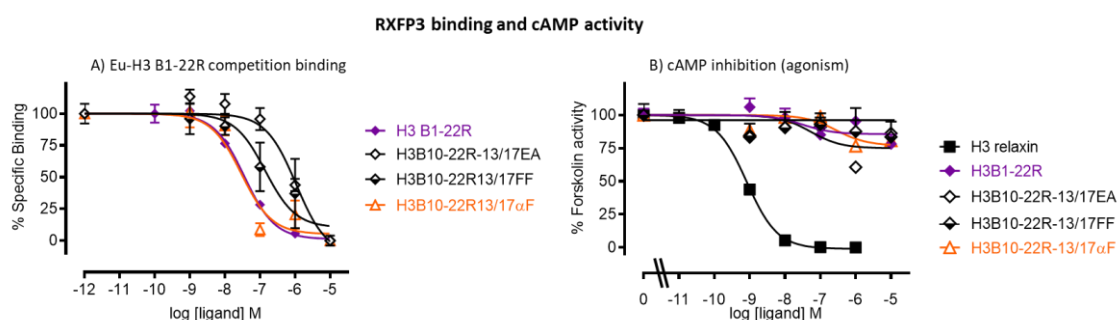


D) H3B10-22R-13/17αF

**Figure 4.2.10:** Primary structure of RXFP3 antagonist peptide. A) RXFP3 single-chain antagonist H3B1-22R, B) control peptide H3B10-22R-13/17EA, C) control peptide with phenylalanine H3B10-22R-13/17FF and D)  $\alpha$ -methyl phenylalanine analogue H3B10-22R-13/17 $\alpha$ F.

#### 4.2.5.1 RXFP3 binding and cAMP activity of antagonist analogues

Eu-H3B1-22R competition binding assay in CHO-K1-RXFP3 cells suggests that incorporation of  $\alpha$ -methyl phenylalanine in i, i+4 manner at positions 13 and 17 have similar binding as H3B1-22R, whereas control peptides (H3B10-22R-13/17FF and H3B10-22R-13/17EA) demonstrated a lower binding affinity (**Figure 4.2.11A, Table 4.2.3**).



**Figure 4.2.11:** RXFP3 binding and activity of antagonist analogues. A) Competition binding curve with 5nM of Eu-B1-22R in CHO-K1-RXFP3 cells; B) cAMP activity as measured by cAMP changes in CHO-K1-RXFP3 cells. The data are the results of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

Then these analogues were tested for their ability to activate the RXFP3 receptor by measured by their ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP3 cells. As expected, none of the analogues was able to activate RXFP3 (**Figure 4.2.11B, Table 4.2.3**), suggesting that the target peptide might be an RXFP3 antagonist.

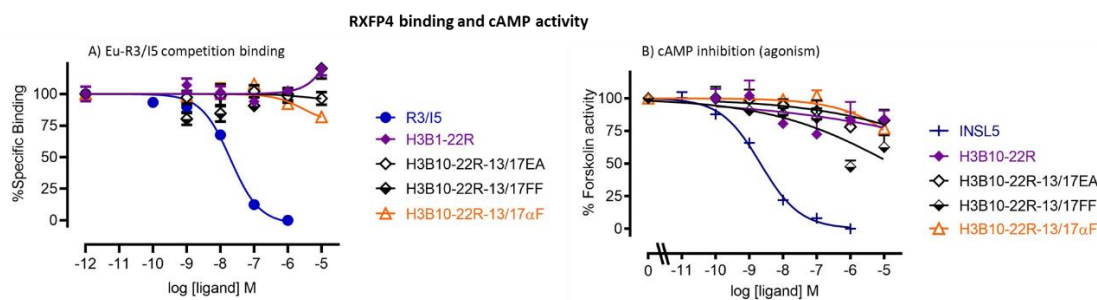
**Table 4.2.3:** Binding (pKi) and cAMP activity (pEC<sub>50</sub>) of RXFP3 antagonist analogues

| Analogues        | RXFP3<br>Eu-H3B 1-22R<br>pKi (n) | RXFP3<br>cAMP<br>pEC <sub>50</sub> (n) | RXFP4<br>Eu-R3/I5<br>pKi (n) | RXFP4<br>cAMP<br>pEC <sub>50</sub> (n) |
|------------------|----------------------------------|----------------------------------------|------------------------------|----------------------------------------|
| H3B1-22R         | 7.64 ± 0.09<br>(4)               | No activity<br>(3)                     | No binding<br>(3)            | No activity<br>(3)                     |
| H3B10-22R13/17EA | 6.08 ± 0.10<br>(3)**             | No activity<br>(3)                     | No binding<br>(3)            | No activity<br>(3)                     |
| H3B10-22R13/17FF | 6.67 ± 0.54<br>(3)*              | No activity<br>(4)                     | No binding<br>(3)            | No activity<br>(3)                     |
| H3B10-22R13/17αF | 7.60 ± 0.04<br>(3)               | No activity<br>(3)                     | No binding<br>(3)            | <5 (4)                                 |

\*p<0.05, \*\*p<0.01 vs H3 B1-22R

#### 4.2.5.2 RXFP4 binding and cAMP activity of antagonist analogues

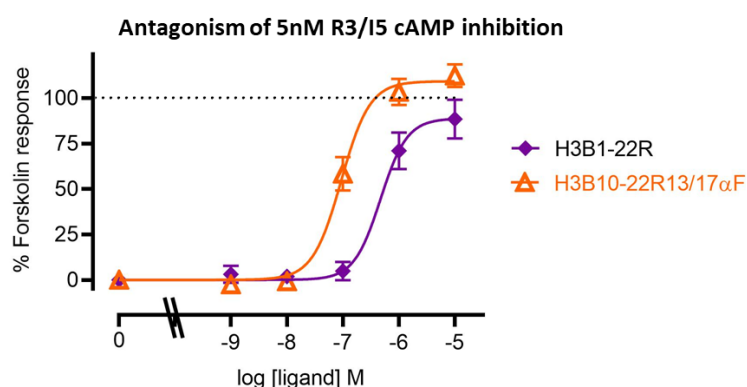
Eu-R3/I5 competition binding assay in CHO-K1-RXFP4 cells data suggest that none of the analogues binds to RXFP4 receptor (**Figure 4.2.12A, Table 4.2.3**). Subsequently, these analogues were tested for their ability to activate the RXFP4 receptor by measuring their ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP4 cells. As expected, none of these analogues has significant activity at the RXFP4 receptor (**Figure 4.2.12B, Table 4.2.3**). Therefore, these antagonist analogues are selective for the RXFP3 receptor.



**Figure 4.2.12:** RXFP4 binding and activity of antagonist analogues. A) Competition binding curve with 5nM of Eu-R3/I5 in CHO-K1-RXFP4 cells; B) cAMP activity as measured by cAMP changes in CHO-K1-RXFP4 cells. The data are the results of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

#### 4.2.5.3 RXFP3 antagonist assay

The best analogue, H3B10-22R-13/17αF, was tested for its ability to antagonise the function of R3/I5, an RXFP3 agonist. H3B10-22R-13/17αF analogue acts as an RXFP3 antagonist as evidenced by its ability to block the function of 5 nM R3/I5 that is inhibition of the forskolin-induced cAMP activity (**Figure 4.2.13**, **Table 4.2.4**). Importantly, H3B10-22R-13/17αF demonstrated higher potency and efficacy than H3B1-22R, possibly due to its higher stability in the 6-hour cAMP activity assay.



**Figure 4.2.13:** RXFP3 antagonism assay, the ability of H3B10-22R-13/17αF to antagonise 5nM R3/I5.

**Table 4.2.4:** Antagonism potency (pIC<sub>50</sub>) and efficacy of RXFP3 antagonist analogues

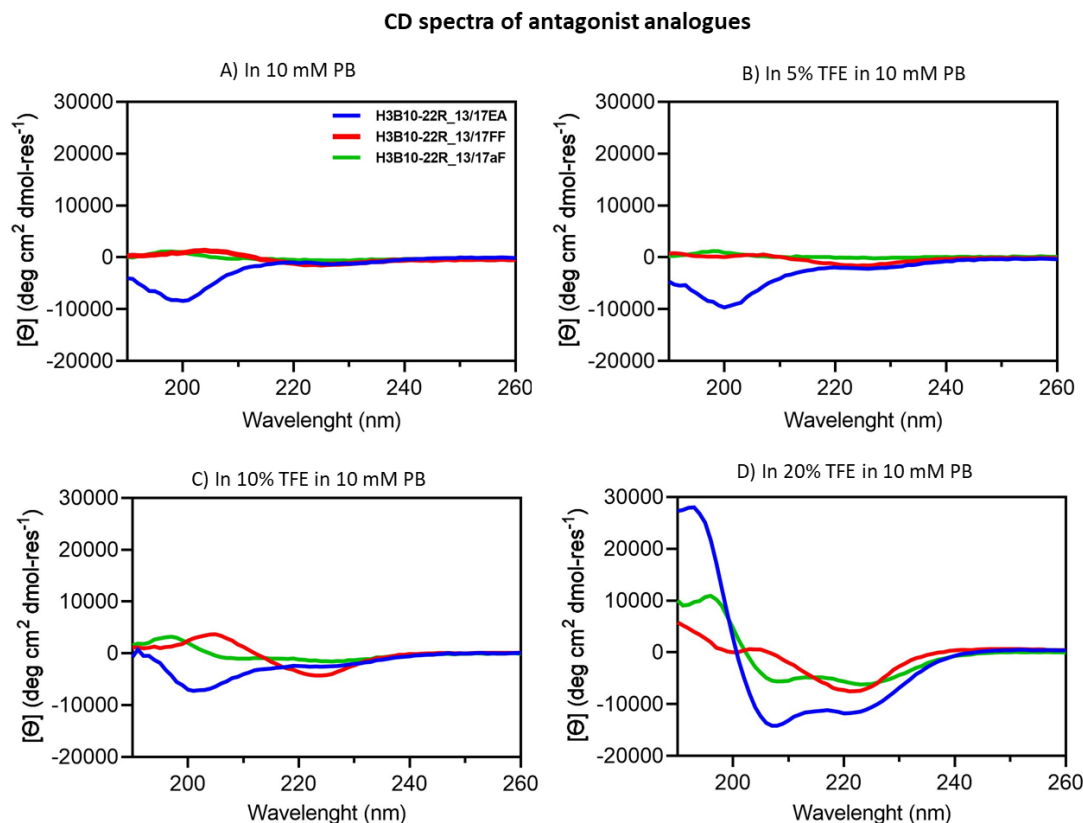
| <b>Analogues</b> | <b>RXFP3 Antagonist<br/>potency pIC<sub>50</sub><br/>(n)</b> | <b>RXFP3 Antagonist<br/>efficacy (%)</b> |
|------------------|--------------------------------------------------------------|------------------------------------------|
| H3B1-22R         | 6.25 ± 0.10<br>(3)                                           | 90.9 ± 12.8 (3)                          |
| H3B10-22R13/17αF | 6.98 ± 0.16<br>(4)**                                         | 110.6 ± 6.7 (4)                          |

\*\*p<0.01 vs H3 B1-22R

#### 4.2.5.4 Structural studies of antagonist analogues

The H3B1-22R single-chain antagonist has a random coil structure in solution<sup>18</sup>. I conducted CD spectroscopy studies for single-chain antagonist analogues. In 10 mM PB, 5% TFE and 10% TFE, none of the peptides are helical (**Figure 4.2.14 A-C**). H3B10-22R-13/17αF and H3B1-22R-13/17EA showed double minima at 208 and 222 nm wavelength in the presence of 20% TFE (**Figure 4.2.14D**), suggesting that they have the propensity to adopt the correct conformation upon binding to the RXFP3 receptor in a similar manner to the agonist peptides.





**Figure 4.2.14:** Circular dichroism spectra of alpha antagonist analogues. A) In 10 mM PB none of the analogues are helical, B) in 5% TFE in 10 mM PB no helicity is seen C) in 10% TFE in 10 mM PB H3B10-22R-13/17αF is slightly helical and D) in 20% TFE in 10 mM PB H3B10-22R-13/17αF and H3B10-22R-13/17EA are helical.

## 4.2.6 Conclusion

I have developed a chemical method that can overcome all the limitations of the RCM reaction to produce high yielding active single-chain agonist and antagonist of RXFP3. H3B10-27-13/17αF analogue is 20-fold more stable in serum compared with the control peptide (H3B10-27-13/17EA). H3B10-22R-13/17αF antagonist is only 14 amino acid long compared to the previously reported 23 residues antagonist<sup>18</sup>. These analogues can be produced in high yields, which can facilitate animal studies. These analogues will help in identifying the function of RXFP3 and could be drug leads. **Table 4.2.5** below summarises the novel analogues developed in this chapter.

**Table 4.2.5:** Summary of RXFP3 analogues

| Previously reported                                                                           | New                                                                 |
|-----------------------------------------------------------------------------------------------|---------------------------------------------------------------------|
| <p><b>Agonist</b></p> <p>Peptide 5</p>                                                        | <p><b>Agonist</b></p> <p>H3B10-27-13/17<math>\alpha</math>F</p>     |
| <p><b>Antagonist</b></p> <p>R-A-A-P-Y-G-V-R-L-S-G-R-E-F-I-R-A-V-I-F-T-S-R</p> <p>H3B1-22R</p> | <p><b>Antagonist</b></p> <p>H3B10-22R-13/17<math>\alpha</math>F</p> |

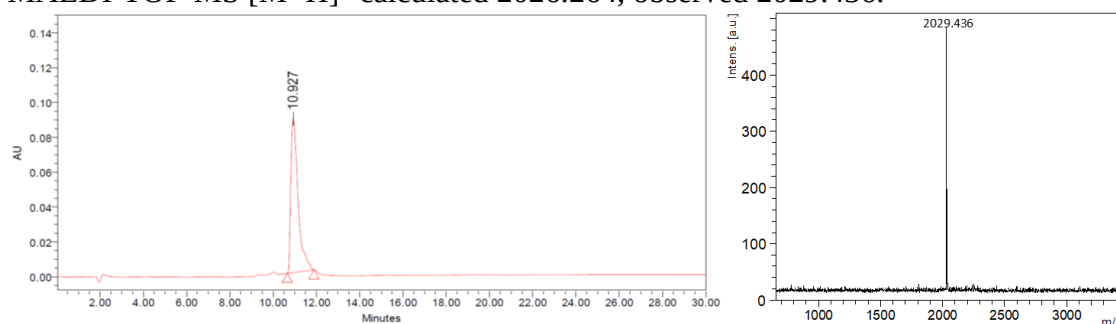
## 4.2.7 Supporting information

### 4.2.7.1 Analytical RP-HPLC and MALDI-TOF-MS of all compounds

#### 1. H3B10-27-13/17EA

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r = 10.927$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2026.264, observed 2029.436.

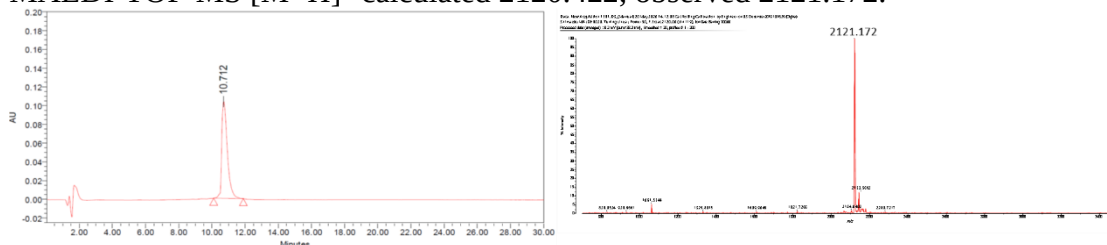


**Figure S4.2.1:** RP-HPLC profile of H3B10-27-13/17EA (left) and MALDI-TOF-MS (right).

#### 2. H3B10-27-13/17FF

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t_r = 10.712$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2120.422, observed 2121.172.

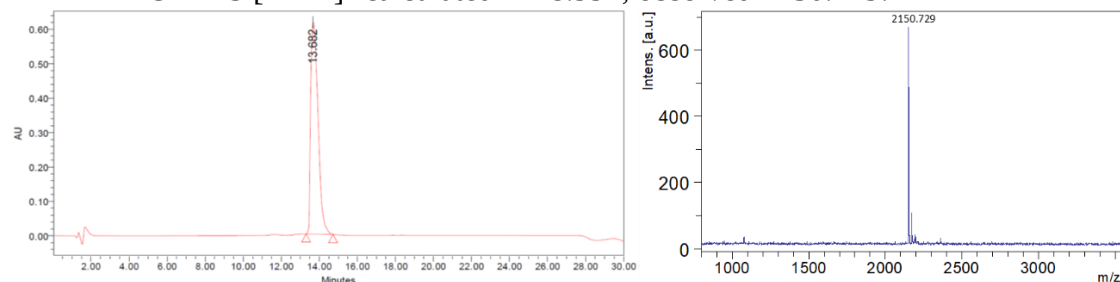


**Figure S4.2.2:** RP-HPLC profile of H3B10-27-13/17FF (left) and MALDI-TOF-MS (right).

### 3. H3B10-27-13/17 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 30-60% in 30 minutes,  $t_r$  = 13.682 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2148.991, observed 2150.729.

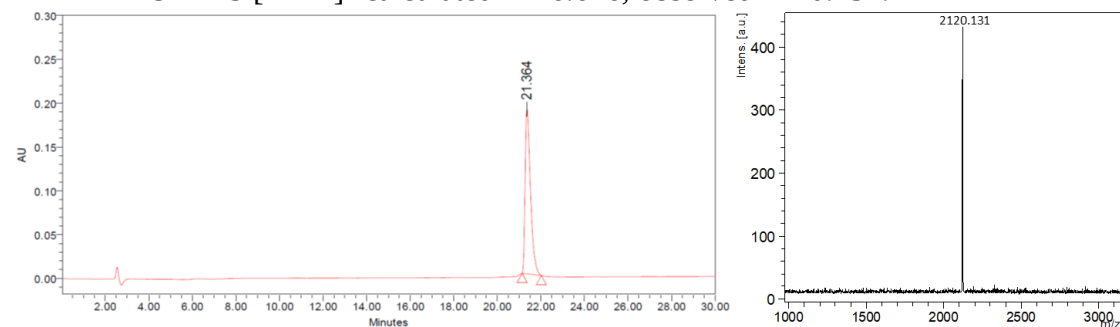


**Figure S4.2.3:** RP-HPLC profile of H3B10-27-13/17 $\alpha$ F (left) and MALDI-TOF-MS (right).

### 4. H3B10-27-13E/17 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 21.364 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2116.646, observed 2120.131.

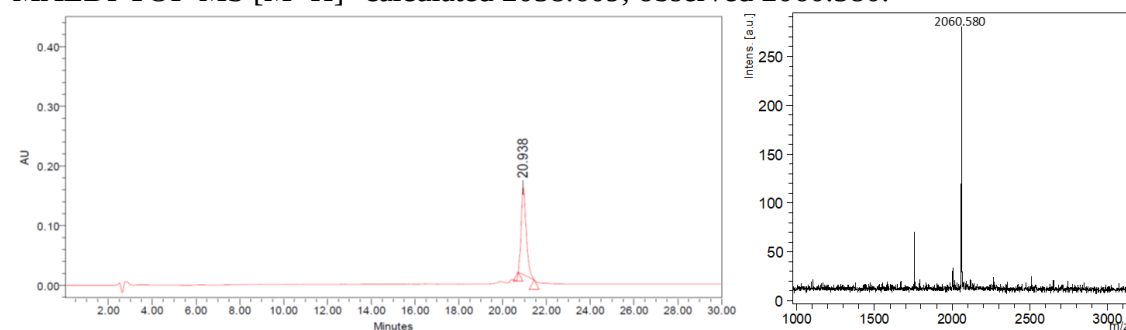


**Figure S4.2.4:** RP-HPLC profile of H3B10-27-13E/17 $\alpha$ F (left) and MALDI-TOF-MS (right).

### 5. H3B10-27-13 $\alpha$ F/17A

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 20.938 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2058.609, observed 2060.580.

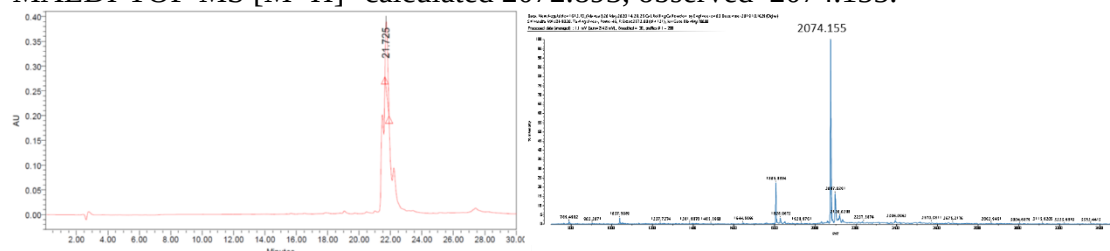


**Figure S4.2.5:** RP-HPLC profile of H3B10-27-13 $\alpha$ F/17A (left) and MALDI-TOF-MS (right).

## 6. H3B10-27-13/17 $\alpha$ F-14A

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r$  = 21.725 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2072.893, observed 2074.155.

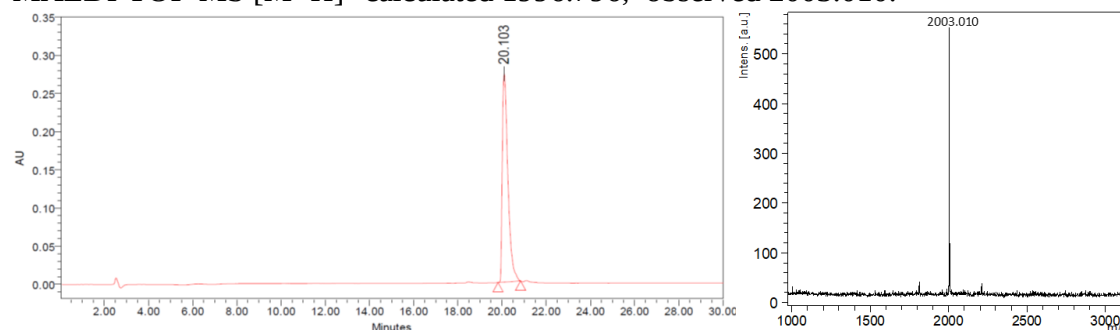


**Figure S4.2.6:** RP-HPLC profile of H3B10-27-13/17 $\alpha$ F-14A (left) and MALDI-TOF-MS (right).

## 7. H3B10-27-13/17 $\alpha$ F-14/20A

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 20.103 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 1996.796, observed 2003.010.

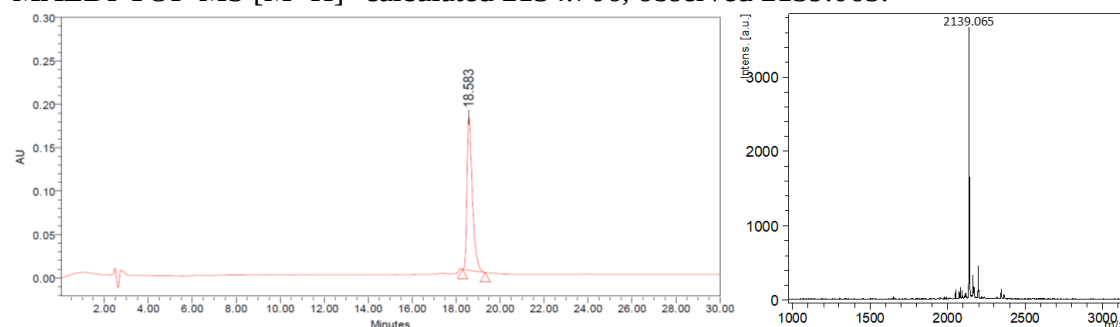


**Figure S4.2.7:** RP-HPLC profile of H3B10-27-13/17 $\alpha$ F-14/20A (left) and MALDI-TOF-MS (right).

## 8. H3B10-27-13F/17 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r$  = 18.583 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2134.706, observed 2139.065.

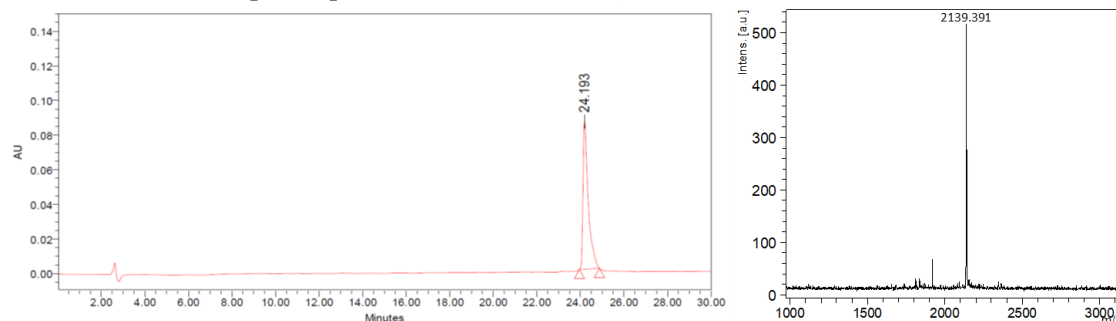


**Figure S4.2.8:** RP-HPLC profile of H3B10-27-13F/17 $\alpha$ F (left) and MALDI-TOF-MS (right).

### 9. H3B10-27-13 $\alpha$ F/17F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 24.193 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2134.706, observed 2139.391.

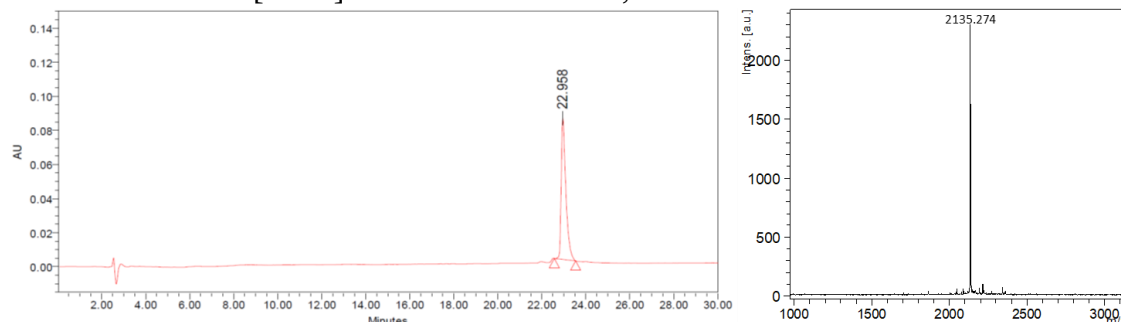


**Figure S4.2.9:** RP-HPLC profile of H3B10-27-13 $\alpha$ F/17F (left) and MALDI-TOF-MS (right).

### 10. H3B10-27-13E-14/17 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 22.958 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2130.930, observed 2135.274.

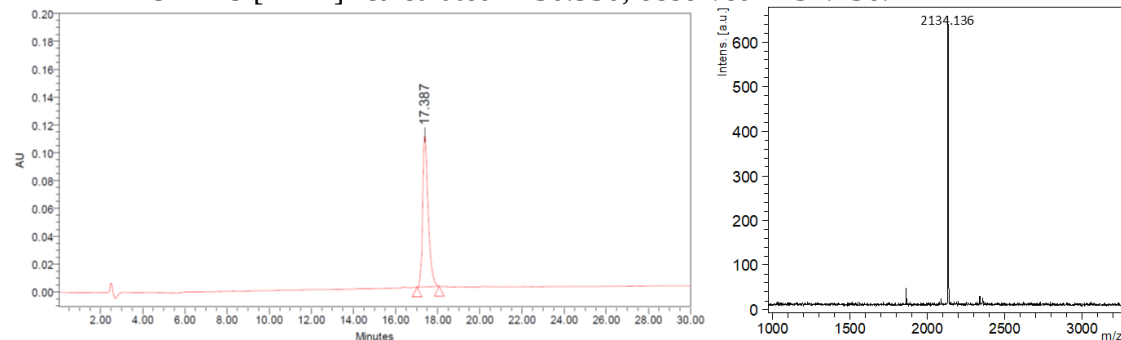


**Figure S4.2.10:** RP-HPLC profile of H3B10-27-13E-14/17 $\alpha$ F (left) and MALDI-TOF-MS (right).

### 11. H3B10-27-13E-17/20 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r$  = 17.387 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2130.930, observed 2134.136.

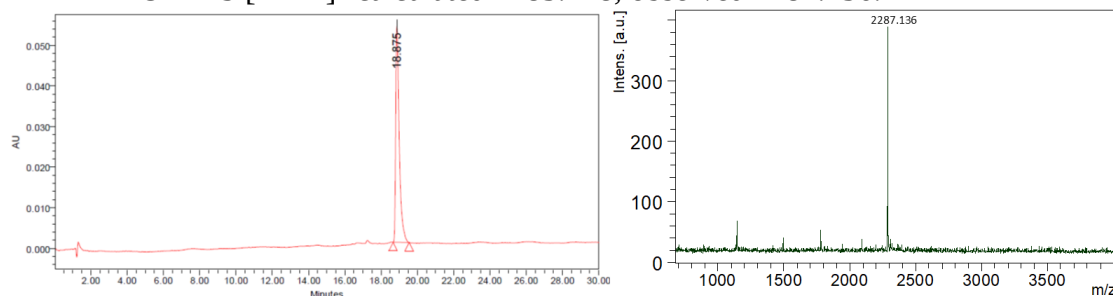


**Figure S4.2.11:** RP-HPLC profile of H3B10-27-13E-17/20 $\alpha$ F (left) and MALDI-TOF-MS (right).

#### 12. H3B10-27-13/17-21/25 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r$  = 18.875 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 2283.728, observed 2287.136.

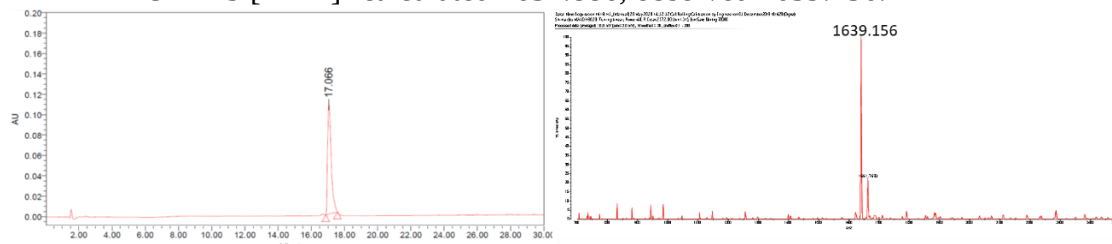


**Figure S4.2.12:** RP-HPLC profile of H3B10-27-13/17-21/25 $\alpha$ F (left) and MALDI-TOF-MS (right).

#### 13. H3B10-22R-13/17EA

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-45% in 30 minutes,  $t_r$  = 17.066 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 1637.886, observed 1639.156.

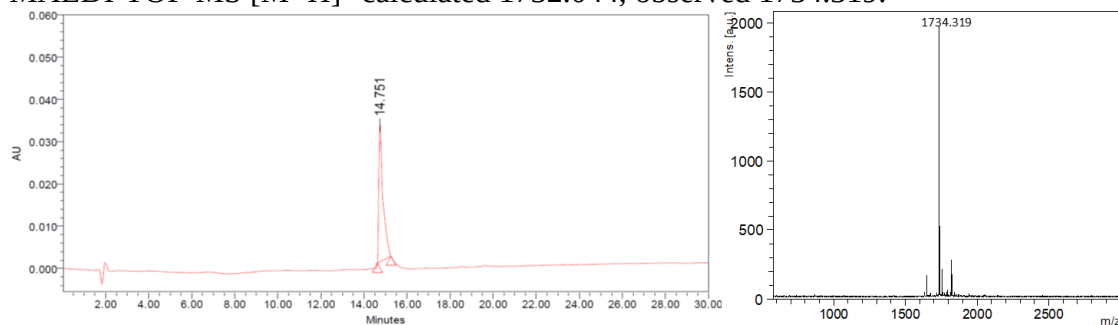


**Figure S4.2.13:** RP-HPLC profile of H3B10-22R-13/17EA (left) and MALDI-TOF-MS (right).

#### 14. H3B10-22R-13/17FF

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 25-55% in 30 minutes,  $t_r$  = 14.751 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 1732.044, observed 1734.319.

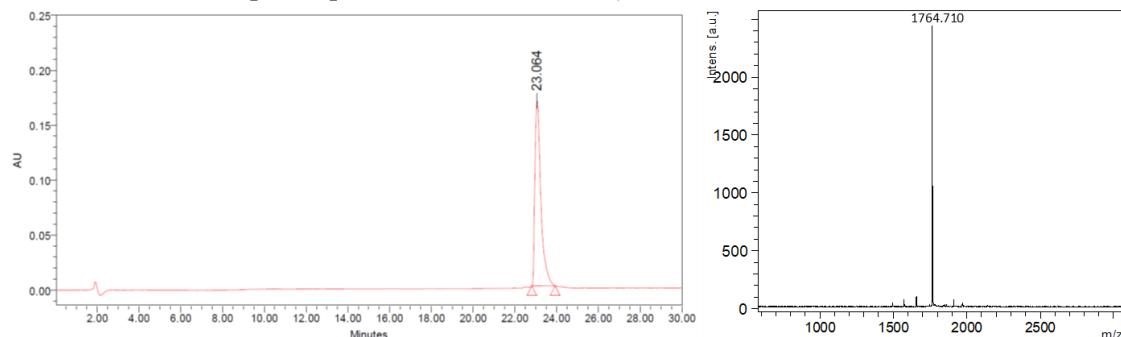


**Figure S4.2.14:** RP-HPLC profile of H3B10-22R-13/17FF (left) and MALDI-TOF-MS (right).

### 15. H3B10-22R-13/17 $\alpha$ F

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t_r$  = 23.064 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 1760.612, observed 1764.710.



**Figure S4.2.15:** RP-HPLC profile of H3B10-22R-13/17 $\alpha$ F (left) and MALDI-TOF-MS (right).

**Table S4.2.1:** Summary of peptide characterisation by MALDI-TOF MS and HPLC

| Analogues                        | MALDI TOF MS |            | HPLC        | Purity (%) | Yield (%) |
|----------------------------------|--------------|------------|-------------|------------|-----------|
|                                  | Observed*    | Calculated | $t_r$ (min) |            |           |
| H3B10-27-13/17EA                 | 2029.436     | 2026.264   | 10.927      | 99         | 50        |
| H3B10-27-13/17FF                 | 2121.172     | 2120.422   | 10.717      | 99.39      | 67        |
| H3B10-27-13/17 $\alpha$ F        | 2150.729     | 2148.991   | 13.682      | 99.95      | 60        |
| H3B10-27-13E/17 $\alpha$ F       | 2120.131     | 2116.646   | 21.364      | 99.96      | 50        |
| H3B10-27-13 $\alpha$ F/17A       | 2060.580     | 2058.609   | 20.938      | 97.5       | 45        |
| H3B10-27-13/17 $\alpha$ F-14A    | 2074.155     | 2072.893   | 21.725      | 78.25      | 55        |
| H3B10-27-13/17 $\alpha$ F-14/20A | 2003.010     | 1996.796   | 20.103      | 99.63      | 40        |
| H3B10-27-13F/17 $\alpha$ F       | 2139.065     | 2134.706   | 18.583      | 99.38      | 43        |
| H3B10-27-13 $\alpha$ F/17F       | 2139.391     | 2134.706   | 24.193      | 100        | 53        |
| H3B10-27-13E-14/17 $\alpha$ F    | 2135.274     | 2130.930   | 22.958      | 99.31      | 50        |
| H3B10-27-13E-17/20 $\alpha$ F    | 2134.136     | 2130.930   | 17.387      | 100        | 45        |
| H3B10-27-13/17-21/25 $\alpha$ F  | 2287.136     | 2283.728   | 18.875      | 100        | 50        |



|                            |          |          |        |       |    |
|----------------------------|----------|----------|--------|-------|----|
| H3B10-22R-13/17EA          | 1639.156 | 1637.886 | 17.066 | 99.3  | 56 |
| H3B10-22R-13/17FF          | 1734.319 | 1732.044 | 14.751 | 99.93 | 65 |
| H3B10-22R-13/17 $\alpha$ F | 1764.710 | 1760.612 | 23.064 | 99.82 | 60 |

\* Observed molecular weights were determined by MALDI-TOF MS of the purified peptides

#### 4.2.8 References

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## **Chapter 5 SAR studies on RXFP4 ligands: INSL5, analogue 13, and $\Delta$ R3/I5**

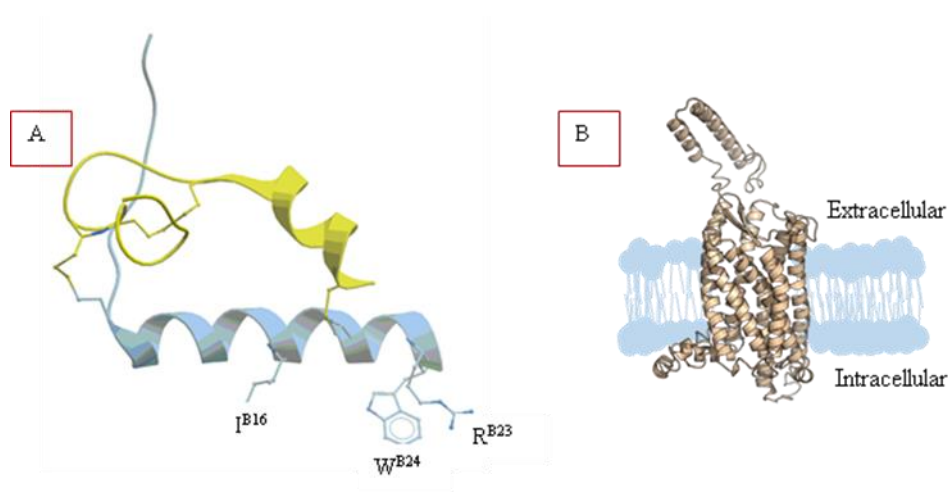
### **5.1 Abstract**

Insulin-like peptide 5 (INSL5) is an essential pharma target for treating human conditions such as anorexia and diabetes. However, INSL5 with two chains and three disulfide bridges is an extremely difficult peptide to assemble by chemical or recombinant means. In a recent study, our lab developed a simplified INSL5 analogue **13**, which is a relaxin family peptide receptor 4 (RXFP4)-specific agonist. To date, however, no RXFP4-specific antagonist (peptide or small molecule) has been reported in the literature. The focus of this study was to utilise the non-specific RXFP3/RXFP4 antagonist  $\Delta$ R3/I5 as a template to rationally design an RXFP4 specific antagonist. Unexpectedly, we demonstrated that  $\Delta$ R3/I5 exhibited partial agonism at RXFP4 when expressed in CHO cells which is associated with only partial antagonism of INSL5 analogue activation. In an attempt to improve RXFP4 specificity and antagonist activity, we designed and chemically synthesised a series of analogues of  $\Delta$ R3/I5. While all the chimeric analogues still demonstrated partial agonism at RXFP4, one peptide (Analogue 17) exhibited significantly improved RXFP4 specificity. Importantly, analogue 17 has a simplified structure which is more amenable to chemical synthesis. Therefore, analogue 17 is an ideal template for further development into a specific high-affinity RXFP4 antagonist which will be an important tool to probe the physiological role of RXFP4/INSL5 axis.

## 5.2 Introduction

The gut endocrine system is a rich source of peptides with therapeutic potential<sup>1-3</sup>. The demonstrated involvement of altered gut hormone profiles in both the success of bariatric surgery and the treatment of metabolic disorders implies that greater understanding and informed manipulation of gut hormones may lead to meaningful therapies for conditions such as anorexia, obesity, and diabetes<sup>4,5</sup>.

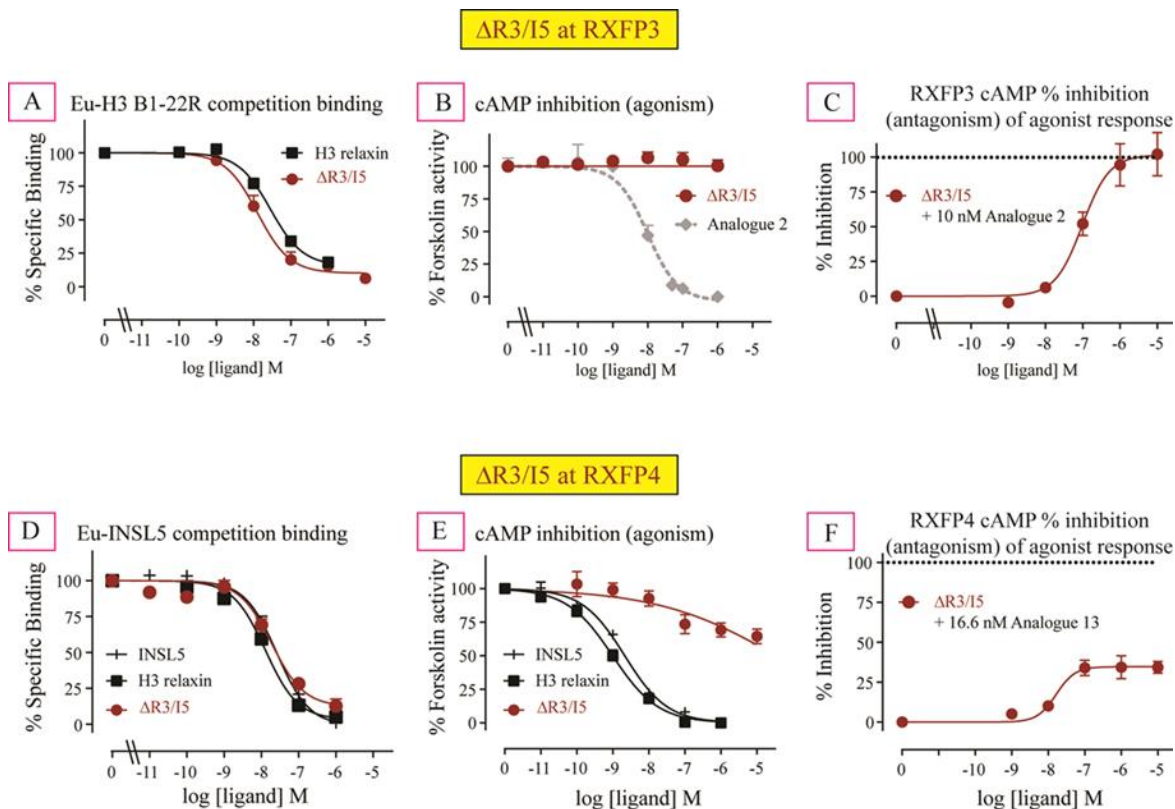
Insulin-like peptide 5 (INSL5, **Figure 5.1A, Table 5.1**) is a novel gut hormone produced by colonic L-cells<sup>6</sup>. Its target G protein-coupled receptor, Relaxin Family Peptide Receptor (RXFP4, **Figure 5.1B**) is expressed in the enteric nervous system<sup>6</sup>. The first biological data in RXFP4 knockout mice suggested a potential role of INSL5 in appetite regulation<sup>6</sup>. However, the primary source of INSL5 comes from a gut region (colonic enteroendocrine L cells<sup>6</sup>) that has only a minor influence on feeding. Therefore, there is a debate on the physiological role of INSL5 and RXFP4. The recent data in the literature also suggest that INSL5/RXFP4 system may be a potential therapeutic target for Type 2 diabetes<sup>7</sup>.



**Figure 5.1:** NMR structure of human INSL5 (PDB: 2KBC) with primary binding residues in the B-chain (I<sup>B16</sup>, R<sup>B23</sup>, W<sup>B24</sup>) highlighted. The image was generated by Molsoft LLC software (ICM-browser Pro(x64); ActiveICM (version 1.2-4); [www.molsoft.com](http://www.molsoft.com)). (B) Hypothetical structure of the RXFP4 receptor generated with Pymol.

Studies on the biology of INSL5 have been limited by the lack of sufficient quantities of the native peptide and the absence of small molecule RXFP4-specific agonists. Synthesis of both human INSL5 and mouse INSL5 has proven to be extraordinarily challenging by either chemical<sup>8,9</sup> or recombinant means<sup>10</sup>. The A-chain is insoluble, and B-chain is aggregating in nature, and therefore, both synthesis and purification were found to be very difficult, resulting in very poor yield<sup>8,9</sup>. We recently undertook structure-activity relationship (SAR) studies and utilised the knowledge to engineer a simplified, potent RXFP4 agonist peptide, analogue 13 (**Table 5.1**)<sup>11</sup>. The peptide is an INSL5 analogue that has a simplified A-chain connected to the B-chain by two disulfide bonds and is readily synthesised in 17.5 fold higher yield than INSL5<sup>11</sup>. A small-molecule agonist was recently reported<sup>12</sup> which has nanomolar potency both at RXFP4, and RXFP3, a receptor for human relaxin-3 (H3 relaxin)<sup>12</sup>. However, to date, no RXFP4-specific antagonist (peptide or small molecule) has been reported in the literature, and such a compound would be enormously valuable to understand the physiology of INSL5 and validate RXFP4 as a potential therapeutic target for the treatment of human conditions such as obesity and diabetes.

Some peptide ligands of GPCRs utilise amino acid residues for high-affinity binding, which are distinct from residues involved in activation. One way of generating antagonist peptide ligands is to delete the amino acids responsible for receptor activation while not affecting the binding interaction. This method was successful for developing antagonist for related receptors, RXFP2<sup>13,14</sup> (endogenous receptor for insulin-like peptide 3, INSL3) and RXFP3<sup>15,16</sup>. This was possible as the amino acids involved in activation did not greatly contribute to binding affinity, and thus deletion of these residues resulted in receptor antagonists. However, extensive SAR studies on INSL5 to date have been unable to separate distinct binding and activation domains<sup>17</sup>. While R<sup>B23</sup> and W<sup>B24</sup> in INSL5 are responsible for RXFP4 activation in a similar manner to the same residues in H3 relaxin, in INSL5 these residues also contribute significantly to the RXFP4 binding interaction<sup>17</sup>. Therefore, here, we explored alternative options to develop RXFP4-selective antagonists.



**Figure 5.2:** Binding, agonist and antagonist activities of  $\Delta R3/I5$  in CHO-K1-RXFP3 cells. (A) Competition binding curve of  $\Delta R3/I5$  competing with 5 nM of Eu-B1-22R; (B) Agonist activity of  $\Delta R3/I5$  as measured by cAMP changes; (C) Ability of  $\Delta R3/I5$  to antagonise 10 nM analogue 2-induced inhibition of cAMP activity. Binding, agonist and antagonist activities of  $\Delta R3/I5$  in CHO-K1-RXFP4 cells. (D) Competition binding curve of  $\Delta R3/I5$  competing with 5 nM of Eu-INSL5; (E) Agonist activity of  $\Delta R3/I5$  as measured by cAMP changes; (F) Ability of  $\Delta R3/I5$  to antagonise 16.6 nM analogue 13-induced inhibition of cAMP activity. The data are the result of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

A chimeric peptide consisting of the A-chain of INSL5 and C-terminally truncated B-chain of H3 relaxin,  $\Delta R3/I5$  (Table 5.1), was reported in the literature a decade ago<sup>16</sup>. *In vitro* pharmacological studies showed that  $\Delta R3/I5$  is a high-affinity antagonist for both RXFP3 and RXFP4 and it has been used successfully in rodent brain studies to demonstrate specific roles of RXFP3 as RXFP4 is not expressed in the brain<sup>16</sup>. In this study we have

|    | Analogues                                      | Sequences                                                                                                                                                                                                                                    | RXFP3                              |                                                             | RXFP4                               |                                                             |
|----|------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|-------------------------------------------------------------|-------------------------------------|-------------------------------------------------------------|
|    |                                                |                                                                                                                                                                                                                                              | Binding<br>pKi<br>(Ki)<br>(n)      | Activity<br>pEC <sub>50</sub><br>(EC <sub>50</sub> )<br>(n) | Binding<br>pKi<br>(Ki)<br>(n)       | Activity<br>pEC <sub>50</sub><br>(EC <sub>50</sub> )<br>(n) |
| 2  | Relaxin3<br>(R3)                               | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-G-G-S-R-W<br>D-V-L-A-G-L-S-S-S-C-C-K-W-G-C-S-K-S-E-I-S-S-L-C                                                                                                                                     | 7.64 ± 0.09<br>(22.9 nM)<br>(4)    | 9.08 ± 0.07<br>(0.83 nM)<br>(5)                             | 8.64 ± 0.15<br>(2.29 nM)<br>(3)     | 8.94 ± 0.13<br>(1.15 nM)<br>(4)                             |
|    | Analogue2<br>(A2)                              | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-G-G-S-R-W<br>C-K-W-G-A-S-K-S-E-I-S-S-L-C                                                                                                                                                         | 7.72 ± 0.29<br>(19.05 nM)<br>(3)   | 8.43 ± 0.24<br>(3.71 nM)<br>(4)                             | 7.1 ± 0.07<br>(79.43 nM)<br>(4)     | 7.7 ± 0.07<br>(19.95 nM)<br>(4)                             |
| 13 | INSL5<br>(I5)                                  | K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C                                                                                                                                                 | ND                                 | No activity<br>(3)                                          | 8.39 ± 0.14<br>(4.07 nM)<br>(3)     | 8.67 ± 0.08<br>(2.14 nM)<br>(5)                             |
|    | INSL5:<br>A(8-21)T15K<br>(Analogue 13)         | K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                               | ND                                 | ND                                                          | 7.53 ± 0.19<br>(29.51 nM)<br>(3)    | 7.68 ± 0.04<br>(20.90 nM)<br>(3)                            |
|    | ΔR3B<br>ΔI5B                                   | <div> <div>4</div> <div>5</div> <div>12</div> <div>17</div> <div>21</div> <div>1</div> <div>2</div> <div>9</div> <div>14</div> <div>18</div> </div> R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-R |                                    |                                                             |                                     |                                                             |
|    | ΔR3/I5                                         | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C                                                                                                                                                   | 8.01 ± 0.10<br>(9.77 nM)<br>(3)    | No activity<br>(3)                                          | 8.34 ± .08<br>(4.57 nM)<br>(3)      | Weak partial<br>agonist<br>(5)                              |
| 14 | ΔR3(R12L)/I5                                   | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-L-G-C-S-M-T-D-L-S-A-L-C                                                                                                                                                   | 7.62 ± 0.05<br>(23.99 nM)<br>(3)   | No activity<br>(3)                                          | 7.62 ± 0.25<br>(23.99 nM)<br>(3)**  | Weak partial<br>agonist<br>(6)                              |
| 15 | ΔR3/<br>I5A(8-21)T15K                          | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 7.81 ± 0.19<br>(15.49 nM)<br>(3)   | No activity<br>(4)                                          | 7.64 ± 0.09<br>(22.90 nM)<br>(3)**  | Weak partial<br>agonist<br>(5)                              |
| 16 | ΔR3B(T21I)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 7.61 ± 0.11<br>(24.55 nM)<br>(3)   | No activity<br>(4)                                          | 7.30 ± 0.18<br>(50.12 nM)<br>(3)*** | Weak partial<br>agonist<br>(4)                              |
| 17 | ΔR3B(A17T)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-T-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 7.72 ± 0.14<br>(19.05 nM)<br>(3)   | No activity<br>(3)                                          | 7.39 ± 0.07<br>(40.74 nM)<br>(3)*** | Weak partial<br>agonist<br>(4)                              |
| 18 | ΔR3B(R12L)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 7.59 ± 0.09<br>(25.70 nM)<br>(3)*  | No activity<br>(3)                                          | 7.83 ± 0.06<br>(14.79 nM)<br>(3)*   | Weak partial<br>agonist<br>(3)                              |
| 19 | ΔR3B(P4K)/<br>I5A(8-21)T15K                    | R-A-A-K-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 8.1 ± 0.20<br>(7.94 nM)<br>(3)     | No activity<br>(3)                                          | 7.99 ± 0.06<br>(10.23 nM)<br>(3)    | Weak partial<br>agonist<br>(4)                              |
| 20 | ΔR3B(P4KY5ER<br>12LA17TT21I)/<br>I5A(8-21)T15K | R-A-A-K-E-G-V-R-L-C-G-L-E-F-I-R-T-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                                                                                                                                                                 | 7.31 ± 0.17<br>(48.98 nM)<br>(3)** | No activity<br>(4)                                          | 6.88 ± 0.08<br>(131.82nM)<br>(3)*** | Weak partial<br>agonist<br>(4)                              |

**Table 5.1:** Primary structure of INSL5/H3 relaxin, their minimised analogues and chimeric peptides and their receptor binding affinity (pKi/Ki) and cAMP activation (pEC<sub>50</sub>/EC<sub>50</sub>) on the RXFP4/RXFP3 receptors. The different amino acids in the B-chain of INSL5 and H3 relaxin with chemically distinct side chains are boxed. Z is pyroglutamate which is a cyclic form of glutamic acid or glutamine that is produced by the loss of a water molecule. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  vs ΔR3/I5.

utilised ΔR3/I5 peptide as a template to rationally design an RXFP4 antagonist based on our understanding of RXFP3 and RXFP4 structure-function relationships. Unexpectedly, ΔR3/I5 demonstrated weak partial agonism of cAMP activation in RXFP4 expressing cells which



was associated with only partial antagonism of INSL5 analogue mediated cAMP activation. Rational design of synthetic  $\Delta R3/I5$  analogues demonstrated improvements in RXFP4 specificity however still demonstrated weak partial agonism associated with only partial RXFP4 antagonist activity.

### 5.3 Results and discussion

The human relaxin family is comprised of seven peptide hormones that include H1 relaxin, H2 relaxin, H3-relaxin, INSL3, INSL4, INSL5, and INSL6. H2 relaxin, H3-relaxin, INSL3 and INSL5 exert various biological functions through their GPCR receptors RXFP1-4, respectively<sup>18</sup>. While INSL5 is a selective ligand (agonist) for RXFP4, other family peptides are non-selective agonists for RXFP1-RXFP4. H3 relaxin, for example, is an endogenous ligand for RXFP3. However, it also strongly interacts with RXFP1 and RXFP4. To fully understand the role of relaxin family peptides, the development of selective agonists and antagonists is crucial. Recent reports have demonstrated that such receptor-selective agonists and antagonists can be achieved by making chimeric peptides<sup>16,19,20</sup>.  $\Delta R3/I5$  is an example of such a chimeric peptide. While H3 relaxin binds three receptors (RXFP1, RXFP3, and RXFP4), the chimeric  $\Delta R3/I5$  interacts with RXFP3 and RXFP4 only where it is a high-affinity antagonist. This study reports further modification of this chimeric peptide  $\Delta R3/I5$  leading to improvements in RXFP4 selectivity.

### 5.4 Design and synthesis of $\Delta R3/I5$ and $\Delta R3/I5$ -based chimeric analogues

Instead of applying conventional alanine scanning to determine which residues in  $\Delta R3/I5$  are responsible for selectivity between RXFP3 and RXFP4, I have rationally designed and chemically assembled several chimeric analogues of INSL5 and H3 relaxin (**Table 5.1**) based on our knowledge of the INSL5/RXFP4 and H3 relaxin/RXFP3 interactions<sup>18</sup>.

I synthesised seven  $\Delta R3/I5$ -based analogues (**14-20**) and have utilised five previously reported peptides (H3 relaxin<sup>21</sup>, INSL5<sup>8</sup>, analogue 2<sup>22</sup>, analogue 13<sup>11</sup> and  $\Delta R3/I5$ <sup>16</sup>) as

controls (**Table 5.1**). I have used H3 relaxin and analogue **2** as RXFP3 agonists, INSL5 and analogue **13** as RXFP4-selective agonists, and  $\Delta$ R3/I5 as a non-selective antagonist for both RXFP3 and RXFP4 (**Table 5.1**). All the analogues were made with free C-termini (carboxylic acid) as we have previously shown that INSL5 and H3 relaxin analogues with amidated C-termini were less active compared with native INSL5 or H3 relaxin with free C-termini<sup>9</sup>. All linear single-chain peptides (A and B chains) were synthesised by Fmoc solid-phase peptide synthesis using automated peptide synthesiser (Liberty Blue) and then connected them by disulfide bonds in solution. The peptides were purified by reverse-phase high-performance liquid chromatography, and high final purity (93-99%) was confirmed by MALDI-TOF MS and RP-HPLC analysis (Supplementary information, **Table S5.1**). The concentration of peptide samples for pharmacological testing was confirmed by using Direct Detect assay-free sample cards and the Direct Detect spectrometer (please see section 2.8 for methods in chapter 2).

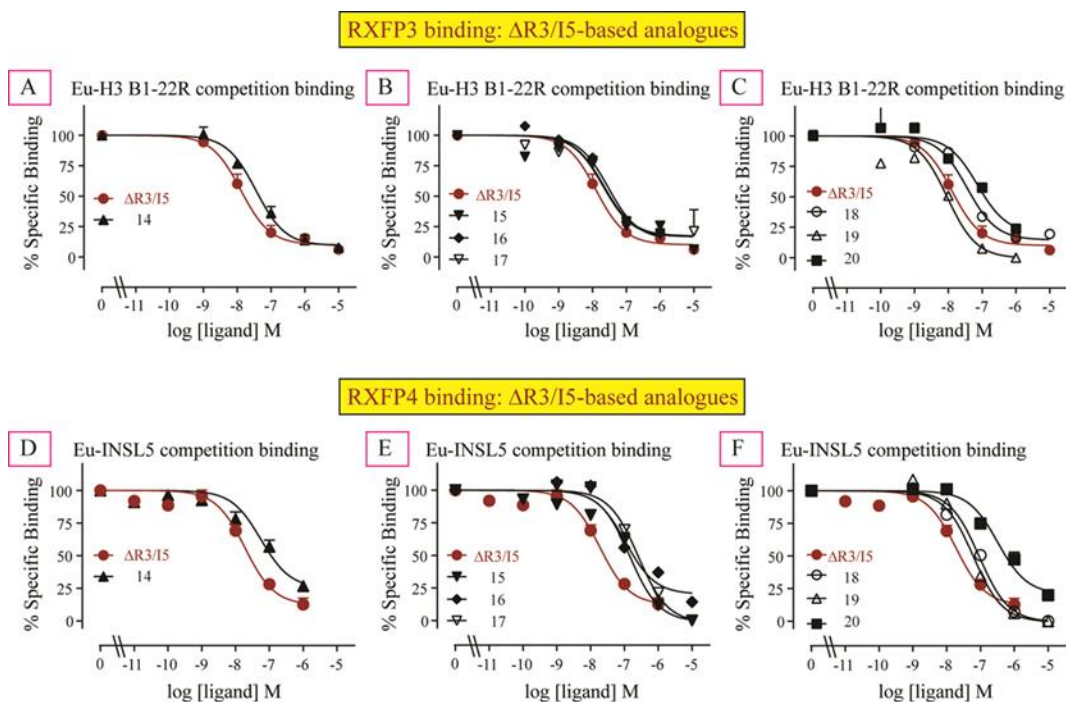
## 5.5 $\Delta$ R3/I5 – a previous study

$\Delta$ R3/I5 was originally reported to strongly bind to RXFP3 and RXFP4 but showed no agonist activity (NA) at either of these receptors<sup>16</sup> suggesting that  $\Delta$ R3/I5 is an antagonist for both RXFP3 and RXFP4. The antagonism of RXFP3 and RXFP4 was compared, and the results showed that  $\Delta$ R3/I5 dose-dependently shifted the agonism curves to the right confirming  $\Delta$ R3/I5 to be an antagonist for both RXFP3 and RXFP4<sup>16</sup>. Here we have chemically synthesised and pharmacologically characterised  $\Delta$ R3/I5 for binding and cAMP potency in CHO cells stably expressing RXFP3 (CHO-K1-RXFP3) or RXFP4 (CHO-K1-RXFP4).

## 5.6 The pharmacological activity of $\Delta$ R3/I5 at RXFP3

We have first tested our synthetic  $\Delta$ R3/I5 in competition binding assays CHO-K1-RXFP3 cells.  $\Delta$ R3/I5 exhibited high affinity binding at RXFP3 with an affinity higher than the native ligand H3 relaxin as previously reported ( $K_i$  of 9.77 nM compared to 22.9 nM; **Figure 5.2A**, **Table 5.1**). The  $\Delta$ R3/I5 was then tested for its ability to activate the RXFP3 receptor as

measured via its ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP3 cells (**Figure 5.2B**). Consistent with the previous report,  $\Delta R3/I5$  was unable to activate RXFP3 (**Figure 5.2B**, **Table 5.1**) and displayed potent antagonistic activity ( $IC_{50}$  of 117.49 nM,  $E_{max} = 100\%$ ; **Figure 5.2C**, **Table 5.2**) at RXFP3 evident from its ability to block 10 nM analogue 2 (RXFP3 agonist) inhibition of the forskolin-induced cAMP activity.



**Figure 5.3:** Competition binding curves for  $\Delta R3/I5$  and novel chimeric analogues 14-20, (A-C) in comparison to Eu-B1-22R in CHO-K1-RXFP3-expressing cells and (D-F) in comparison to Eu-INSL5 in RXFP4-expressing cells. The data are the result of  $n=3-4$  independent experiments and are expressed as mean  $\pm$  SEM.

|    | Analogues                                              | Sequences                                                                                                                               | RXFP3                                                                 |                         | RXFP4                                                                 |                         |
|----|--------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|-------------------------|-----------------------------------------------------------------------|-------------------------|
|    |                                                        |                                                                                                                                         | Antagonist potency<br>pIC <sub>50</sub><br>(IC <sub>50</sub> )<br>(n) | E <sub>max</sub><br>(n) | Antagonist potency<br>pIC <sub>50</sub><br>(IC <sub>50</sub> )<br>(n) | E <sub>max</sub><br>(n) |
|    | $\Delta$ R3/I5                                         | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R} \\ \text{Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C} \end{array}$ | 6.93 ± 0.21<br>(117.49 nM)<br>(3)                                     | 100.1 ± 3.8<br>(3)      | 7.76 ± 0.16<br>(17.38 nM)<br>(3)                                      | 36.7 ± 7.4<br>(3)       |
| 14 | $\Delta$ R3(R12L)/I5                                   | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R} \\ \text{Z-D-L-Q-T-L-C-C-T-L-G-C-S-M-T-D-L-S-A-L-C} \end{array}$ | 6.58 ± 0.21<br>(263.03 nM)<br>(3)                                     | 66.5 ± 14.8<br>(3)**    | 6.48 ± 0.25<br>(311.13 nM)<br>(3)***                                  | 37.3 ± 7.6<br>(3)       |
| 15 | $\Delta$ R3/<br>I5A(8-21)T15K                          | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 43.5 ± 12.1<br>(3)****  | 6.27 ± 0.05<br>(537.03 nM)<br>(3)***                                  | 49.4 ± 6.2<br>(3)       |
| 16 | $\Delta$ R3B(T21I)/<br>I5A(8-21)T15K                   | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-I-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 55.8 ± 3.4<br>(3)***    | <5<br>(<10 $\mu$ M)<br>(4)                                            | 2.1 ± 4.7<br>(4)**      |
| 17 | $\Delta$ R3B(A17T)/<br>I5A(8-21)T15K                   | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-T-V-I-F-T-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 34.8 ± 0.9<br>(3)***    | 7.46 ± 0.26<br>(34.67 nM)<br>(4)                                      | 36 ± 10.0<br>(4)        |
| 18 | $\Delta$ R3B(R12L)/<br>I5A(8-21)T15K                   | $\begin{array}{c} \text{R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 47.0 ± 7.3<br>(3)***    | 6.67 ± 0.09<br>(213.80 nM)<br>(3)**                                   | 56.2 ± 11.8<br>(3)      |
| 19 | $\Delta$ R3B(P4K)/<br>I5A(8-21)T15K                    | $\begin{array}{c} \text{R-A-A-K-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 76.3 ± 1.8<br>(3)*      | 7.10 ± 0.12<br>(79.43 nM)<br>(4)*                                     | 71.3 ± 4.8<br>(4)*      |
| 20 | $\Delta$ R3B(P4KY5ER12LA<br>17TT21I)/<br>I5A(8-21)T15K | $\begin{array}{c} \text{R-A-A-K-E-G-V-R-L-C-G-L-E-F-I-R-T-V-I-F-I-C-R} \\ \text{C-T-D-G-A-S-M-K-D-L-S-A-L-C} \end{array}$               | <5<br>(<10 $\mu$ M)<br>(3)                                            | 19.9 ± 2.3<br>(3)***    | <5<br>(<10 $\mu$ M)<br>(4)                                            | 8.1 ± 9.5<br>(4)*       |

**Table 5.2:** Primary structure of  $\Delta$ R3/I5 and novel chimeric peptides and their antagonist potency (pIC<sub>50</sub>/IC<sub>50</sub>) and efficacy on the RXFP4/RXFP3 receptors. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  vs  $\Delta$ R3/I5.

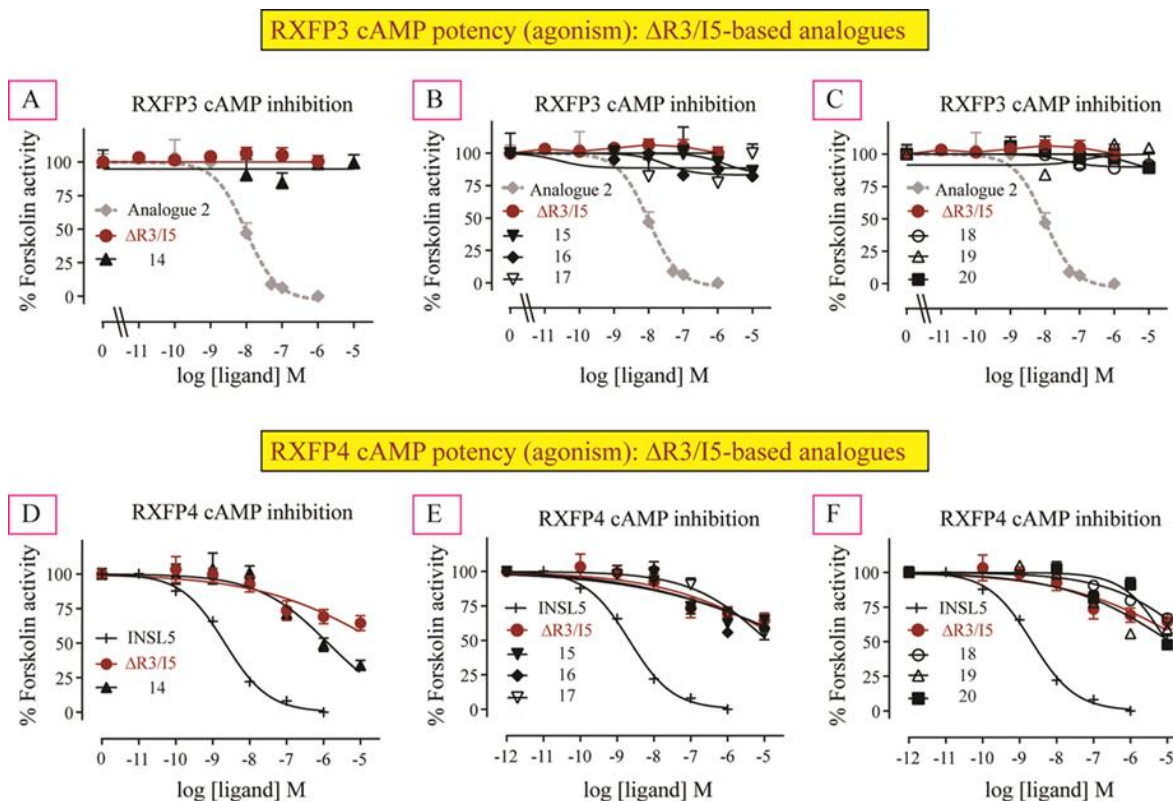
## 5.7 The pharmacological activity of $\Delta$ R3/I5 at RXFP4

We tested  $\Delta$ R3/I5 in competition binding assays on human CHO-K1-RXFP4 cells.  $\Delta$ R3/I5 showed high affinity binding to RXFP4 with an affinity similar to the native agonist INSL5 (K<sub>i</sub> of 4.57 nM compared to 2.14 nM; **Figure 5.2D**, **Table 5.1**). However, to our surprise,  $\Delta$ R3/I5 acted as a weak partial agonist at RXFP4 (**Figure 5.2E**, **Table 5.1**). This result seems to contradict the original published report where  $\Delta$ R3/I5 was described as having no activity at human RXFP4 although dose response curves were not presented<sup>16</sup>. Notably, the original

study utilised SK-N-MC/CRE- $\beta$ -gal cells expressing human RXFP4 hence although the reporter gene system was similar, the studies were done in a completely different cell system. Importantly, consistent with this partial agonist activity, antagonism assays utilising synthetic  $\Delta$ R3/I5 demonstrated that the peptide was only able to partially block INSL5 agonist mediated cAMP activation ( $E_{max}$  = 36.7% of response,  $IC_{50}$  of 17.38 nM, **Figure 5.2F**, **Table 5.2**).

This is the first report of  $\Delta$ R3/I5 showing partial agonism and antagonism of cAMP activation at human RXFP4. Notably, although  $\Delta$ R3/I5 shows no agonist activity and is a full antagonist of H3 relaxin in CHO-K1-RXFP3 cells utilising the CRE-reporter gene assay<sup>23</sup> and in SK-N-MC/CRE- $\beta$ -gal cells expressing human RXFP3<sup>16</sup> two other reports have shown that  $\Delta$ R3/I5 is a partial agonist of RXFP3. Kocan et al. demonstrated that  $\Delta$ R3/I5 was a weak partial agonist of p38MAPK, ERK1/2 and cAMP signalling but also was able to act as an antagonist of these pathways<sup>24</sup>. Another study demonstrated that  $\Delta$ R3/I5 is a partial agonist in assays of [35S]-GTP $\gamma$ S binding, cAMP activation and dynamic mass redistribution (DMR) in HEK-293 cells expressing rat and human RXFP3<sup>25</sup>.

In this study, we have attempted to modify the  $\Delta$ R3/I5 peptide to achieve RXFP4-specificity and improve the peptides antagonist properties at RXFP4.



**Figure 5.4:** Agonist activities of novel chimeric analogues 14-20 compared with  $\Delta$ R3/I5 in CHO-K1-RXFP3 cells (A-C) and CHO-K1-RXFP4 cells (D-F) as measured by changes in cAMP activity. The data are the result of  $n=3-4$  independent experiments and are expressed as mean  $\pm$  SEM.

## 5.8 Novel $\Delta$ R3/I5-based analogues

H3 relaxin interacts with both RXFP3 and RXFP4 by utilising the same primary binding residues in the B-chain<sup>16</sup>: R<sup>B8</sup>, R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>. INSL5, on the other hand, interacts with RXFP4 by utilising primary binding residues in the B-chain: I<sup>B16</sup>, R<sup>B23</sup> and W<sup>B24</sup> in the B-chain<sup>17,26</sup>. Given that H3 relaxin interacts with both RXFP3 and RXFP4 receptors and INSL5 interacts only with RXFP4 using B-chain specific residues, we speculated that we would be able to rationally design RXFP4-selective antagonist by modifying the B-chain of  $\Delta$ R3/I5. Our strategy was to replace non-conservative residues of the B-chain of  $\Delta$ R3/I5 with the corresponding residues of the B-chain of INSL5. We have compared the amino acid

sequences of C-terminally deleted H3 B-chain ( $\Delta$ H3B) in  $\Delta$ R3/I5 with that of equivalent INSL5 B-chain ( $\Delta$ INSL5B) (**Table 5.1**). There are 23 residues in the  $\Delta$ H3B and 21 residues in the corresponding  $\Delta$ INSL5B. However, of these, five residues involve differences with chemically distinct side chains (boxed in **Table 5.1**). We systematically replaced those five distinct residues in the B-chain of  $\Delta$ R3/I5 with the corresponding residues of INSL5B.

## 5.9 Binding of $\Delta$ R3/I5-based analogues at RXFP3 and RXFP4 cells

### 5.9.1 Three disulfide bonds containing $\Delta$ R3/I5-based analogue 14

R<sup>B12</sup> in the B-chain of  $\Delta$ R3/I5 was previously reported to be one of the residues important for binding to both RXFP3 and RXFP4<sup>16</sup>. The corresponding residue L<sup>B9</sup> in the B-chain of INSL5, on the other hand, was reported to be one of the most critical residues by which INSL5 exhibits selectivity for RXFP4 over RXFP3<sup>27</sup>. Therefore, in the first attempt, we replaced the R<sup>B12</sup> residues in the B-chain of  $\Delta$ R3/I5 with corresponding L<sup>B9</sup> residue of INSL5 B-chain in order to achieve selectivity for RXFP4 over RXFP3 (**Table 5.1**). To our surprise, the resulting analogue **14** did not show significantly reduced affinity for RXFP3 (K<sub>i</sub> of 23.99 nM; **Figure 5.3A, Table 5.1**), but did show significantly less affinity for RXFP4 (K<sub>i</sub> of 23.99 nM; **Figure 5.3D, Table 5.1**) compared with  $\Delta$ R3/I5. The fact that the agonist peptide R3/I5 with the same mutation (R<sup>B12</sup>/L<sup>B9</sup>) was shown to exhibit ~1600 less RXFP3 potency without losing much RXFP4 potency<sup>27</sup> suggest that the binding mode of the agonist (R3/I5) peptide for RXFP3 and RXFP4 might be different to that of the antagonist peptide ( $\Delta$ R3/I5).

### 5.9.2 Two disulfide bonds containing $\Delta$ R3/I5-based analogue 15

The current lead antagonist  $\Delta$ R3/I5 or mutated analogue **14** has two chains (A and B chain) and three disulfide bonds with 43 residues (**Table 5.1**). Chemical synthesis of this peptide is tedious, time-consuming, and low yielding (3.3%) (Supplementary information, **Table S5.1**). We, therefore, decided to simplify the structure of  $\Delta$ R3/I5 so that we can facilitate SAR

studies. In this context, we replaced the A-chain (21 residues) of  $\Delta R3/I5$  with the modified A-chain (14 residues) of the simplified RXFP4 agonist, analogue **13**<sup>11</sup> (**Table 5.1**). The resulting analogue **15** has only two-disulfide bridges and is high yielding (17.4%) (Supplementary information, **Table S5.1**). Excitingly, analogue **15** showed high affinity for both RXFP3 (K<sub>i</sub> of 15.49 nM; **Figure 5.3B, C, Table 5.1**) and RXFP4 (K<sub>i</sub> of 22.90 nM; **Figure 5.2E, Table 5.1**). Therefore, we have used this high yielding chimeric peptide template **15** for further SAR studies as outlined below.

### 5.9.3 Two disulfide bonds containing $\Delta R3/I5$ -based analogues 16-20

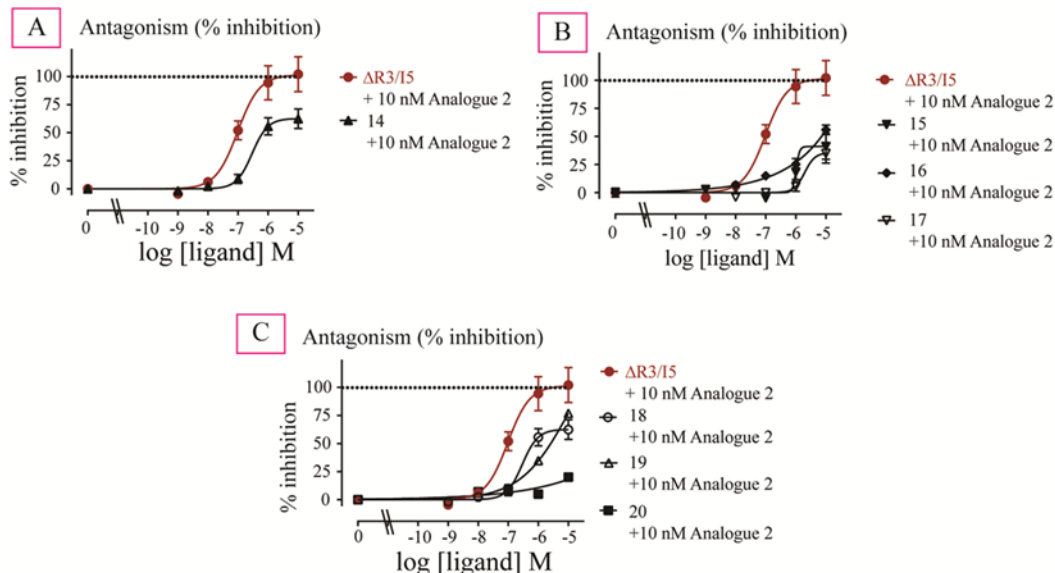
The analogues (**16-19**) derived from analogue **15** by single amino acid replacement showed a strong binding affinity for both RXFP3 (K<sub>i</sub> of 7.94 nM to 25.70 nM; **Figure 5.3B, C, Table 5.1**) and RXFP4 (K<sub>i</sub> 10.23 nM to 50.12 nM; **Figure 5.3E, F, Table 5.1**). When compared with analogue **15**, it is clear that non-conservative residues between the  $\Delta B$ -chain of INSL5 and H3 relaxin have minimal or no contribution to RXFP3/RXFP4 binding. However, when all five distinct residues in the B-chain of analogue **15** were replaced with corresponding residues of the INSL5 B-chain, the resulting analogue **20** showed significantly less affinity compared with  $\Delta R3/I5$  for both RXFP3 (K<sub>i</sub> of 48.98 nM; **Figure 5.3C, Table 5.1**) and RXFP4 (K<sub>i</sub> of 131.82 nM; **Figure 5.3F, Table 5.1**) that might be due to accumulated loss contributed by each mutation or due to destabilisation of the overall structure.

### 5.10 Agonism potency of $\Delta R3/I5$ -based analogues at RXFP3 and RXFP4 cells

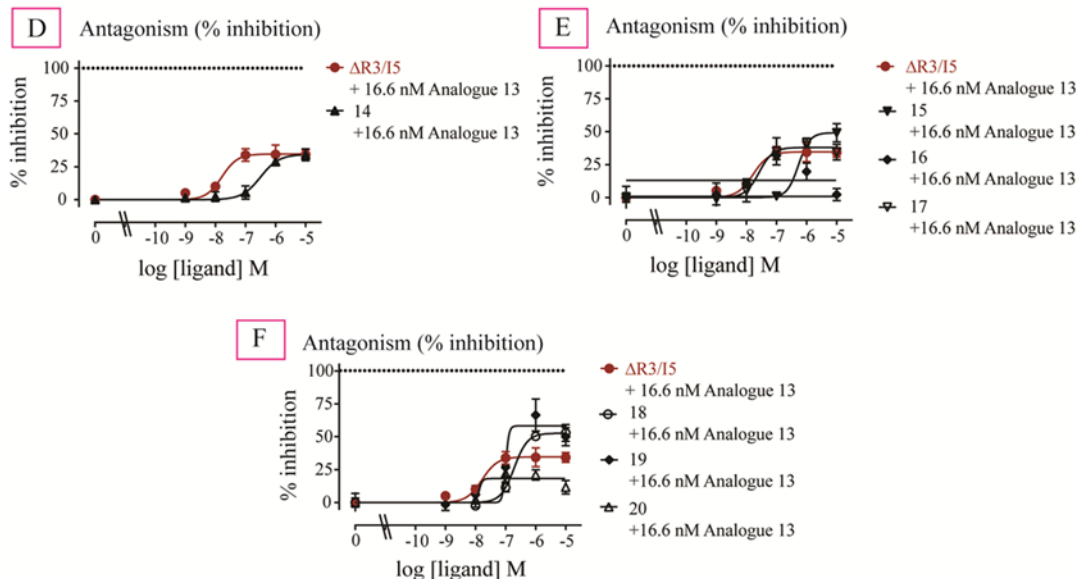
Like  $\Delta R3/I5$ , all the analogues (**14-20**) demonstrated no significant activation of RXFP3 (**Figure 5.4A-C, Table 5.1**) but acted as weak partial agonists at RXFP4 (**Figure 5.4D-F, Table 5.1**) suggesting that they are likely to be antagonists for both RXFP3 and RXFP4.



RXFP3 cAMP % inhibition (antagonism) of agonist response:  $\Delta R3/I5$ -based analogues

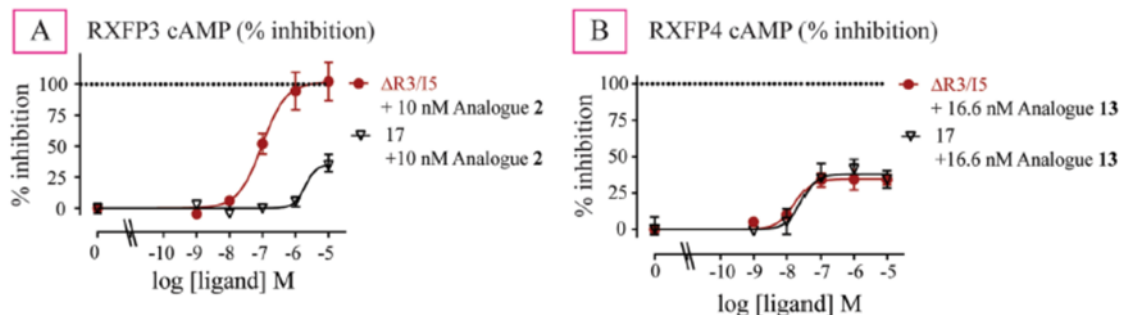


RXFP4 cAMP % inhibition (antagonism) of agonist response:  $\Delta R3/I5$ -based analogues



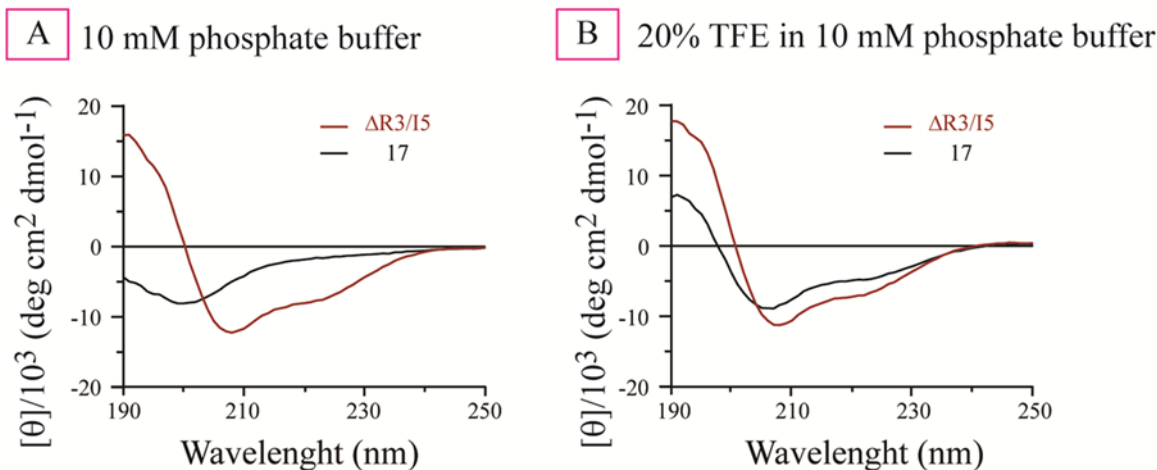
**Figure 5.5:** Antagonist activities of novel chimeric analogues 14-20 compared with  $\Delta R3/I5$  in CHO-K1-RXFP3 and CHO-K1-RXFP4 cells. (A-C) The ability of  $\Delta R3/I5$  and analogues 14-20 to antagonise 10 nM analogue 2-induced inhibition of cAMP activity. (D-F) The ability of  $\Delta R3/I5$  and analogues 14-20 to antagonise 16.6 nM analogue 13-induced inhibition of cAMP activity. The data are the result of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

Analogue 17 is RXFP4-selective antagonist



**Figure 5.6:** Antagonist activity of novel chimeric analogue 17 compared with  $\Delta R3/I5$  in CHO-K1-RXFP3 and CHO-K1-RXFP4 cells. (A) The ability of  $\Delta R3/I5$  and analogues **17** to antagonise 10 nM analogue **2**-induced inhibition of cAMP activity. (B) The ability of  $\Delta R3/I5$  and analogues **17** to antagonise 16.6 nM analogue **13**-induced inhibition of cAMP activity. The data are the result of n=3-4 independent experiments and are expressed as mean  $\pm$  SEM.

Analogue 17 forms induced-helix



**Figure 5.7:** CD spectra of analogue 17 compared with  $\Delta R3/I5$ . CD was performed in 10 mM phosphate buffer at pH 7.5 (A) and 20% TFE (B).

### 5.11 Antagonism potency of $\Delta$ R3/I5-based analogues at RXFP3 and RXFP4 cells

Interestingly, despite high binding affinity and no activity at RXFP3, all the analogues (**14-20**) showed significantly less antagonistic potency and efficacy (**Figure 5.5A-C, Table 5.2**) compared with  $\Delta$ R3/I5. This result was initially encouraging, as it suggested some RXFP4-selectivity. However, when tested in RXFP4 cells, all analogues except two (**17** and **19**) showed less antagonistic potency compared with  $\Delta$ R3/I5 (**Figure 5.5D-F, Table 5.2**). Interestingly, analogues **17** and **19** showed high antagonistic potency (**17**: IC<sub>50</sub> of 34.67 nM, **19**: IC<sub>50</sub> of 79.43 nM; **Figure 5.5E, F, Table 5.2**) with analogue **19** showing higher efficacy (E<sub>max</sub> of 71.3%) than  $\Delta$ R3/I5. Importantly, while analogue **17** has  $\Delta$ R3/I5-like antagonist potency for RXFP4, it was found to be more selective for RXFP4 over RXFP3 (**Figure 5.6, Table 5.2**) compared with both  $\Delta$ R3/I5 and analogue **19**. However, all of the chimeric  $\Delta$ R3/I5 analogues still demonstrated partial agonist activity like the parent  $\Delta$ R3/I5 peptide and importantly were only able to partially antagonise RXFP4 agonist activation. Nonetheless, analogue **17** demonstrates improved selectivity for RXFP4 and has a simpler structure compared with  $\Delta$ R3/I5 which enables its preparation in higher yield (yield of  $\Delta$ R3/I5=3.3% and yield of analogue **17**=17.4%; Supplementary information).

### 5.12 Structural study of $\Delta$ R3/I5 and analogue **17**

While the structure of the  $\Delta$ R3/I5 peptide has not been determined, the NMR solution structure of the agonist peptide R3/I5 has been solved<sup>28</sup>. The NMR solution structure of R3/I5 revealed that the INSL5 A-chain adopts a conformation similar to the H3 relaxin A-chain, and thus can structurally support a native-like conformation of the H3 relaxin B-chain through which it interacts with both RXFP3 and RXFP4. Truncations of the A-chain and/or removal of the intra-A-chain disulfide bond has been previously used to successfully simplifying the structure while maintaining affinity and efficacy of several relaxin peptides<sup>11,22,29,30</sup>. However, extensive truncation or removal of the disulfide bond often leads to a compromised overall structure<sup>29,30</sup>. Such minimised peptides are generally challenging to study by NMR due to the increased structural flexibility resulting in spectral line

broadening<sup>31</sup> and tendencies of aggregation<sup>32</sup>. To investigate the conformation of analogue **17** in solution, and to compare it to that of  $\Delta$ R3/I5, we, therefore, decided to carry out circular dichroism (CD) studies. Consistent with the solution NMR structure of R3/I5, our synthetic  $\Delta$ R3/I5 demonstrated clear  $\alpha$ -helical structure in aqueous buffer with characteristic double minima in the spectrum at 208 and 222 nm (**Figure 5.7A**). In contrast, analogue **17** showed spectra representing principally random coil structure (**Figure 5.7A**). As analogue **17** has strong RXFP4 binding affinity ( $IC_{50}$  of 34.67 nM), we postulate that analogue **17** adopted an appropriate conformation upon binding to the receptor. To confirm this hypothesis, we have studied CD spectroscopy of these peptides in 20% trifluoroethanol (TFE) in phosphate buffer. Trifluoroethanol (TFE) is known to stabilise the  $\alpha$ -helical structure of peptide and proteins by improving hydrogen bonding. While 20% TFE did not significantly affect the secondary structure of  $\Delta$ R3/I5 (**Figure 5.7B**), it induced helical structure in analogue **17**, suggesting that analogue **17** has a tendency to form  $\alpha$  helix. This finding is consistent with our previous studies where the truncated INSL5 analogues **6**<sup>30</sup> and **13**<sup>11</sup> exhibited compromised fold but showed very high RXFP4 affinity<sup>30</sup>. A similar trend was also observed for an H3 relaxin analogue where the A-chain was truncated up to 9 residues at the *N*-terminus, and the resulting analogue with compromised structure was found not to significantly affect binding or activation of RXFP3<sup>29</sup>.

### 5.13 Conclusion

In conclusion, we have demonstrated for the first time that the RXFP3/RXFP4 antagonist  $\Delta$ R3/I5 exhibits partial agonism at RXFP4 when expressed in CHO cells which is associated with only partial antagonism of INSL5 analogue activation. In an attempt to develop an RXFP4-specific antagonist we have designed and chemically synthesised a series of chimeric simplified analogues of  $\Delta$ R3/I5. All of the chimeric analogues still demonstrated partial agonism at RXFP4, however analogue **17** exhibited improved RXFP4 specificity. Moreover, analogue **17** has a simplified structure which enables its synthesis in higher yields than  $\Delta$ R3/I5. Therefore, analogue **17** is a potential template for further SAR to improve RXFP4

antagonistic efficacy and facilitate pre-clinical studies to probe the physiological role of RXFP4/INSL5 axis. **Table 5.3** below summarises the analogue developed in this chapter.

**Table 5.3:** Summary of RXFP4 analogue

| Previously reported                                                                                                                      | New                                                                                                                             |
|------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| <div><div>R·A·A·P·Y·G·V·R·L·C·G·R·E·F·I·R·A·V·I·F·T·C·R</div><div>Z·D·L·Q·T·L·C·C·T·D·G·C·S·M·T·D·L·S·A·L·C</div><div>ΔR3/I5</div></div> | <div><div>R·A·A·P·Y·G·V·R·L·C·G·R·E·F·I·R·T·V·I·F·T·C·R</div><div>C·T·D·G·A·S·M·K·D·L·S·A·L·C</div><div>Analogue 17</div></div> |

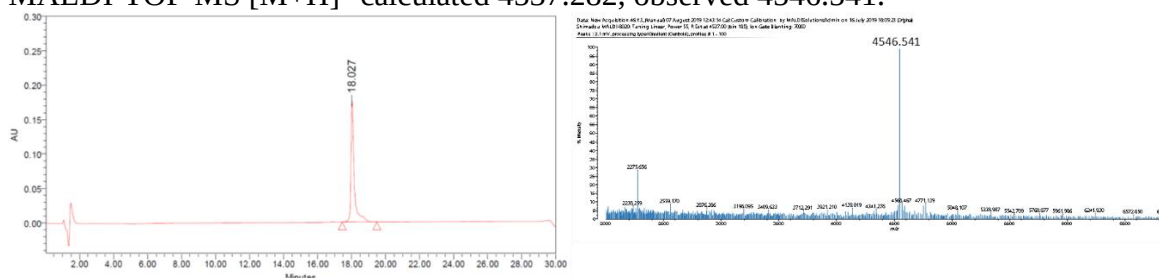
## 5.14 Supplementary information

### 5.14.1 Analytical RP-HPLC and MALDI-TOF-MS of all compounds

#### 1. Analogue 2 (A2)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% in 30 minutes,  $t = 18.027$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4537.282, observed 4546.541.

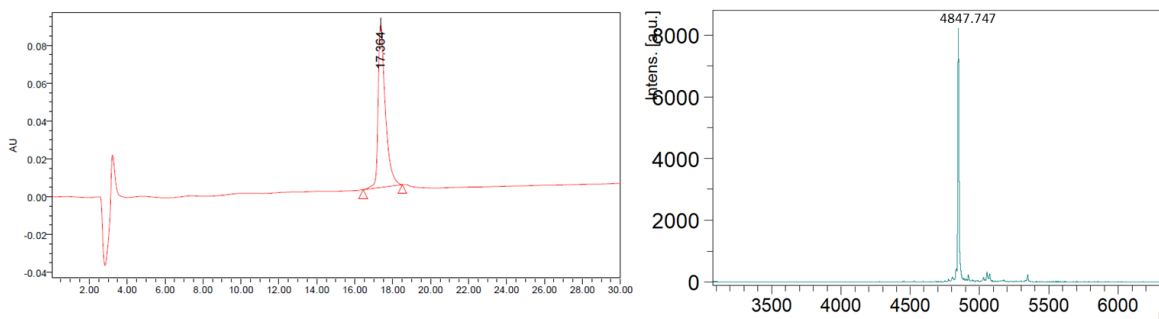


**Figure S5.1:** RP-HPLC profile of A2 (left) and MALDI-TOF-MS (right).

#### 2. $\Delta R3/I5$

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t = 17.364$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4852.698, observed 4847.747.

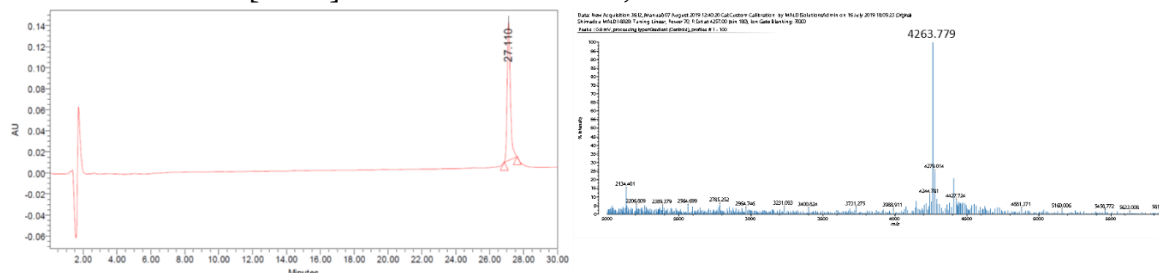


**Figure S5.2:** RP-HPLC profile of  $\Delta R3/I5$  (left) and MALDI-TOF-MS (right).

### 3. INSL5:A(8-21)T15K (Analogue 13)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 15-55% over 45 minutes,  $t = 27.110$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4263.929, observed 4263.779

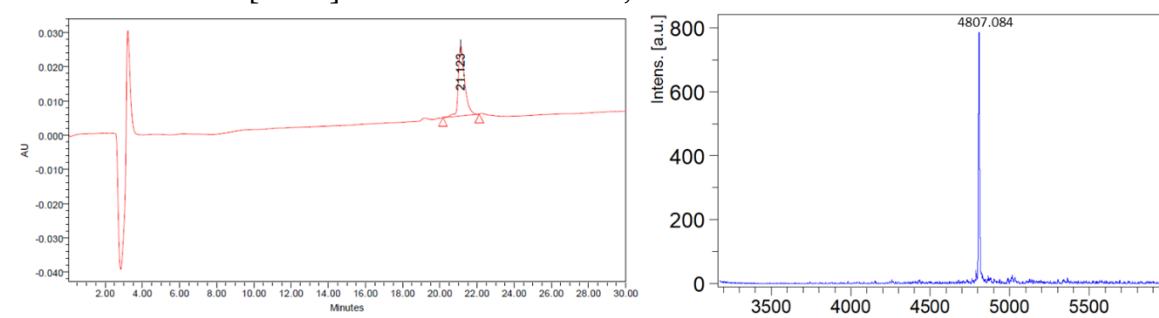


**Figure S5.3:** RP-HPLC profile of INSL5:A(8-21)T15K (left) and MALDI-TOF-MS (right).

### 4. $\Delta R3(R12L)/I5$ (Analogue 14)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t = 21.123$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4807.741, observed 4807.084.

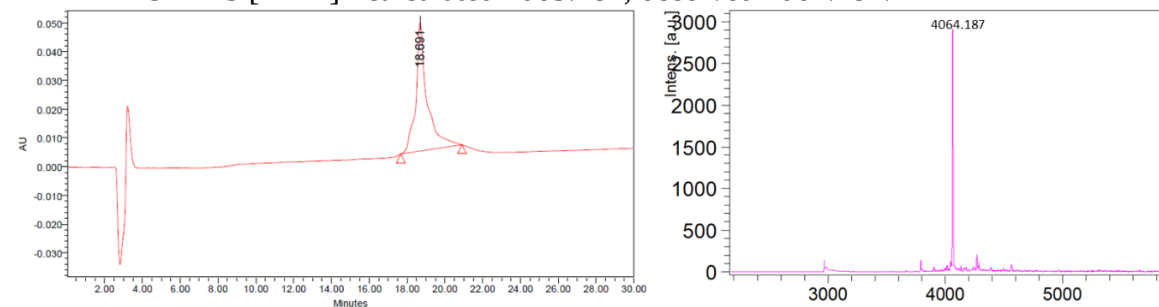


**Figure S5.4:** RP-HPLC profile of  $\Delta R3(R12L)/I5$  (left) and MALDI-TOF-MS (right).

### 5. $\Delta R3B/I5A(8-21)T15K$ (Analogue 15)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t = 18.691$  min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4065.787, observed 4064.187.



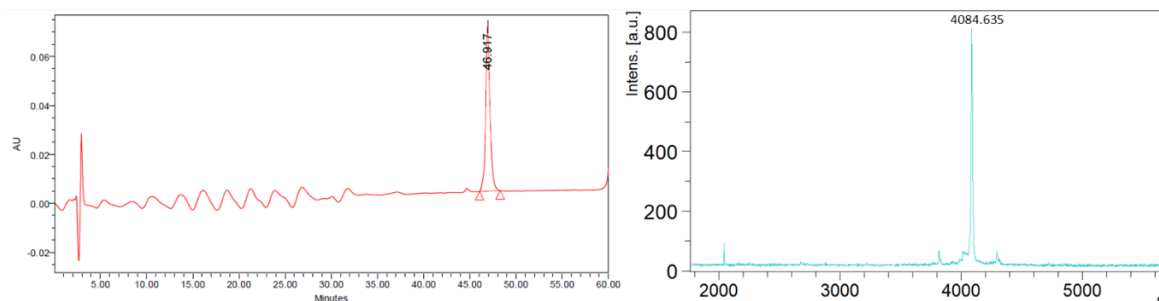
**Figure S5.5:** RP-HPLC profile of  $\Delta R3B/I5A(8-21)T15K$  (left) and MALDI-TOF-MS (right).

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#### 6. $\Delta$ R3B(T21I)/I5A(8-21)T15K (Analogue 16)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 10-40% over 60 minutes,  $t_r$ =46.917 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4077.841, observed 4084.635.



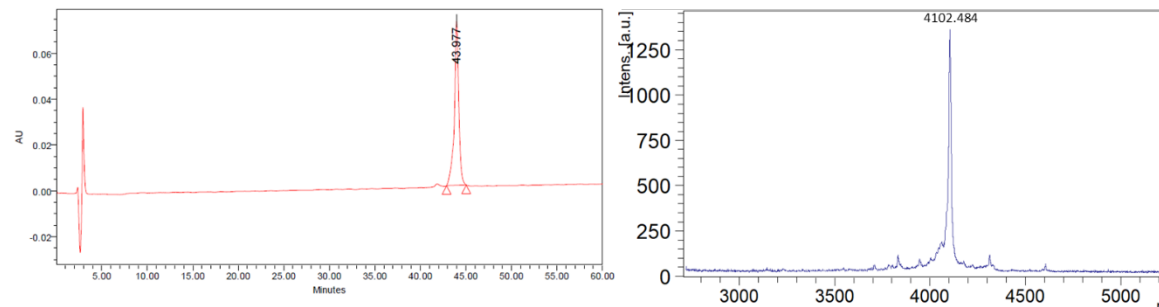
**Figure S5.6:** RP-HPLC profile of  $\Delta$ R3B(T21I)/I5A(8-21)T15K (left) and MALDI-TOF-MS (right).

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#### 7. $\Delta$ R3B(A17T)/I5A(8-21)T15K (Analogue 17)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 10-40% over 60 minutes,  $t_r$ =43.977 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4095.813, observed 4102.484.



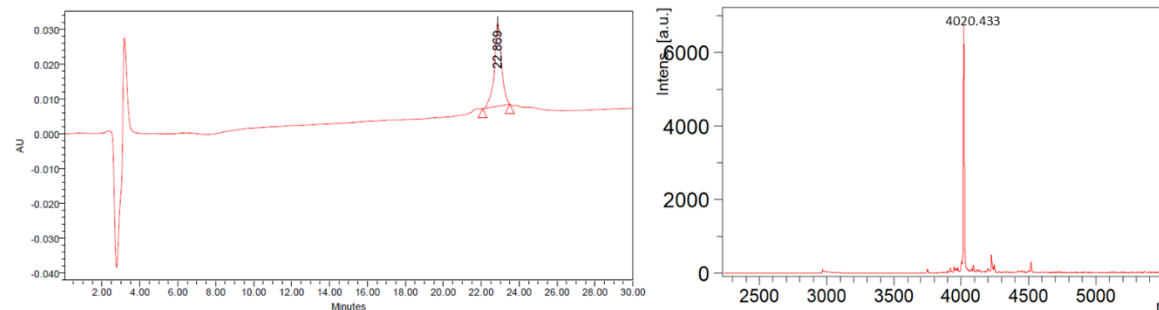
**Figure S5.7:** RP-HPLC profile of  $\Delta$ R3B(A17T)/I5A(8-21)T15K (left) and MALDI-TOF-MS (right).

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#### 8. $\Delta$ R3B(R12L)/I5A(8-21)T15K (Analogue 18)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t_r$ =22.869 min.

MALDI-TOF-MS  $[M+H]^+$  calculated 4022.759 observed 4020.433.



**Figure S5.8:** RP-HPLC profile of  $\Delta$ R3B(R12L)/I5A(8-21)T15K (left) and MALDI-TOF-MS (right).

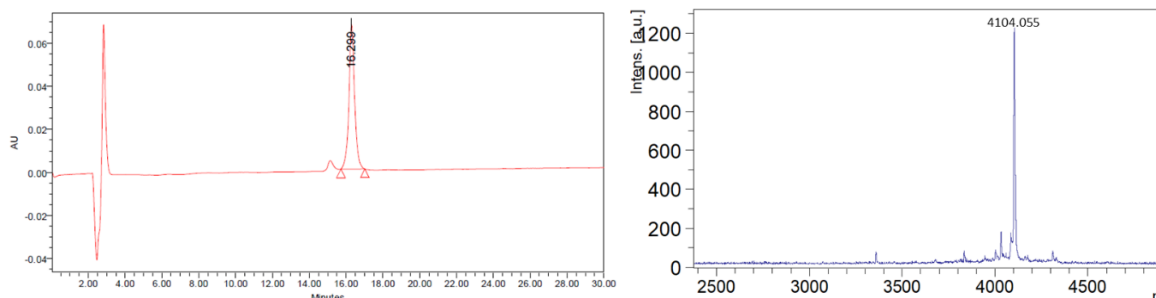
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### 9. $\Delta$ R3B(P4K)/I5A(8-21)T15K (Analogue 19)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t_r$ =16.299 min.

MALDI-TOF-MS [M+H]<sup>+</sup> calculated 4096.844, observed 4104.055.

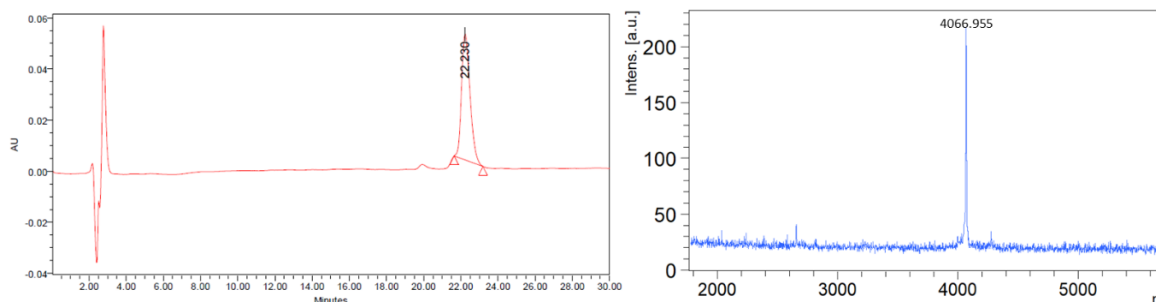


**Figure S5.9:** RP-HPLC profile of  $\Delta$ R3B(P4K)/I5A(8-21)T15K (left) and MALDI-TOF-MS (right).

### 10. $\Delta$ R3B(P4KY5ER12LA17T21I)/I5A(8-21)T15K (Analogue 20)

RP-HPLC carried out as described in the methods chapter 2 using the elution gradient: buffer B 20-50% over 30 minutes,  $t_r$ =22.230 min.

MALDI-TOF-MS [M+H]<sup>+</sup> calculated 4061.837, observed 4066.955.



**Figure S5.10:** RP-HPLC profile of  $\Delta$ R3B(P4KY5ER12LA17T21I)/I5A(8-21)T15K (left) and MALDI-TOF-MS (right).

**Table S5.1:** Summary of peptide characterisation by MALDI-TOF MS and HPLC

| Analogues      | MALDI TOF MS |            | HPLC<br>$t_r$ (min) | Purity (%) | Yield (%) |
|----------------|--------------|------------|---------------------|------------|-----------|
|                | Observed*    | Calculated |                     |            |           |
| Analogue 2     | 4546.541     | 4537.282   | 18.027              | 99.47      | 15.45     |
| Analogue 13    | 4263.779     | 4263.929   | 27.11               | 99.63      | 14        |
| $\Delta$ R3/I5 | 4847.747     | 4852.698   | 17.364              | 99.33      | 3.3       |
| Analogue 14    | 4807.084     | 4807.741   | 21.123              | 93.79      | 20.28     |

|                    |          |          |        |       |      |
|--------------------|----------|----------|--------|-------|------|
| Analogue <b>15</b> | 4064.187 | 4065.787 | 18.691 | 96.22 | 45   |
| Analogue <b>16</b> | 4084.635 | 4077.841 | 46.917 | 98.88 | 12   |
| Analogue <b>17</b> | 4102.015 | 4095.813 | 43.977 | 99.34 | 17.4 |
| Analogue <b>18</b> | 4020.433 | 4022.759 | 22.869 | 96.49 | 38.1 |
| Analogue <b>19</b> | 4104.055 | 4096.844 | 16.299 | 93.23 | 8    |
| Analogue <b>20</b> | 4066.955 | 4061.837 | 46.917 | 97.53 | 10.7 |

\* Observed molecular weights were determined by MALDI-TOF MS of the purified peptides

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## Chapter 6 Conclusion and future directions

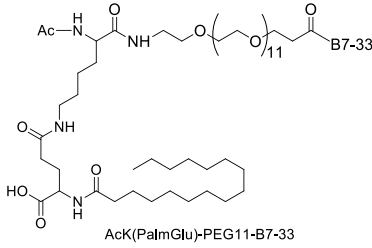
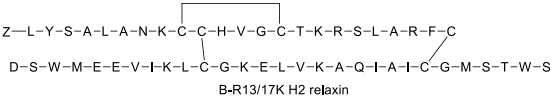
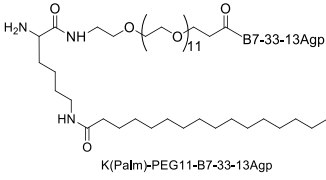
### 6.1 Abstract

Relaxin family peptides interact with the G protein-coupled receptors (GPCRs), Relaxin Family Peptide Receptors (RXFPs)<sup>1</sup>. GPCRs are considered to be the most important class of proteins for drug discovery, with over 30% of clinically marketed drugs target these receptors<sup>2,3</sup>. RXFPs are potential targets for the treatment of human disease conditions, including fibrosis, and heart failure (RXFP1), neurological disorders (RXFP3), anorexia, obesity, and constipation (RXFP4). However, for chronic treatment, it would be beneficial to have longer-acting versions of relaxin ligands. As complex insulin-like heterodimeric structures, relaxin peptides are difficult to modify to improve their PK properties<sup>4</sup>. Recently, our laboratory has developed and published target-specific single-chain or simplified peptidomimetics of these complex relaxin family peptides. I have used B7-33 (RXFP1 agonist)<sup>5</sup>, peptide 5 (RXFP3 agonist)<sup>6</sup>, H3B1-22R (RXFP3 antagonist)<sup>7</sup> and  $\Delta$ R3/I5 (RXFP3/4 antagonist)<sup>8</sup> as templates to further optimise them by minimising the structure, and improving the half-life, and receptor selectivity. I also developed a novel single-chain RXFP1 antagonist for the first time. My compounds are important research tools and scaffolds for drug development.

6.2 H2 relaxin/RXFP1

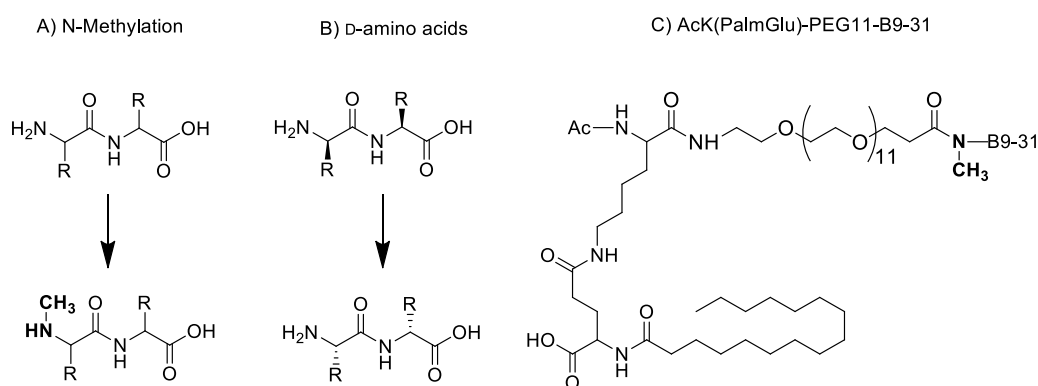
**Conclusion-** In the study described in chapter 3, it was shown that B7-33 could be further truncated up to 4 residues (B9-31) (**Table 6.1**). Furthermore, conjugating a fatty acid chain to B7-33 increases its *in vitro* half-life from 6 minutes to 60 minutes (AcK(PalmGlu)-PEG11-B7-33) (**Table 6.1**). Mimicking important RXFP1 binding residues, such as arginine residues at positions 13 and 17 of the B-chain led to a high yielding single-chain antagonist for RXFP1. These analogues can now be used as tools to understand the function of RXFP1. These agonist and antagonist peptides also can serve as templates for drug design for the treatment of fibrosis and fibrosis-related diseases, heart failure (agonists), and prostate, and other cancers (antagonists). **Table 6.1** below summarises the analogues made in for RXFP1 receptor.

**Table 6.1:** Summary of RXFP1 analogues

| Summary for H2 relaxin/RXFP1                                                                                |                                                                                                                   |
|-------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| Reported RXFP1 single-chain agonist <sup>5</sup>                                                            | New RXFP1 single-chain agonist                                                                                    |
| V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R-S-L<br>B7-33                                              | K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R<br>B9-31                                                            |
| Reported B7-33 (with short half-life) <sup>5</sup>                                                          | New long-acting B7-33 analogue                                                                                    |
| V-I-K-L-S-G-R-E-L-V-R-A-Q-I-A-I-S-G-M-S-T-W-S-K-R-S-L<br>B7-33                                              | <br>AcK(PalmGlu)-PEG11-B7-33  |
| Reported RXFP1 antagonist <sup>9</sup>                                                                      | New RXFP1 single chain antagonist                                                                                 |
| <br>B-R13/17K H2 relaxin | <br>K(Palm)-PEG11-B7-33-13Agp |



**Future direction** - All the SAR information on B7-33 will be used to construct the smallest possible analogue with improved *in vivo* half-life. For example, B9-31 will be used as a template and be stabilised against proteolytic degradation by using N-methylation (**Figure 6.1A**) or by changing the chirality of amino acids (D-amino acids) (**Figure 6.1B**). Then K(PalmGlu)-PEG11 will be conjugated at the *N*-terminus of enzyme stabilised B9-31 (**Figure 6.1C**). Then half-life of the lipidated analogue, AcK(PalmGlu)-PEG11-B9-31 (**Figure 6.1C**) will be evaluated both *in vitro* and *in vivo* animal models. Also, we will do toxicity test for the lead peptide. The single-chain antagonist peptide (**Table 6.1**) needs to be validated in animal models of prostate cancer.



**Figure 6.1:** A) N-methylation, B) D-amino acid, and C) lipidated analogue.

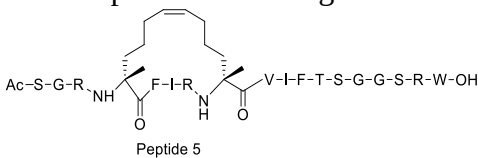
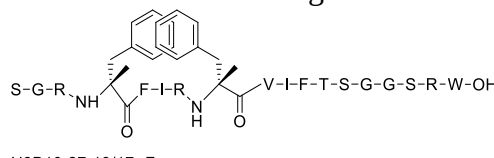
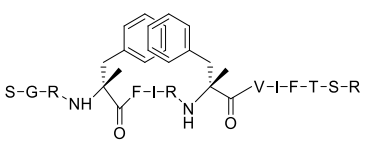
### 6.3 H3 relaxin/RXFP3

**Conclusion** - In the study described in chapter 4 – Part 1, I have chemically prepared four novel two chains, two disulfide-bonded A2 analogues bearing *C*-terminal modifications. In stark contrast to H3 relaxin, modifications at the *C*-terminus of the B-chain of A2 is tolerable for RXFP3 cAMP potency.

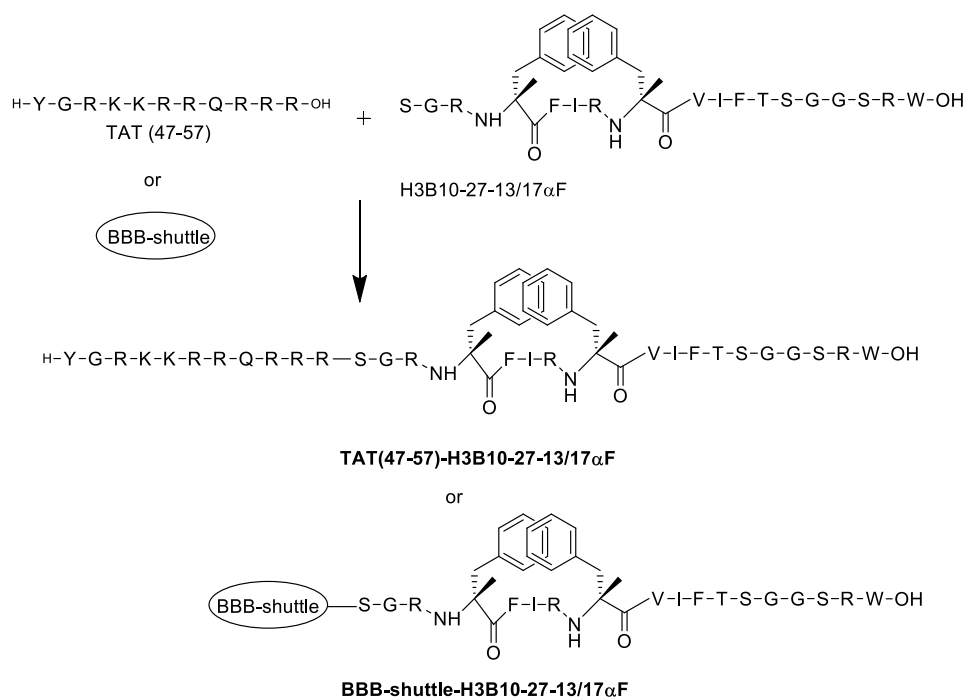
In the study described in chapter 4 – Part 2, I have developed a novel  $\pi$ - $\pi$  stapling method to create  $\alpha$ -helical peptides. I have used two unnatural amino acids (alpha-methyl phenylalanine) at positions 13 and 17 to stabilise peptide structure. My  $\pi$ - $\pi$  stapling method has advantages (e.g. high yielding, one product) over other stapling methods such as all-hydrocarbon stapling method (e.g. low yielding, two isomers). Using my method, I have developed a high yielding single-chain agonist (H3B10-27-13/17 $\alpha$ F) and antagonist

(H3B10-22R-13/17 $\alpha$ F) for RXFP3. These analogues exhibited over twenty-fold more stability in human serum compared with linear and non-stapled peptides. These analogues will help to understand the physiological function of RXFP3 and also will be drug leads for the treatment of eating and addictive disorders. **Table 6.2** below summarises the reported and new analogues made for targeting the RXFP3 receptor.

**Table 6.2:** Summary of RXFP3 analogues

| Summary for H3 relaxin/RXFP3                                                                                                                 |                                                                                                                                                             |
|----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>Reported RXFP3 agonist<sup>6</sup></p>  <p>Peptide 5</p> | <p>New RXFP3 agonist</p>  <p>H3B10-27-13/17<math>\alpha</math>F</p>       |
| <p>Reported RXFP3 antagonist<sup>7</sup></p> <p>R-A-A-P-Y-G-V-R-L-S-G-R-E-F-I-R-A-V-I-F-T-S-R</p> <p>H3B1-22R</p>                            | <p>New RXFP3 antagonist</p>  <p>H3B10-22R-13/17<math>\alpha</math>F</p> |

**Future direction** – The C-terminally modified analogues are expected to have improved stability in serum and animal models which needs to be investigated in the future. The parent neuropeptide H3 relaxin and its target receptor RXFP3 are co-expressed in the brain. Therefore, the analogues I have developed will be evaluated to see if they can cross the blood-brain barrier. If not, they will be conjugated to blood-brain carrier shuttle or cell-penetrating peptides (e.g. TAT) (**Figure 6.2**) which can help peptide to cross blood-brain barrier<sup>10,11</sup>. Solution NMR structures of H3B10-27-13/17 $\alpha$ F and H3B10-22R-13/17 $\alpha$ F analogues will be evaluated to determine their 3D structure. Future work would also focus on extending our  $\pi$ - $\pi$  stapling strategy to create helical mimetics of other bioactive peptides.



**Figure 6.2:** Proposed protocol for the synthesis of novel RXFP3 agonist/antagonist analogue to cross BBB.

## 6.4 INSL5/RXFP4

**Conclusion** - In the study described in chapter 5, I have demonstrated for the first time that the chimeric peptide  $\Delta$ R3/I5 is a partial agonist, not an antagonist as previously reported<sup>8</sup>. I have shown that my analogue **17** is better than  $\Delta$ R3/I5 in a sense that analogue **17** exhibited improved RXFP4 specificity. Moreover, analogue **17** has a simplified structure that enables its synthesis in higher yields than  $\Delta$ R3/I5. **Table 6.3** below summarises the reported and novel RXFP4 antagonists.

**Table 6.3:** Summary of RXFP4 analogues

## Summary for INSL5/RXFP4

Reported non-selective RXFP3 and  
RXFP4-specific antagonist<sup>8</sup>

R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R

Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C

ΔR3/I5

New RXFP4-selective antagonist

R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-T-V-I-F-T-C-R

C-T-D-G-A-S-M-K-D-L-S-A-L-C

Analogue 17

**Future direction** – Further SAR studies required to develop a high-affinity full antagonist or a single-chain agonist and/ or antagonist.

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# **Publications**



## Single chain peptide agonists of relaxin receptors

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### ABSTRACT

There are seven human relaxin family peptides that have two chains (A and B) and three disulfide bonds. The target receptors for four of these peptides are known as relaxin family peptide receptors, RXFP1–RXFP4. Detailed structure-activity relationship (SAR) studies of relaxin family peptides have been reported over the years and have led to the design of new analogs with agonistic and antagonistic properties. This review briefly summarizes the SAR of human relaxin 2 (H2 relaxin) and human relaxin 3 (H3 relaxin) leading to the design and development of single-B-chain only agonists, B7-33 and peptide 5. The physiological functions of these new peptides agonists in cellular and animal models are also described.

### 1. Introduction

There are seven insulin-like relaxin family peptides in humans that includes human relaxin 1 (H1 relaxin), human relaxin 2 (H2 relaxin), human relaxin 3 (H3 relaxin), insulin-like peptide 3 (INSL3 also known as relaxin-like factor or Leydig insulin-like peptide), insulin-like peptide 4 (INSL4), insulin-like peptide 5 (INSL5) and insulin-like peptide 6 (INSL6) (Hossain and Wade, 2014; Bathgate et al., 2002, 2013; Bathgate et al., 2013a,b; Patil et al., 2017; Hossain and Bathgate, 2017; Adham et al., 1993; Chang et al., 2000; Chassin et al., 1995; Conklin et al., 1999; Crawford et al., 1984; Hudson et al., 1983). These peptides are known to have two peptide chains (A and B) linked by two inter-chain disulfide bonds and one intra-A-chain disulfide bond (Hossain and Wade, 2014; Büllsbach and Schwabe, 2002; Liu et al., 2003). The relaxin peptides, INSL3 and INSL5 bind to and activate G protein-coupled receptors (GPCRs) that are now known as relaxin family peptide receptors (RXFPs) (Bathgate et al., 2006). The receptors for INSL4 and INSL6 are unknown (Halls et al., 2007).

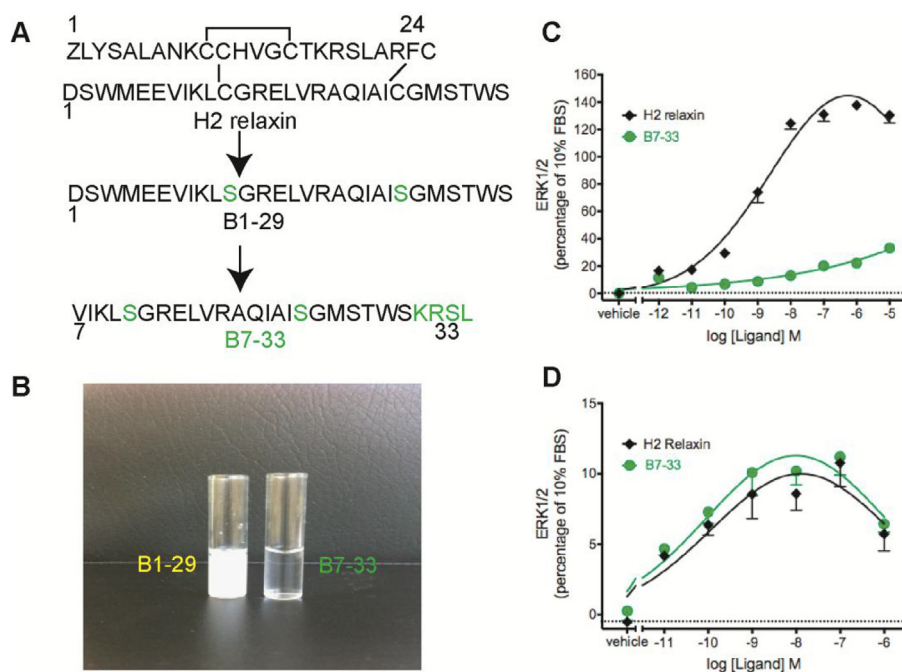
Relaxin family peptides have potential therapeutic applications for treating many human diseases including heart failure, fibrosis, stress-anxiety, and eating and colon motility disorders (Bathgate et al., 2013a,b). Despite their enormous promise as drugs, as they are peptides, they have short serum half-lives (Hossain et al., 2015; Nair et al., 2015; Haugaard-Kedstrom et al., 2015) which limits their ability to be used for chronic treatment. Each of the peptides can also cross-react with other RXFP receptors with various affinities (Bathgate et al.,

2013a,b) which, while probably not related to their biological function, has unknown consequences for drug targeting. Additionally, their complex, two-chain (A and B chains), three disulfide-bonded structure (with ~50 amino acids) make them expensive to manufacture and difficult to easily modify to improve their pharmacokinetic properties (e.g., bioavailability, half-life).

It would be ideal to develop single chain variants of these peptides which were receptor specific and would therefore be more easily modified to improve their pharmacokinetic properties. Developing a receptor-specific simple peptide scaffold requires an understanding of the mechanism of peptide binding and activation. This is complicated for relaxin peptides and receptors due to the complexity of both the peptide and receptor. Extensive structure-activity relationship studies (SAR) on H2 relaxin/RXFP1 and H3 relaxin/RXFP3 carried out over the years have led to a better understanding of these interactions and the development of B-chain only agonists for RXFP1 (Hossain et al., 2016) and RXFP3 (Hojo et al., 2016), and antagonists for RXFP2 (Del Borgo et al., 2006) and RXFP3 (Haugaard-Kedstrom et al., 2011). Quite unrelated to H2 relaxin, a 34 amino acid long single-chain peptide that targets the RXFP1 receptor was recently identified using a computational approach (Shemesh et al., 2008). This novel peptide, also known as CGEN25009 (P74), exhibited anti-fibrotic and anti-inflammatory properties both *in vitro* and *in vivo* (Shemesh et al., 2009; Pini et al., 2010). However, this review summarizes the current knowledge on the development of H2 and H3 relaxin-based single-B-chain only agonists for RXFP1 and RXFP3, and their biological activities in cellular and

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**Fig. 1.** Design, solubility and *in vitro* activity of B7-33. (A) Design of B7-33. Truncation of six residues from the N-terminus and elongation of 4 residues (KRSL) at the C-terminus of B1-29 resulted in a soluble B-chain derivative of H2 relaxin, B7-33. (B) The native H2 B-chain (B1-29) is insoluble (left vial) whereas the B7-33 is soluble (right vial) at concentration 4 mg mL<sup>-1</sup> H<sub>2</sub>O. (C) B7-33 weakly activated pERK1/2 in stably expressing RXFP1 (HEK-293T) cells but (D) strongly activated pERK1/2 in cells endogenously expressing RXFP1 (rat renal myofibroblasts) as shown by concentration–response curves. Figure adapted from Hossain et al. *Chem Sci* (2016).

animal models (Hossain et al., 2016; Hojo et al., 2016).

## 2. H2 relaxin and RXFP1

The H2 relaxin peptide contains a 24 residue A-chain and a 29 residue B-chain [Fig. 1A]. The endogenous receptor for H2 relaxin is RXFP1 (formerly called LGR7 (Hsu et al., 2002)) that consist of classical 7-transmembrane spanning region (TM), a large extracellular domain containing ten leucine-rich repeats (LRRs) and an N-terminal low-density lipoprotein type A (LDLa) module. In addition to RXFP1, H2 relaxin can also bind and activate RXFP2 (LGR8) (Kumagai et al., 2002), the native receptor for insulin-like peptide 3 (INSL3) and has also been reported to interact with RXFP3 (GPCR 135) (Liu et al., 2003), the receptor for relaxin-3.

### 2.1. Structure-activity relationship studies

Extensive SAR data (Sudo et al., 2003; Diepenhorst et al., 2014; Hossain et al., 2008, 2011, 2012) suggests multiple interactions between the peptide A and B-chains and the receptor necessary for RXFP1 activation. Such a complex mechanism of H2 relaxin/RXFP1 interaction poses considerable challenges for modifying and minimizing the size of the peptide without altering its biological activity. However, as the B-chain of H2 relaxin contains the primary binding site (R<sup>B13</sup>, R<sup>B17</sup>, I<sup>B20</sup>), and the A-chain is believed to contain a weaker secondary binding site, there were several attempts to engineer B-chain only ligands for RXFP1 (Hossain et al., 2016; Del Borgo et al., 2005).

### 2.2. Design of a single B-chain mimetic of H2 relaxin, B7-33

The earlier attempts to mimic the helical B-chain of H2 relaxin was not successful in that all the single-B-chain analogues were found to be inactive despite some of them exhibited helical structure (Del Borgo et al., 2005). The native H2 B-chain with 29 residues (B1-29) and its cyclic derivatives were found to be insoluble (Fig. 1B). Based on SAR studies data (Hossain et al., 2008, 2011) an N-terminus truncated, and C-terminus elongated B-chain specific peptide, B7-33 was developed (Fig. 1A) (Hossain et al., 2016). The highly positively charged peptide B7-33 was freely water-soluble unlike B1-29 and its derivatives (Fig. 1B). B7-33 was then tested in several cell and animal models in

comparison to H2 relaxin as outlined below.

### 2.3. The activity of H2 relaxin and B7-33 in cells or *ex vivo*

Upon activation by H2 relaxin, RXFP1 increases activity at a variety of intracellular signalling pathways including cyclic adenosine monophosphate (cAMP) and extracellular signal-regulated kinases 1 and 2 (ERK1/2). However, the particular pathways activated depend on the type of cell being stimulated. For example, HEK293T cells expressing RXFP1 (HEK-RXFP1 cells) show robust increases in cAMP activity after relaxin stimulation (Halls et al., 2006; Hsu et al., 2002; Valkovic et al., 2018) whereas fibroblasts expressing RXFP1 show little or no cAMP activity after relaxin stimulation (Mookerjee et al., 2009; Samuel et al., 2004). Additionally, chronic (48 h or more) treatment of fibroblasts with relaxin impedes the signalling of transforming growth factor beta 1 (TGF-β1) in order to abrogate fibrosis, such as by suppressing the activation of fibroblasts into myofibroblasts, and upregulating the collagen-degrading matrix metalloproteinases (MMPs) (Samuel et al., 2017). In rat renal myofibroblasts, H2 relaxin-induced upregulation of MMPs is dependent on RXFP1, ERK1/2, nitric oxide, and cyclic guanosine monophosphate signalling (Chow et al., 2012). The anti-fibrotic effects of relaxin are also dependent on complexes formed between RXFP1 and the angiotensin II type 2 receptor (AT<sub>2</sub>R) (Chow et al., 2014).

H2 relaxin and B7-33 were initially compared for activity in recombinant HEK-RXFP1 cells (Hossain et al., 2016). Whereas H2 relaxin potently increased cAMP accumulation, B7-33 had a substantially weaker potency (H2 relaxin pEC<sub>50</sub>: 10.49 ± 0.13; B7-33 pEC<sub>50</sub>: 5.12 ± 0.06), which is consistent with the weak affinity of B7-33 in these cells, although it was still a full agonist (Hossain et al., 2016). Similarly, B7-33 was also a weak full agonist for cAMP accumulation in THP-1 cells. Unlike H2 relaxin, which binds at RXFP2 (Hsu et al., 2002) albeit with low affinity, B7-33 did not bind to HEK-RXFP2 cells even at high micromolar concentration (Hossain et al., 2016).

H2 relaxin and B7-33 were also compared for their ability to induce phosphorylation of ERK1/2. In HEK-RXFP1 cells, both agonists produced rapid and transient ERK1/2 phosphorylation, but in this case, B7-33 showed substantially lower efficacy as well as potency (Fig. 1C) (Hossain et al., 2016). However, when tested for ERK1/2 phosphorylation in rat renal myofibroblasts, B7-33 surprisingly showed

comparable efficacy and potency to H2 relaxin (Fig. 1D). This was unlikely to be related to differences in RXFP1 between species, as B7-33 still only showed weak cAMP activity in HEK293T cells expressing mouse or rat RXFP1 (Hossain et al., 2016). Similarly, B7-33 induced upregulation of MMP-2 to a similar degree as H2 relaxin after a 72-h treatment, which occurred via RXFP1 as demonstrated by using an RXFP1 antagonist (Hossain et al., 2016). Additionally, AT<sub>2</sub>R antagonist also blocked the ability of B7-33 to upregulate MMP-2 in rat renal myofibroblasts which demonstrates H2-like similar mechanism of action (Chow et al., 2014).

The actions of B7-33 and H2 relaxin was compared in an ex vivo model of vascular disease using mouse mesenteric arteries pre-incubated in placental trophoblast conditioned media to induce endothelial dysfunction, characteristic of preeclampsia (Marshall et al., 2017). Co-incubation of these arteries in trophoblast conditioned media with B7-33 or H2 relaxin prevented the development of endothelial dysfunction (Marshall et al., 2017). Further studies of B7-33 signalling networks in diverse cellular backgrounds will enhance our understanding of the molecular mechanisms behind the actions of B7-33. Based on these findings in fibroblasts, and particularly its high potency in activating pERK1/2, and MMPs, the efficacy of B7-33 in several animal models was examined as outlined below.

#### 2.4. The activity of B7-33 and H2 relaxin in animal models

**Myocardial infarction (MI)-induced heart failure model:** The efficacy of B7-33 in a pathophysiologically relevant rat model of MI-induced heart failure was examined (Fig. 2A–C) (Hossain et al., 2016). Systemic administration of B7-33 or H2 relaxin, after eight weeks of MI-induced injury, over a four week treatment period, significantly reduced the build-up of MI-induced collagen confirming that B7-33 had a similar anti-fibrotic activity to H2 relaxin in experimental heart failure. Furthermore, improved left ventricular (LV) function evidenced by a significant reduction in left ventricular end-diastolic pressure (LVEDP) was also observed.

**Cardiomyopathy-related cardiac fibrosis model:** The efficacy of systemic B7-33 administration (throughout the onset of injury; from days 1–14) in a mouse model of cardiomyopathy-related cardiac fibrosis was investigated (Fig. 2D–F) (Hossain et al., 2016) and showed that B7-33 had similar efficacy to H2 relaxin in preventing isoproterenol (ISO)-induced cardiac fibrosis, confirming again its potential as a drug candidate for the treatment of fibrosis and related dysfunction associated with heart failure.

**Mouse models with ovalbumin (OVA)-induced chronic allergic airways disease (AAD):** Finally, the anti-remodelling and anti-fibrotic effects of B7-33 given by intranasal administration in a nine-week OVA-induced murine model of chronic AAD *in vivo* was assessed (Fig. 2G–I) (Hossain et al., 2016). When administered daily from weeks 9–11, B7-33 significantly diminished airway epithelial thickening, total lung collagen concentration (a measure of fibrosis) and airway hyperresponsiveness (a measure of lung dysfunction) to a similar extent to native H2 relaxin, confirming that it also had therapeutic potential for chronic inflammatory lung diseases.

The most recent study compared the rapid vascular effects of an acute bolus injection of B7-33 compared with H2 relaxin (Marshall et al., 2017). Male Wistar rats received a tail vein injection of placebo, B7-33 or H2 relaxin (equimolar concentration). Three hours later vascular function in the mesenteric artery was assessed by wire myography. B7-33 and serelaxin selectively enhanced bradykinin-mediated endothelium-dependent relaxation in the rat mesenteric artery by increasing endothelium-derived hyperpolarization.

Like H2 relaxin, B7-33 (i.p. vs. s.c. vs. i.n.) showed clear efficacy in all animal models (rat and mouse) tested to date in chronic or acute settings. However the mechanism by which B7-33 is achieving its potent cell specific actions via RXFP1 are still to be determined.

### 3. H3 relaxin and RXFP3

H3 relaxin is predicted to have an A chain of 24 residues and a B chain of 27 residues [Fig. 3A] based on the native pig relaxin-3 peptide which was extracted from brain (Liu et al., 2003). It is a neuropeptide which is co-expressed in the brain with its receptor RXFP3 (also known as GPCR 135) (Liu et al., 2003). H3 relaxin also strongly binds to and activates RXFP1, the native receptor for H2 relaxin, and RXFP4 (GPCR 142) (Liu et al., 2005a,b), the native receptor for INSL5. However the physiological significance of these interactions are not known, especially as RXFP4 is not expressed in the brain.

#### 3.1. Structure-activity relationship studies

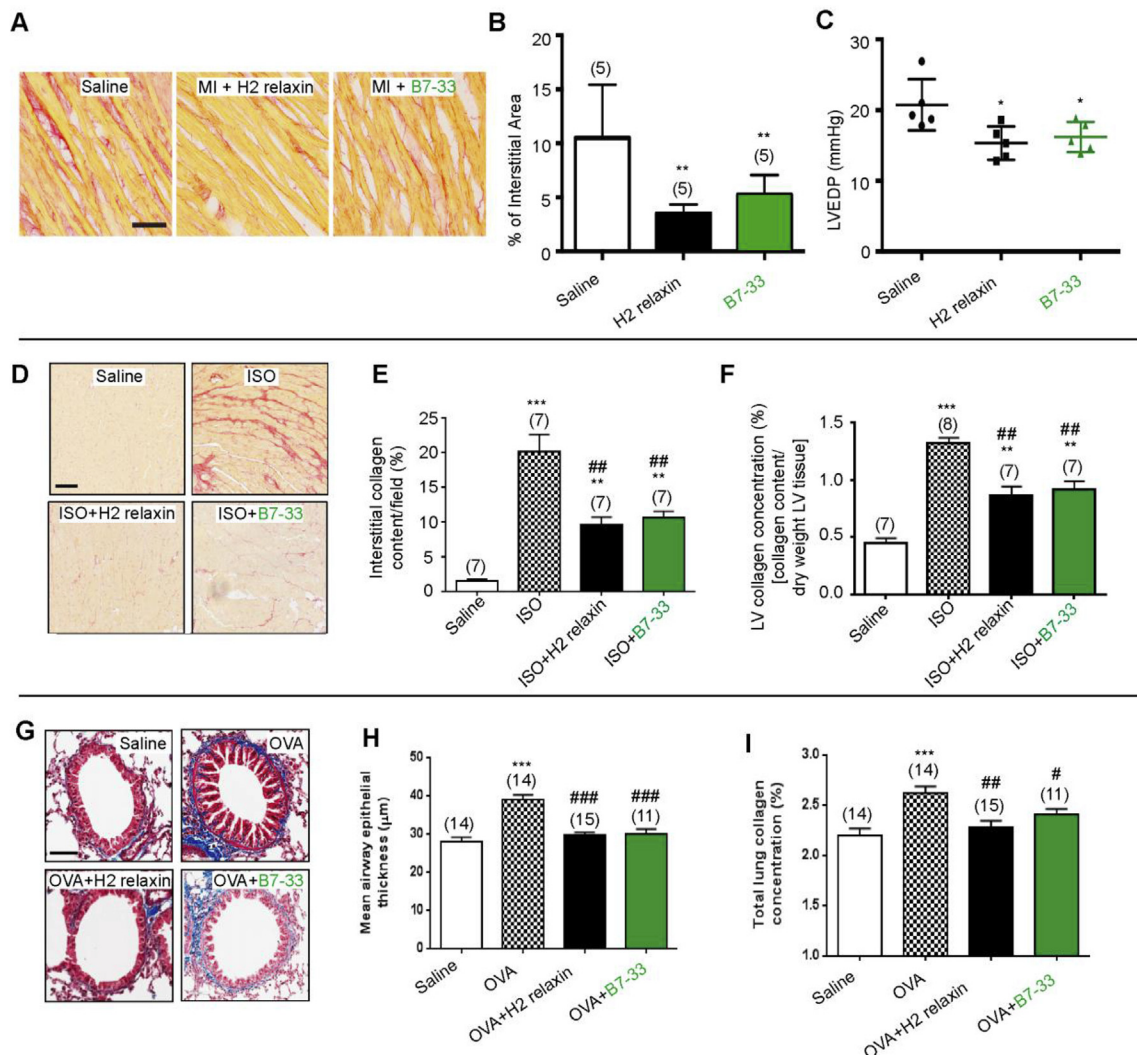
SAR studies on H3 relaxin (Hossain et al., 2008, 2009; Kuei et al., 2007; Liu et al., 2005a,b) and the RXFP3 receptor and modeling of the H3 relaxin/RXFP3 complex (Wong et al., 2018; Bathgate et al., 2013a,b; Zhang et al., 2014) have shown that the primary binding site is located within the surface of the helical domain in the B-chain. The single B-chain alone has been shown to bind to RXFP3 albeit with considerably lower affinity and potency compared to native H3 relaxin (Liu et al., 2003). Although the  $\alpha$ -helix is a predominant secondary structure in the B-chain of H3 relaxin, the single B-chain alone does not adopt an  $\alpha$ -helical conformation (Haugaard-Kedström et al., 2011). Consequently, the low affinity of the B-chain was thought to be due to the lack of this secondary  $\alpha$ -helical structure that is maintained by the A-chain linked by two disulfide bridges (Rosengren et al., 2006).

#### 3.2. Design of a single B-chain mimetic of H3 relaxin, Peptide 5

There were attempts of utilizing judiciously placed salt bridges, and single and bis-cysteine bridges (Shabanpoor et al., 2012) as potential means of stabilizing the bioactive conformations of the B-chain. Unfortunately, these approaches were not successful in that the constrained analogues did not show any improvement in helical structure (Shabanpoor et al., 2012). From structure-activity relationship studies on the H3 relaxin peptide, the primary binding site (R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>) is located in the same surface of the helical domain in the B-chain of H3 relaxin (Kuei et al., 2007). Also, the activation domain (R<sup>B26</sup> and W<sup>B27</sup>) is located at the C-terminus of the B-chain. A classical helical wheel representation reveals that two faces of the helix with the active site (R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>) is exposed on one face and the hydrophobic residues that interact with the A-chain in the native structure is exposed on the second face. Based on this analysis, Jayakody et al. carried out ring closing metathesis reaction to staple B18 and B22 positions and B14 and B18 positions (i, i + 4) (Jayakody et al., 2016). These stapled analogs showed poor RXFP3 activation (Jayakody et al., 2016). Hojo et al. stapled positions B13 and B17, and B17 and B21 and deleted the N-terminal-seven residues of the B-chain which was previously found to be not crucial for RXFP3 activity (Shabanpoor et al., 2012). The best position to mimic the B-chain was found to be B13 and B17. Stapling these positions (B13 and B17) together with the truncation at the N-terminus resulted in an 18 residue peptide, peptide 5 (Fig. 3B), which was shown to have helical structure (Hojo et al., 2016). Peptide 5 was then tested *in vitro* and *in vivo* and compared its bioactivity with H3 relaxin as outlined below.

#### 3.3. The activity of H3 relaxin and peptide 5 in cells

Upon activation, RXFP3 couples to G $\alpha_{i/o}$  proteins to inhibit the production of cAMP (Liu et al., 2003; Van der Westhuizen et al., 2005) and induce phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 (Kocan et al., 2014; Van der Westhuizen et al., 2007). The endogenous ligand for RXFP3, H3 relaxin, can also bind to and activate the related G $\alpha_{i/o}$ -coupled receptor RXFP4 (Liu et al., 2005a,b), and the G $\alpha_s$ -coupled receptor RXFP1 (Bathgate et al., 2002; Sudo et al., 2003).



**Fig. 2.** *In vivo* anti-fibrotic activity of B7-33. (A–C) B7-33 significantly reduced MI-induced fibrosis and improved heart function. (A) Representative photomicrographs of collagen stained with picrosirius red in LVs of the heart of rat. (B) H2 relaxin and B7-33 significantly reduced LV fibrosis post-MI compared with saline treated rats. (C) Animals treated with H2 relaxin or B7-33 were also associated with reduced LVEDP compared to saline-treated animals. Numbers in parentheses represent the number of animals used per group. \* $p < 0.05$ , \*\* $p < 0.01$  vs. saline group. Scale bar 100  $\mu$ m. (D–F) B7-33 prevented ISO-induced cardiac fibrosis of mice. (D) Collagen was stained with picrosirius red to determine (E) % interstitial LV collagen and assessed by hydroxyproline to determine (F) total LV collagen concentration. B7-33 ( $0.25 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) significantly prevented LV fibrosis to a similar extent as native H2 relaxin ( $0.5 \text{ mg kg}^{-1} \text{ d}^{-1}$ ) by day 14 post-ISO-induced injury. Numbers in parentheses represent the number of animals used per group. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. saline (control) group; ## $p < 0.01$  vs. ISO group. Scale bar = 50  $\mu$ m. (G–I) B7-33 reduced OVA-induced airway/lung fibrosis and improved airway function. In (G) representative photomicrographs of Masson's trichrome-stained airway/lung sections of mice. Scale bar = 100  $\mu$ m; In (H) subepithelial collagen thickness (mm) in the lamina reticularis as mean  $\pm$  SEM. In (I) total lung collagen (hydroxyproline) content (a measure of fibrosis) from each of the groups studied. numbers in parentheses represent number of mice analysed per group; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. saline group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. OVA group. Figure adapted from Hossain et al. *Chem Sci* (2016).

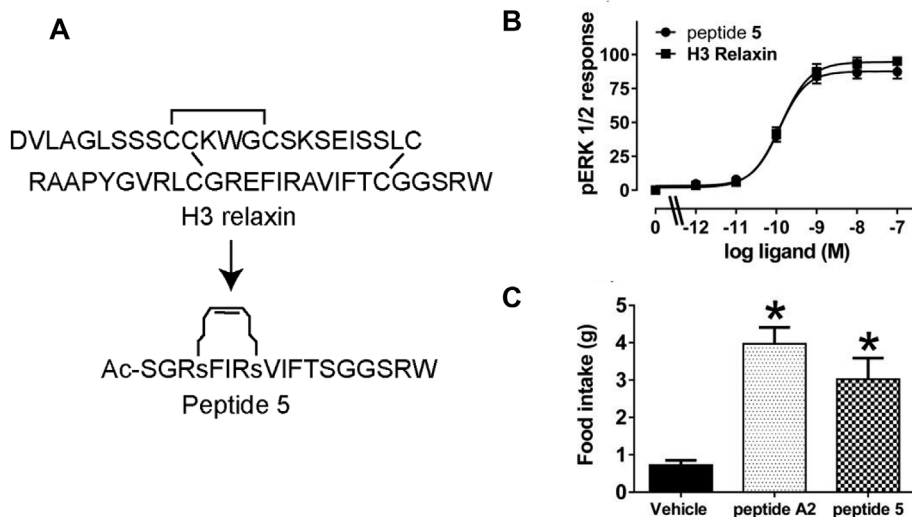
H3 relaxin and peptide 5 were compared for their ability to activate RXFP3 in CHO-K1 cells stably expressing RXFP3 (CHO-RXFP3 cells). Peptide 5 was a full agonist for inhibition of forskolin-stimulated cAMP activity and was only slightly less potent than H3 relaxin (H3 relaxin  $\text{pEC}_{50}$ :  $9.08 \pm 0.07$ ; peptide 5  $\text{pEC}_{50}$ :  $8.48 \pm 0.06$ ) (Hojo et al., 2016). Similarly, both agonists were measured for their ability to induce ERK1/2 phosphorylation, which demonstrated that peptide 5 is a full agonist for pERK1/2, and is equipotent to H3 relaxin (H3 relaxin  $\text{pEC}_{50}$ :  $9.83 \pm 0.10$ ; peptide 5  $\text{pEC}_{50}$ :  $9.92 \pm 0.10$ ) (Fig. 3B) (Hojo et al., 2016). As peptide 5 showed only slightly weaker cAMP inhibition than H3 relaxin in CHO-RXFP3 cells, which was likely a result of the experimental conditions (6-h ligand stimulation), and showed equal activity to H3 relaxin for ERK1/2 phosphorylation, peptide 5 is considered to be a true mimetic of H3 relaxin, rather than a biased agonist for RXFP3 (Hojo et al., 2016). H3 relaxin and peptide 5 were also

compared for activity at RXFP4 expressed in CHO-K1 cells (CHO-RXFP4 cells), and RXFP1 expressed in HEK293T cells (HEK-RXFP1 cells). At RXFP4, peptide 5 was about 100 times less potent than H3 relaxin for cAMP inhibition, consistent with the weaker affinity of peptide 5 for RXFP4 (H3 relaxin  $\text{pEC}_{50}$ :  $8.94 \pm 0.13$ ; peptide 5  $\text{EC}_{50}$ :  $6.95 \pm 0.10$ ) (Hojo et al., 2016). Additionally, whereas H3 relaxin binds to and activates RXFP1 to increase cAMP activity, peptide 5 showed little or no cAMP activity at RXFP1 (Hojo et al., 2016). Thus, in comparison to H3 relaxin, peptide 5 is a high-affinity agonist at RXFP3, a weaker agonist at RXFP4, and has weak or no activity at RXFP1.

#### 3.4. The activity of peptide 5 and H3 relaxin in animals

Effects of icv administration of H3 relaxin agonist (A2) and peptide 5 on food intake was measured (Hojo et al., 2016) (Fig. 3C). Peptide 5





**Fig. 3.** Design, *in vitro* and *in vivo* activity of peptide 5. (A) Design of stapled peptide 5. Hydrocarbon stapling of the truncated B-chain of H3 relaxin at positions 13 and 17 resulted in peptide 5. (B) Effect of H3 relaxin and peptide 5 on ERK1/2 kinase phosphorylation in CHO-K1-RXFP3 cells. (C) Effect of icv injection of peptide 5 (n = 12), H3 relaxin agonist (A2) (n = 6) and vehicle (n = 12) on food intake in satiated rats. \**p* < 0.001 compared to vehicle treatment. Figure adapted from Hojo et al. *J Med Chem* (2016).

significantly stimulated food intake, compared to control, similar to RXFP3 agonist, A2 (Hojo et al., 2016) (Fig. 3C).

#### 4. Conclusion

B7-33 and peptide 5 are important research tools to dissect the physiological roles of their target receptors, RXFP1 and RXFP3 respectively. As single-chain peptides, they are easier scaffolds to improve their therapeutic values (e.g improved half-life or reduced immunogenicity) by industry-standard techniques such as PEGylation (Swierczewska et al., 2015), lipidation (Zhang et al., 2012), or glycosylation (Tatsumi et al., 2012). However, the pharmacokinetics or immunogenicity of these single-chain peptides remain to be studied. While SAR studies on H2 relaxin have suggested that both the A- and B-chain are essential for RXFP1 activity, the discovery of water-soluble B-chain only agonist, B7-33, highlights that there is still a lot unknown about the H2 relaxin-RXFP1 interaction, particularly in the different cellular backgrounds. B7-33 is selective for RXFP1 over RXFP2. B7-33 showed clear efficacy in every animal models tested suggesting that the peptide is an ideal template for further development as a cost-effective vasoactive therapeutic in cardiovascular diseases including fibrosis. The B-chain of H3 relaxin, on the other hand, is the sole determinant for RXFP3 binding and activation, and thus a B-chain only agonist, peptide 5, was possible to design and develop by judiciously placed hydrocarbon stapling. This peptide 5 is a full agonist and truly mimicked the activity of H3 relaxin both *in vitro* and *in vivo*. Peptide 5 is selective for RXFP3/RXFP4 over RXFP1.

#### Conflicts of interest

RADB and MAH are inventors on the patent application PCT/AU2015/050184, Modified Relaxin B chain peptides. All other authors declare that there are no conflicts of interest.

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OPEN

# Engineering of chimeric peptides as antagonists for the G protein-coupled receptor, RXFP4

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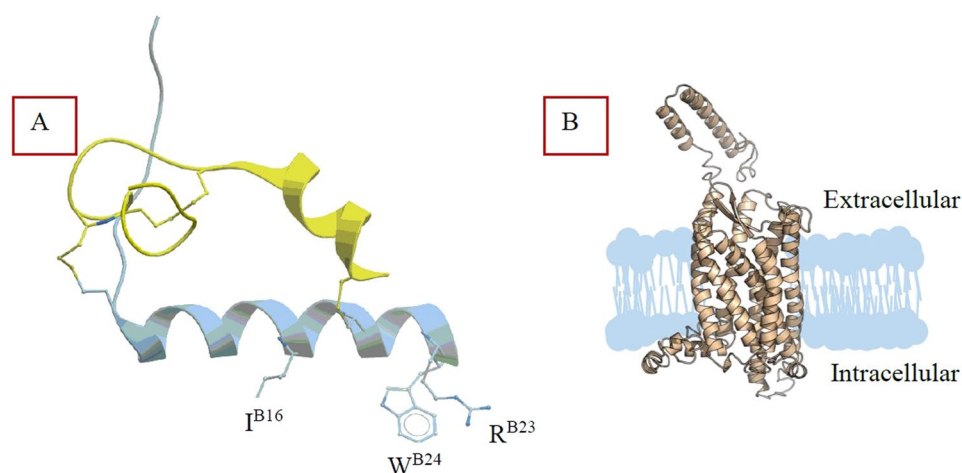
Insulin-like peptide 5 (INSL5) is a very important pharma target for treating human conditions such as anorexia and diabetes. However, INSL5 with two chains and three disulfide bridges is an extremely difficult peptide to assemble by chemical or recombinant means. In a recent study, we were able to engineer a simplified INSL5 analogue 13 which is a relaxin family peptide receptor 4 (RXFP4)-specific agonist. To date, however, no RXFP4-specific antagonist (peptide or small molecule) has been reported in the literature. The focus of this study was to utilize the non-specific RXFP3/RXFP4 antagonist  $\Delta R3/I5$  as a template to rationally design an RXFP4 specific antagonist. Unexpectedly, we demonstrated that  $\Delta R3/I5$  exhibited partial agonism at RXFP4 when expressed in CHO cells which is associated with only partial antagonism of INSL5 analogue activation. In an attempt to improve RXFP4 specificity and antagonist activity we designed and chemically synthesized a series of analogues of  $\Delta R3/I5$ . While all the chimeric analogues still demonstrated partial agonism at RXFP4, one peptide (Analogue 17) exhibited significantly improved RXFP4 specificity. Importantly, analogue 17 has a simplified structure which is more amenable to chemical synthesis. Therefore, analogue 17 is an ideal template for further development into a specific high affinity RXFP4 antagonist which will be an important tool to probe the physiological role of RXFP4/INSL5 axis.

The gut endocrine system is a rich source of peptides with therapeutic potential<sup>1–3</sup>. The demonstrated involvement of altered gut hormone profiles in both the success of bariatric surgery and the treatment of metabolic disorders implies that greater understanding and informed manipulation of gut hormones may lead to meaningful therapies for conditions such as anorexia, obesity, and diabetes<sup>4,5</sup>.

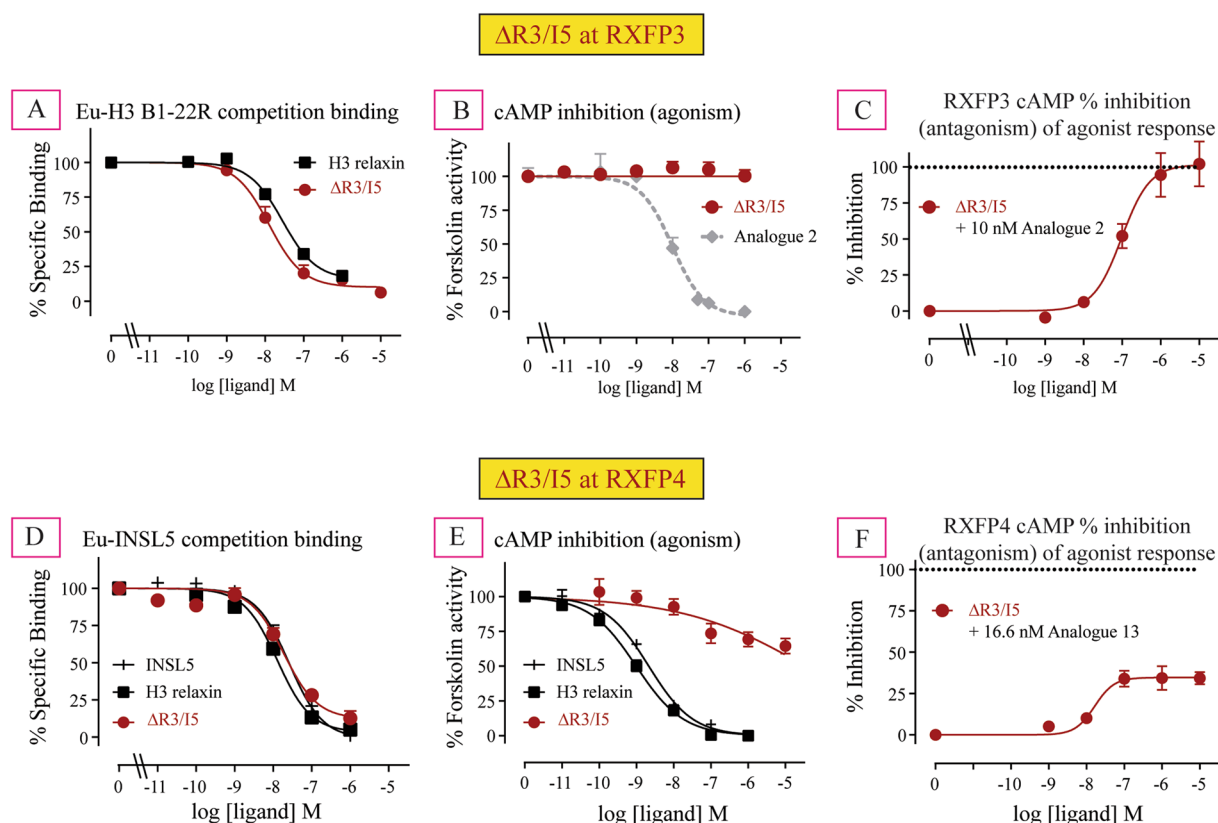
Insulin-like peptide 5 (INSL5, Fig. 1A, Table 1) is a novel gut hormone produced by colonic L-cells<sup>6</sup>. Its target G protein-coupled receptor, Relaxin Family Peptide Receptor (RXFP4, Fig. 1B) is expressed in the enteric nervous system<sup>6</sup>. The first biological data in RXFP4 knockout mice suggested a potential role of INSL5 in appetite regulation<sup>6</sup>. However, the primary source of INSL5 comes from a gut region (colonic enteroendocrine L cells<sup>6</sup>) that has only a minor influence on feeding. Therefore, there is a debate on the physiological role of INSL5 and RXFP4. The recent data in the literature also suggest that INSL5/RXFP4 system may be a potential therapeutic target for Type 2 diabetes<sup>7</sup>.

Studies on the biology of INSL5 have been limited by the lack of sufficient quantities of the native peptide and the absence of small molecule RXFP4-specific agonists. Synthesis of both human INSL5 and mouse INSL5 has proven to be extraordinarily challenging by either chemical<sup>8,9</sup> or recombinant means<sup>10</sup>. The A-chain is insoluble, and B-chain is aggregating in nature, and therefore, both synthesis and purification were found to be very difficult, resulting in very poor yield<sup>8,9</sup>. We recently undertook structure-activity relationship (SAR) studies on INSL5 and utilized the knowledge to engineer a simplified, potent RXFP4 agonist peptide, analogue 13 (Table 1)<sup>11</sup>. The peptide is an INSL5 analogue that has a simplified A-chain connected to the B-chain by two disulfide bonds and is readily synthesized in 17.5 fold higher yield than INSL5<sup>11</sup>. A small-molecule agonist was recently reported<sup>12</sup> which has nanomolar potency both at RXFP4, and RXFP3, a receptor for human relaxin-3 (H3 relaxin)<sup>12</sup>. However, to date, no RXFP4-specific antagonist (peptide or small molecule) has been reported in the literature and such a compound would be enormously valuable to understand the physiology of INSL5 and validate RXFP4 as potential therapeutic target for the treatment of human conditions such as obesity and diabetes.

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**Figure 1.** (A) NMR structure of human INSL5 (PDB: 2KBC) with primary binding residues in the B-chain (I<sup>B16</sup>, R<sup>B23</sup>, W<sup>B24</sup>) highlighted. The image was generated by Molsoft LLC software (ICM-browser Pro(x64); ActiveICM (version 1.2–4); [www.molsoft.com](http://www.molsoft.com)). (B) Hypothetical structure of the RXFP4 receptor generated with Pymol.



**Figure 2.** Binding, agonist and antagonist activities of  $\Delta R3/I5$  in RXFP3 cells. (A) Competition binding curve of  $\Delta R3/I5$  competing with 5 nM of Eu-B1–22R; (B) Agonist activity of  $\Delta R3/I5$  as measured by cAMP changes; (C) Ability of  $\Delta R3/I5$  to antagonize 10 nM analogue 2-induced inhibition of cAMP activity. Binding, agonist and antagonist activities of  $\Delta R3/I5$  in RXFP4 cells. (D) Competition binding curve of  $\Delta R3/I5$  competing with 5 nM of Eu-INSL5; (E) Agonist activity of  $\Delta R3/I5$  as measured by cAMP changes; (F) Ability of  $\Delta R3/I5$  to antagonize 16.6 nM analogue 13-induced inhibition of cAMP activity. The data are the result of  $n = 3-4$  independent experiments and are expressed as mean  $\pm$  SEM.

Some peptide ligands of GPCRs utilize amino acid residues for high affinity binding which are distinct from residues involved in activation. One way of generating antagonist peptide ligands is to delete the amino acids responsible for receptor activation while not affecting the binding interaction. This method was successful for



|    | Analogues                                      | Sequences                                                                                                | RXFP3                              |                                                             | RXFP4                                |                                                             |
|----|------------------------------------------------|----------------------------------------------------------------------------------------------------------|------------------------------------|-------------------------------------------------------------|--------------------------------------|-------------------------------------------------------------|
|    |                                                |                                                                                                          | Binding<br>pKi<br>(Ki)<br>(n)      | Activity<br>pEC <sub>50</sub><br>(EC <sub>50</sub> )<br>(n) | Binding<br>pKi<br>(Ki)<br>(n)        | Activity<br>pEC <sub>50</sub><br>(EC <sub>50</sub> )<br>(n) |
| 2  | Relaxin 3<br>(R3)                              | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-G-G-S-R-W<br>D-V-L-A-G-L-S-S-S-C-C-K-W-G-C-S-K-S-E-I-S-S-L-C | 7.64 ± 0.09<br>(22.9 nM)<br>(4)    | 9.08 ± 0.07<br>(0.83 nM)<br>(5)                             | 8.64 ± 0.15<br>(2.29 nM)<br>(3)      | 8.94 ± 0.13<br>(1.15 nM)<br>(4)                             |
|    | Analogue 2<br>(A2)                             | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-G-G-S-R-W<br>C-K-W-G-S-A-K-S-E-I-S-S-L-C                     | 7.72 ± 0.29<br>(19.05 nM)<br>(3)   | 8.43 ± 0.24<br>(3.71 nM)<br>(4)                             | 7.1 ± 0.07<br>(79.43 nM)<br>(4)      | 7.7 ± 0.07<br>(19.95 nM)<br>(4)                             |
| 13 | INSL5<br>(I5)                                  | K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C             | ND                                 | No activity<br>(3)                                          | 8.39 ± 0.14<br>(4.07 nM)<br>(3)      | 8.67 ± 0.08<br>(2.14 nM)<br>(5)                             |
|    | INSL5:<br>A(8-21)T15K<br>(Analogue 13)         | K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-A-S-S-R-W<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                           | ND                                 | ND                                                          | 7.53 ± 0.19<br>(29.51 nM)<br>(3)     | 7.68 ± 0.04<br>(20.90 nM)<br>(3)                            |
|    | ΔR3B<br>ΔI5B                                   | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>K-E-S-V-R-L-C-G-L-E-Y-I-R-T-V-I-Y-I-C-R                 |                                    |                                                             |                                      |                                                             |
|    | ΔR3/I5                                         | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C               | 8.01 ± 0.10<br>(9.77 nM)<br>(3)    | No activity<br>(3)                                          | 8.34 ± 0.08<br>(4.57 nM)<br>(3)      | Weak<br>partial<br>agonist<br>(5)                           |
| 14 | ΔR3(R12L)/I5                                   | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C               | 7.62 ± 0.05<br>(23.99 nM)<br>(3)   | No activity<br>(3)                                          | 7.62 ± 0.25<br>(23.99 nM)<br>(3)**   | Weak<br>partial<br>agonist<br>(6)                           |
| 15 | ΔR3B/<br>I5A(8-21)T15K                         | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 7.81 ± 0.19<br>(15.49 nM)<br>(3)   | No activity<br>(4)                                          | 7.64 ± 0.09<br>(22.90 nM)<br>(3)**   | Weak<br>partial<br>agonist<br>(5)                           |
| 16 | ΔR3B(T21I)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 7.61 ± 0.11<br>(24.55 nM)<br>(3)   | No activity<br>(4)                                          | 7.30 ± 0.18<br>(50.12 nM)<br>(3)***  | Weak<br>partial<br>agonist<br>(4)                           |
| 17 | ΔR3B(A17T)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-T-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 7.72 ± 0.14<br>(19.05 nM)<br>(3)   | No activity<br>(3)                                          | 7.39 ± 0.07<br>(40.74 nM)<br>(3)***  | Weak<br>partial<br>agonist<br>(4)                           |
| 18 | ΔR3B(R12L)/<br>I5A(8-21)T15K                   | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 7.59 ± 0.09<br>(25.70 nM)<br>(3)*  | No activity<br>(3)                                          | 7.83 ± 0.06<br>(14.79 nM)<br>(3)*    | Weak<br>partial<br>agonist<br>(3)                           |
| 19 | ΔR3B(P4K)/<br>I5A(8-21)T15K                    | R-A-A-K-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 8.1 ± 0.20<br>(7.94 nM)<br>(3)     | No activity<br>(3)                                          | 7.99 ± 0.06<br>(10.23 nM)<br>(3)     | Weak<br>partial<br>agonist<br>(4)                           |
| 20 | ΔR3B(P4KY5E<br>R12LA17TT21I)/<br>I5A(8-21)T15K | R-A-A-K-E-G-V-R-L-C-G-L-E-F-I-R-T-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C                             | 7.31 ± 0.17<br>(48.98 nM)<br>(3)** | No activity<br>(4)                                          | 6.88 ± 0.08<br>(131.82 nM)<br>(3)*** | Weak<br>partial<br>agonist<br>(4)                           |

**Table 1.** Primary structure of INSL5/H3 relaxin, their minimized analogues and chimeric peptides and their receptor binding affinity (pKi/Ki) and cAMP activation (pEC<sub>50</sub>/EC<sub>50</sub>) on the RXFP4/RXFP3 receptors. The different amino acids in the B-chain of INSL5 and H3 relaxin with chemically distinct side chains are boxed. Z is pyroglutamate which is cyclic form of glutamic acid or glutamine that is produced by the loss of a water molecule. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05 vs ΔR3/I5.

developing antagonist for related receptors, RXFP2<sup>13,14</sup> (endogenous receptor for insulin-like peptide 3, INSL3) and RXFP3<sup>15,16</sup>. This was possible as the amino acids involved in activation did not greatly contribute to binding affinity, and thus deletion of these residues resulted in receptor antagonists. However, extensive SAR studies on INSL5 to date have been unable to separate distinct binding and activation domains<sup>17</sup>. While R<sup>B23</sup> and W<sup>B24</sup> in INSL5 are responsible for RXFP4 activation in a similar manner to the same residues in H3 relaxin, in INSL5 these residues also contribute significantly to the RXFP4 binding interaction<sup>17</sup>. Therefore, here, we explored alternative rational design options to develop RXFP4-selective antagonists.

A chimeric peptide consisting of the A-chain of INSL5 and C-terminally truncated B-chain of H3 relaxin, ΔR3/I5 (Table 1), was reported in the literature a decade ago<sup>16</sup>. *In vitro* pharmacological studies showed that ΔR3/I5 is a high-affinity antagonist for both RXFP3 and RXFP4 and it has been used successfully in rodent brain studies to demonstrate specific roles of RXFP3 as RXFP4 is not expressed in the brain<sup>16</sup>. In this study we have



|    | Analogues                                       | Sequences                                                                                  | RXFP3                                                                 |                                   | RXFP4                                                                 |                                |
|----|-------------------------------------------------|--------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|-----------------------------------|-----------------------------------------------------------------------|--------------------------------|
|    |                                                 |                                                                                            | Antagonist potency<br>pIC <sub>50</sub><br>(IC <sub>50</sub> )<br>(n) | Emax<br>(n)                       | Antagonist potency<br>pIC <sub>50</sub><br>(IC <sub>50</sub> )<br>(n) | Emax<br>(n)                    |
|    | $\Delta R3/I5$                                  | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C | 6.93 ± 0.21<br>(117.49 nM)<br>(3)                                     | 100 ± 3.8<br>(3)                  | 7.76 ± 0.16<br>(17.38 nM)<br>(3)                                      | 36.7 ± 7.4<br>(3)              |
| 14 | $\Delta R3(R12L)/I5$                            | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>Z-D-L-Q-T-L-C-C-T-D-G-C-S-M-T-D-L-S-A-L-C | 6.58 ± 0.21<br>(263.03 nM)<br>(3)                                     | 66.5 ± 14.8<br>(3) <sup>**</sup>  | 6.48 ± 0.25<br>(331.13 nM)<br>(3) <sup>***</sup>                      | 37.3 ± 7.6<br>(3)              |
| 15 | $\Delta R3/I5A(8-21)T15K$                       | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 43.5 ± 12.1<br>(3) <sup>***</sup> | <6.27 ± 0.05<br>(537.03 nM)<br>(3) <sup>***</sup>                     | 49.4 ± 6.2<br>(3)              |
| 16 | $\Delta R3B(T21I)/I5A(8-21)T15K$                | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 55.8 ± 3.4<br>(3) <sup>***</sup>  | <5<br>(<10 μM)<br>(4)                                                 | 2.1 ± 4.7<br>(4) <sup>**</sup> |
| 17 | $\Delta R3B(A17T)/I5A(8-21)T15K$                | R-A-A-P-Y-G-V-R-L-C-G-R-E-F-I-R-T-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 34.8 ± 0.9<br>(3) <sup>***</sup>  | 7.46 ± 0.26<br>(34.67 nM)<br>(4)                                      | 36 ± 10<br>(4)                 |
| 18 | $\Delta R3B(R12L)/I5A(8-21)T15K$                | R-A-A-P-Y-G-V-R-L-C-G-L-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 47.0 ± 7.3<br>(3) <sup>***</sup>  | 6.67 ± 0.09<br>(213.80 nM)<br>(3) <sup>**</sup>                       | 56.2 ± 11.8<br>(3)             |
| 19 | $\Delta R3B(P4K)/I5A(8-21)T15K$                 | R-A-A-K-Y-G-V-R-L-C-G-R-E-F-I-R-A-V-I-F-T-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 76.3 ± 1.8<br>(3) <sup>*</sup>    | 7.10 ± 0.12<br>(79.43 nM)<br>(4) <sup>*</sup>                         | 71.3 ± 4.8<br>(4) <sup>*</sup> |
| 20 | $\Delta R3B(P4KY5E R12LA17TT21I)/I5A(8-21)T15K$ | R-A-A-K-E-G-V-R-L-C-G-L-E-F-I-R-T-V-I-F-I-C-R<br>C-T-D-G-A-S-M-K-D-L-S-A-L-C               | <5<br>(<10 μM)<br>(3)                                                 | 19.9 ± 2.3<br>(3) <sup>***</sup>  | <5<br>(<10 μM)<br>(4)                                                 | 8.1 ± 9.5<br>(4) <sup>*</sup>  |

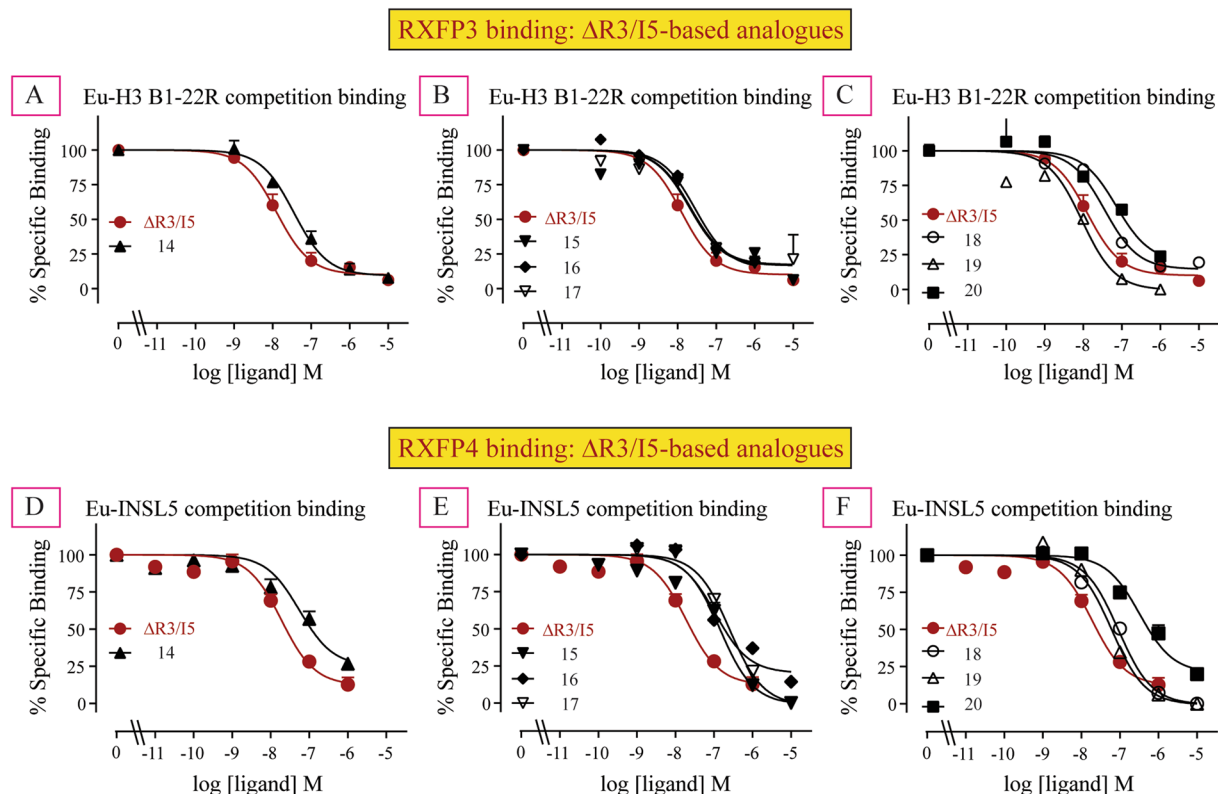
**Table 2.** Primary structure of  $\Delta R3/I5$  and novel chimeric peptides and their antagonist potency (pIC<sub>50</sub>/IC<sub>50</sub>) and efficacy on the RXFP4/RXFP3 receptors. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05 vs  $\Delta R3/I5$ .

utilized the  $\Delta R3/I5$  peptide as a template to rationally design an RXFP4 antagonist based on our understanding of RXFP3 and RXFP4 structure function relationships. Unexpectedly,  $\Delta R3/I5$  demonstrated weak partial agonism of cAMP activation in RXFP4 expressing cells which was associated with only partial antagonism of INSL5 analogue mediated cAMP activation. Rational design of synthetic  $\Delta R3/I5$  analogues demonstrated improvements in RXFP4 specificity however still demonstrated weak partial agonism associated with only partial RXFP4 antagonist activity.

## Results and Discussion

The human relaxin family is comprised of seven peptide hormones that include H1 relaxin, H2 relaxin, H3-relaxin, INSL3, INSL4, INSL5, and INSL6. H2 relaxin, H3-relaxin, INSL3 and INSL5 exert various biological functions through their GPCR receptors RXFP1-4, respectively<sup>18</sup>. While INSL5 is a selective ligand (agonist) for RXFP4, other family peptides are non-selective agonists for RXFP1-RXFP4. H3 relaxin, for example, is an endogenous ligand for RXFP3. However, it also strongly interacts with RXFP1 and RXFP4. To fully understand the role of relaxin family peptides, the development of selective agonists and antagonists is crucial. Recent reports have demonstrated that such receptor-selective agonists and antagonists can be achieved by making chimeric peptides<sup>16,19,20</sup>.  $\Delta R3/I5$  is an example of such a chimeric peptide. While H3 relaxin binds three receptors (RXFP1, RXFP3, and RXFP4), the chimeric  $\Delta R3/I5$  interacts with RXFP3 and RXFP4 only where it is a high affinity antagonist. This study reports further modification of  $\Delta R3/I5$  leading to improvements in RXFP4 selectivity.

**Design and synthesis of  $\Delta R3/I5$  and  $\Delta R3/I5$ -based chimeric analogues.** Instead of applying conventional alanine scanning to determine which residues in  $\Delta R3/I5$  are responsible for selectivity between RXFP3 and RXFP4, we have rationally designed and chemically assembled several chimeric analogues of INSL5 and H3 relaxin (Table 1) based on our knowledge of the INSL5/RXFP4 and H3 relaxin/RXFP3 interactions<sup>18</sup>. We synthesized seven  $\Delta R3/I5$ -based analogues (14–20) and have utilized four previously reported peptides (H3 relaxin<sup>21</sup>, INSL5<sup>8</sup>, analogue 2<sup>22</sup>, analogue 13<sup>11</sup> and  $\Delta R3/I5$ <sup>16</sup>) as controls (Table 1). We have used H3 relaxin and analogue 2 as RXFP3 agonists, INSL5 and analogue 13 as RXFP4-selective agonists, and  $\Delta R3/I5$  as a non-selective antagonist for both RXFP3 and RXFP4 (Table 1). All the analogues were made with free C-termini (carboxylic acid) as we have previously shown that INSL5 and H3 relaxin analogues with amidated C-termini were less active compared with native INSL5 or H3 relaxin with free C-termini<sup>9</sup>. All linear single-chain peptides (A and B chains) were synthesized by Fmoc solid-phase peptide synthesis using automated peptide synthesizer (Liberty Blue) and then



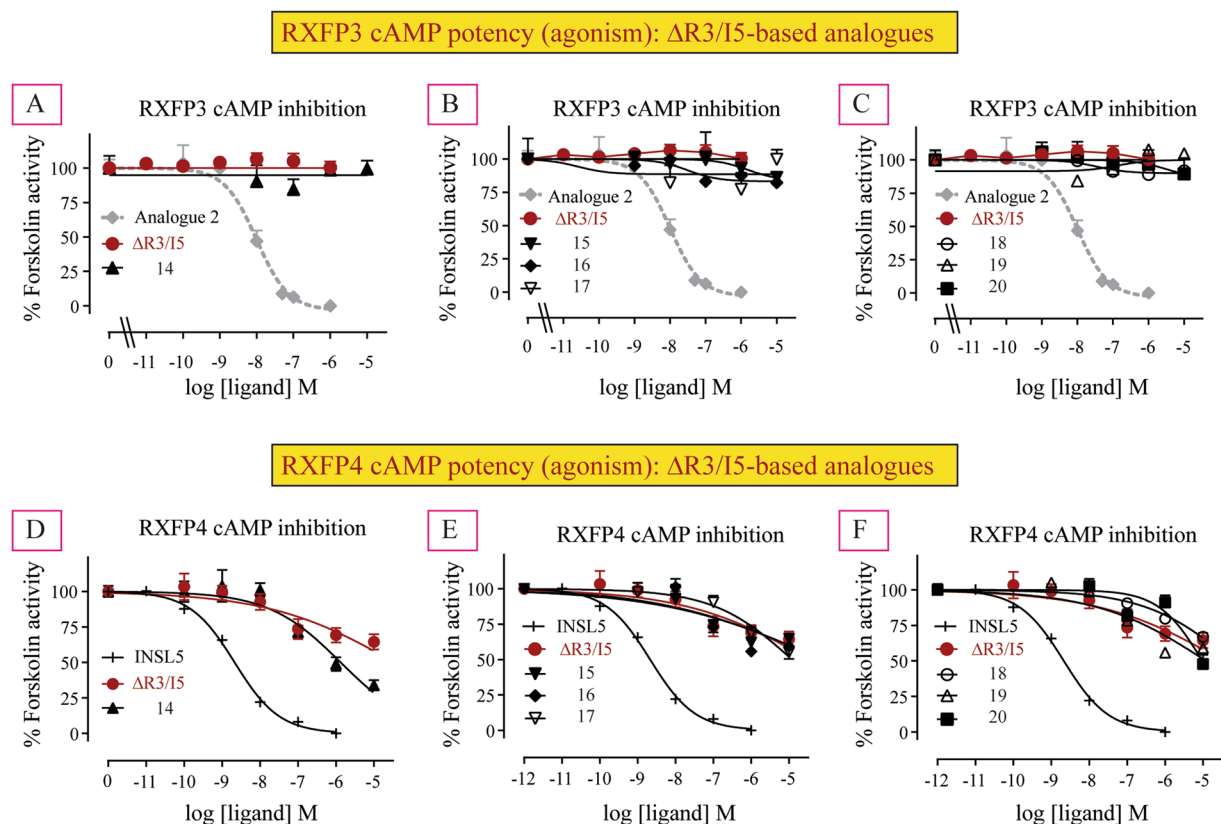
**Figure 3.** Competition binding curves for  $\Delta$ R3/I5 and novel chimeric analogues 14–20, (A–C) in comparison to Eu-B1-22R in RXFP3-expressing cells and (D–F) in comparison to Eu-INSL5 in RXFP4-expressing cells. The data are the result of  $n = 3$ –4 independent experiments and are expressed as mean  $\pm$  SEM.

connected them by disulfide bonds in solution. The peptides were purified by reverse-phase high-performance liquid chromatography, and high final purity (93–99%) was confirmed by MALDI-TOF MS and RP-HPLC analysis (Supplementary Information). The concentration of peptide samples for pharmacological testing was confirmed by using Direct Detect assay-free sample cards and the Direct Detect spectrometer (Supplementary Information).

**$\Delta$ R3/I5 – a previous study.**  $\Delta$ R3/I5 was originally reported to strongly bind to RXFP3 and RXFP4 but showed no agonist activity (NA) at either of these receptors<sup>16</sup> suggesting that  $\Delta$ R3/I5 is an antagonist for both RXFP3 and RXFP4. The antagonism of RXFP3 and RXFP4 was compared, and the results showed that  $\Delta$ R3/I5 dose-dependently shifted the agonism curves to the right confirming  $\Delta$ R3/I5 to be an antagonist for both RXFP3 and RXFP4<sup>16</sup>. Here we have chemically synthesized and pharmacologically characterized  $\Delta$ R3/I5 for binding and cAMP potency in CHO cells stably expressing RXFP3 (CHO-RXFP3) or RXFP4 (CHO-RXFP4).

**The pharmacological activity of  $\Delta$ R3/I5 at RXFP3.** We have first tested our synthetic  $\Delta$ R3/I5 in competition binding assays in CHO-RXFP3 cells.  $\Delta$ R3/I5 exhibited high affinity binding at RXFP3 with an affinity higher than the native ligand H3 relaxin as previously reported ( $K_i$  of 9.77 nM compared to 22.9 nM; Table 1, Fig. 2A). The  $\Delta$ R3/I5 was then tested for its ability to activate the RXFP3 receptor as measured via its ability to inhibit forskolin-stimulated cAMP production in CHO-K1-RXFP3 cells (Fig. 2B). Consistent with the previous report,  $\Delta$ R3/I5 was unable to activate RXFP3 (Fig. 2B, Table 1) and displayed potent antagonistic activity ( $IC_{50}$  of 117.49 nM,  $E_{max} = 100\%$ ; Table 2, Fig. 2C) at RXFP3 evident from its ability to block 10 nM analogue 2 (RXFP3 agonist) inhibition of the forskolin-induced cAMP activity.

**The pharmacological activity of  $\Delta$ R3/I5 at RXFP4.** We tested  $\Delta$ R3/I5 in competition binding assays on human CHO-K1-RXFP4 cells.  $\Delta$ R3/I5 showed high affinity binding to RXFP4 with an affinity similar to the native agonist INSL5 ( $K_i$  of 4.57 nM compared to 2.14 nM; Table 1, Fig. 2D). However, to our surprise,  $\Delta$ R3/I5 acted as a weak partial agonist at RXFP4 (Table 1, Fig. 2E). This result seems to contradict the original published report where  $\Delta$ R3/I5 was described as having no activity at human RXFP4 although dose response curves were not presented<sup>16</sup>. Notably, the original study utilized SK-N-MC/CRE- $\beta$ -gal cells expressing human RXFP4 hence although the reporter gene system was similar the studies were done in a completely different cell system. Importantly, consistent with this partial agonist activity, antagonism assays utilizing synthetic  $\Delta$ R3/I5 demonstrated that the peptide was only able to partially block INSL5 agonist mediated cAMP activation ( $E_{max} = 36.7\%$  of response,  $IC_{50}$  of 17.38 nM, Table 2, Fig. 2F).



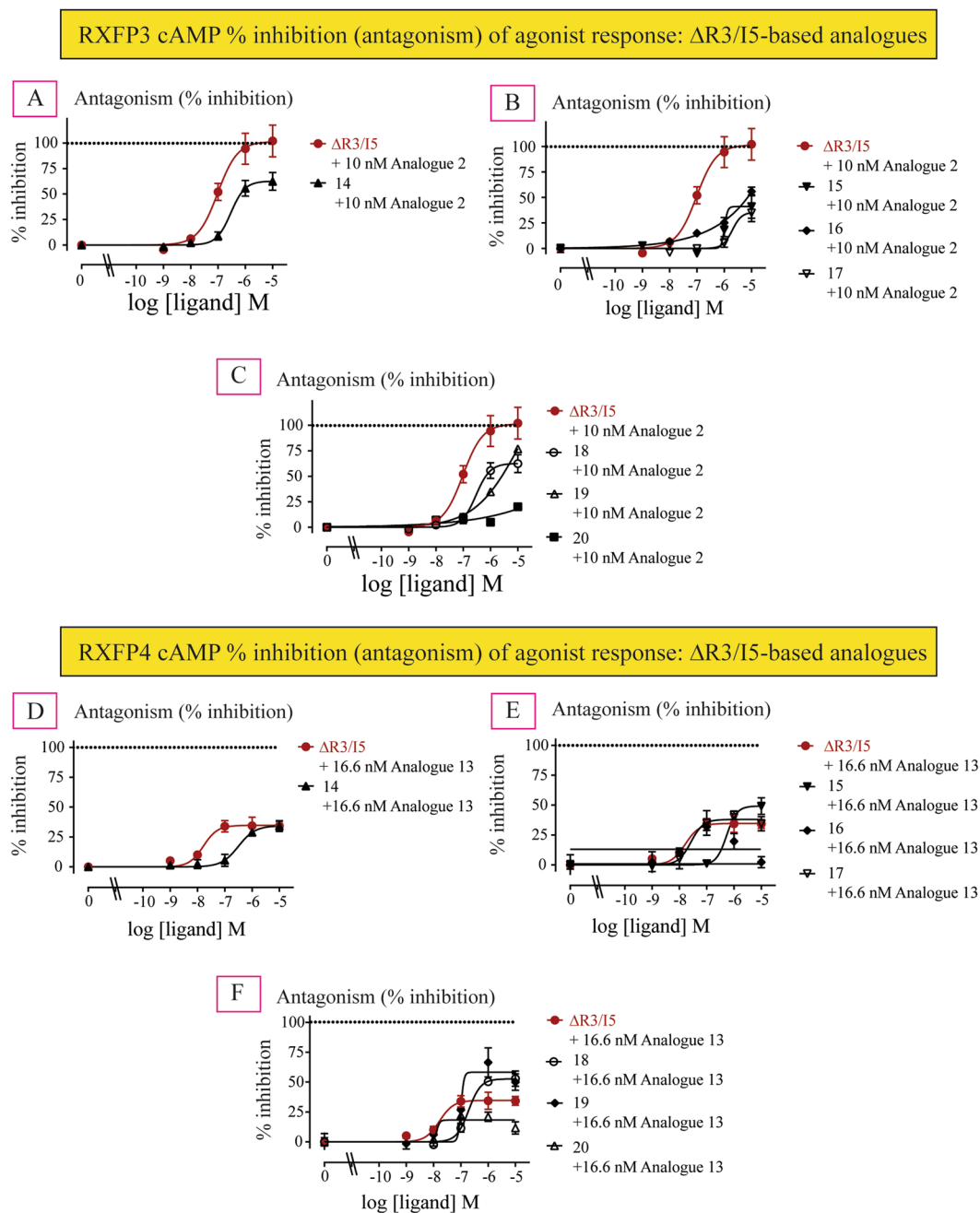
**Figure 4.** Agonist activities of novel chimeric analogues 14–20 compared with  $\Delta R3/I5$  in RXFP3 cells (A–C) and RXFP4 cells (D–F) as measured by changes in cAMP activity. The data are the result of  $n = 3$ –4 independent experiments and are expressed as mean  $\pm$  SEM.

This is the first report of  $\Delta R3/I5$  showing partial agonism and antagonism of cAMP activation at human RXFP4. Notably, although  $\Delta R3/I5$  shows no agonist activity and is a full antagonist of H3 relaxin in CHO-RXFP3 cells utilizing the CRE-reporter gene assay<sup>23</sup> and in SK-N-MC/CRE- $\beta$ -gal cells expressing human RXFP3<sup>16</sup> two other reports have shown that  $\Delta R3/I5$  is a partial agonist of RXFP3. Kocan *et al.* demonstrated that  $\Delta R3/I5$  was a weak partial agonist of p38MAPK, ERK1/2 and cAMP signalling but also was able to act as an antagonist of these pathways<sup>24</sup>. Another study demonstrated that  $\Delta R3/I5$  is a partial agonist in assays of [35S]-GTP $\gamma$ S binding, cAMP activation and dynamic mass redistribution (DMR) in HEK-293s cells expressing rat and human RXFP3<sup>25</sup>.

In this study we have attempted to modify the  $\Delta R3/I5$  peptide to achieve RXFP4-specificity and improve the peptides antagonist properties at RXFP4.

**Novel  $\Delta R3/I5$ -based analogues.** H3 relaxin interacts with both RXFP3 and RXFP4 by utilizing the same primary binding residues in the B-chain<sup>16</sup>: R<sup>B8</sup>, R<sup>B12</sup>, I<sup>B15</sup>, R<sup>B16</sup>, and F<sup>B20</sup>. INSL5, on the other hand, interacts with RXFP4 by utilizing primary binding residues in the B-chain: I<sup>B16</sup>, R<sup>B23</sup> and W<sup>B24</sup> in the B-chain (Fig. 1B)<sup>17,26</sup>. Given that H3 relaxin interacts with both RXFP3 and RXFP4 receptors and INSL5 interacts only with RXFP4 using B-chain specific residues, we speculated that we would be able to rationally design RXFP4-selective antagonist by modifying the B-chain of  $\Delta R3/I5$ . Our strategy was to replace non-conservative residues of the B-chain of  $\Delta R3/I5$  with the corresponding residues of the B-chain of INSL5. We have compared the amino acid sequences of C-terminally deleted H3 B-chain ( $\Delta H3B$ ) in  $\Delta R3/I5$  with that of equivalent INSL5 B-chain ( $\Delta INSL5B$ ) (Table 1). There are 23 residues in the  $\Delta H3B$  and 21 residues in the corresponding  $\Delta INSL5B$ . However, of these, five residues involve differences with chemically distinct side chains (boxed in Table 1). We systematically replaced those five distinct residues in the B-chain of  $\Delta R3/I5$  with the corresponding residues of INSL5B.

**Binding of  $\Delta R3/I5$ -based analogues at RXFP3 and RXFP4 cells.** Three disulfide bond containing  $\Delta R3/I5$ -based analogue 14. R<sup>B12</sup> in the B-chain of  $\Delta R3/I5$  was previously reported to be one of the residues important for binding to both RXFP3 and RXFP4<sup>16</sup>. The corresponding residue L<sup>B9</sup> in the B-chain of INSL5, on the other hand, was reported to be one of the most critical residues by which INSL5 exhibits selectivity for RXFP4 over RXFP3<sup>27</sup>. Therefore, in the first attempt, we replaced the R<sup>B12</sup> residues in the B-chain of  $\Delta R3/I5$  with corresponding L<sup>B9</sup> residue of INSL5 B-chain in order to achieve selectivity for RXFP4 over RXFP3 (Table 1). To our surprise, the resulting analogue 14 did not show significantly reduced affinity for RXFP3 ( $K_i$  of 23.99 nM; Table 1, Fig. 3A), but did show significantly less affinity for RXFP4 ( $K_i$  of 23.99 nM; Table 1, Fig. 3D) compared with  $\Delta R3/I5$ . The fact that the agonist peptide R3/I5 with the same mutation (R<sup>B12</sup>/L<sup>B9</sup>) was shown to exhibit ~1600

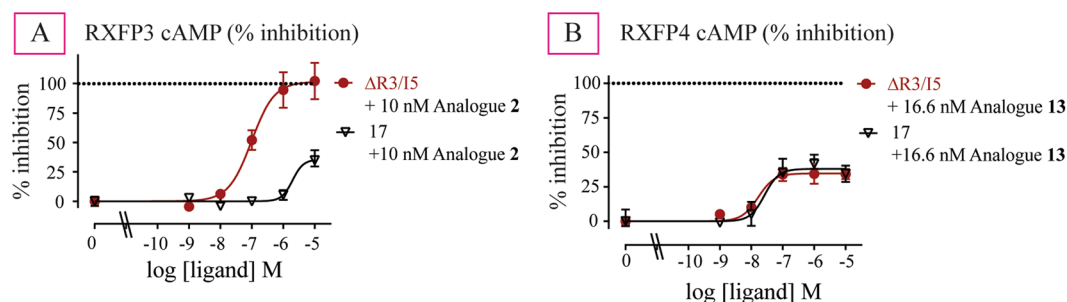


**Figure 5.** Antagonist activities of novel chimeric analogues **14–20** compared with  $\Delta$ R3/I5 in RXFP3 and RXFP4 cells. (**A–C**) Ability of  $\Delta$ R3/I5 and analogues **14–20** to antagonize 10 nM analogue **2**-induced inhibition of cAMP activity. (**D–F**) Ability of  $\Delta$ R3/I5 and analogues **14–20** to antagonize 16.6 nM analogue **13**-induced inhibition of cAMP activity. The data are the result of  $n = 3–4$  independent experiments and are expressed as mean  $\pm$  SEM.

less RXFP3 potency without losing much RXFP4 potency<sup>27</sup> suggest that the binding mode of the agonist (R3/I5) peptide for RXFP3 and RXFP4 might be different to that of the antagonist peptide ( $\Delta$ R3/I5).

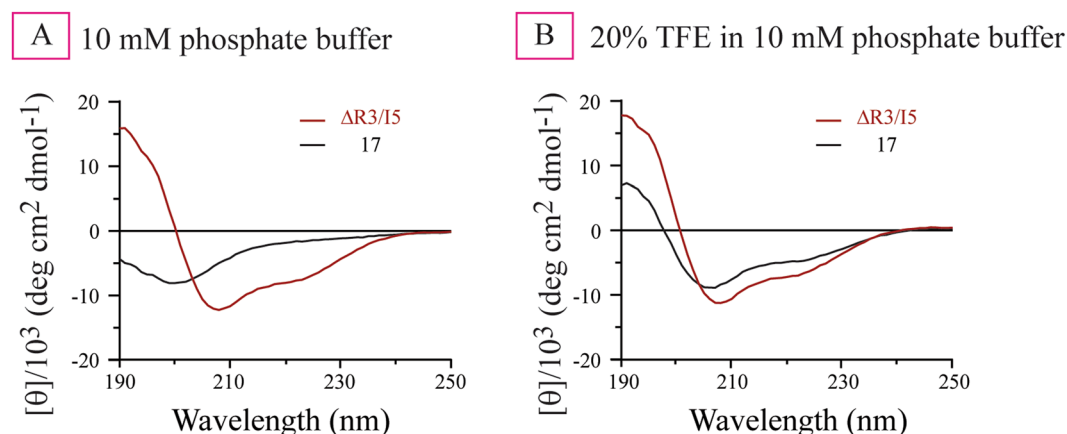
**Two disulfide bond containing  $\Delta$ R3/I5-based analogue **15**.** The current lead antagonist  $\Delta$ R3/I5 or mutated analogue **14** has two chains (A and B chain) and three disulfide bonds with 43 residues (Table 1). Chemical synthesis of this peptide is tedious, time-consuming, and low yielding (3.3%) (Supplementary Information). We therefore decided to simplify the structure of  $\Delta$ R3/I5 so that we can facilitate SAR studies. In this context, we replaced the A-chain (21 residues) of  $\Delta$ R3/I5 with the modified A-chain (14 residues) of the simplified RXFP4 agonist, analogue **13**<sup>11</sup> (Table 1). The resulting analogue **15** has only two-disulfide bridges and is high yielding (17.4%) (Supplementary Information). Excitingly, analogue **15** showed high affinity for both RXFP3 ( $K_i$  of 15.49 nM;

## Analogue 17 is RXFP4-selective antagonist



**Figure 6.** Antagonist activity of novel chimeric analogue 17 compared with  $\Delta R3/I5$  in RXFP3 and RXFP4 cells. (A) Ability of  $\Delta R3/I5$  and analogues 17 to antagonize 10 nM analogue 2-induced inhibition of cAMP activity. (B) Ability of  $\Delta R3/I5$  and analogues 17 to antagonize 16.6 nM analogue 13-induced inhibition of cAMP activity. The data are the result of  $n = 3-4$  independent experiments and are expressed as mean  $\pm$  SEM.

## Analogue 17 forms induced-helix



**Figure 7.** CD spectrum of analogue 17 compared with  $\Delta R3/I5$ . CD was performed in 10 mM PBS buffer at pH 7.5 (A) and in 20% TFE (B).

Table 1, Fig. 3B) and RXFP4 (Ki of 22.90 nM; Table 1, Fig. 3E). Therefore, we have used this high yielding chimeric peptide template 15 for further SAR studies as outlined below.

**Two disulfide bond containing  $\Delta R3/I5$ -based analogues 16–20.** The analogues (16–19) derived from analogue 15 by single amino acid replacement showed strong binding affinity for both RXFP3 (Ki of 7.94 nM to 25.70 nM; Table 1, Fig. 3B,C) and RXFP4 (Ki 10.23 nM to 50.12 nM; Table 1, Fig. 3E,F). When compared with analogue 15, it is clear that non-conservative residues between the  $\Delta B$ -chain of INSL5 and H3 relaxin have minimal or no contribution to RXFP3/RXFP4 binding. However, when all five distinct residues in the B-chain of analogue 15 were replaced with corresponding residues of the INSL5 B-chain, the resulting analogue 20 showed significantly less affinity compared with  $\Delta R3/I5$  for both RXFP3 (Ki of 48.98 nM; Table 1, Fig. 3C) and RXFP4 (Ki of 131.82 nM; Table 1, Fig. 3F) that might be due to accumulated loss contributed by each mutation or due to destabilization of the overall structure.

**Agonism potency of  $\Delta R3/I5$ -based analogues at RXFP3 and RXFP4 cells.** Like  $\Delta R3/I5$ , all the analogues (14–20) demonstrated no significant activation of RXFP3 (Table 1, Fig. 4A–C) but acted as weak partial agonists at RXFP4 (Table 1, Fig. 4D–F) suggesting that they are likely to be antagonists for both RXFP3 and RXFP4.

**Antagonism potency of  $\Delta R3/I5$ -based analogues at RXFP3 and RXFP4 cells.** Interestingly, despite high binding affinity and no activity at RXFP3, all the analogues (14–20) showed significantly less antagonistic potency and efficacy (Fig. 5A–C, Table 2) compared with  $\Delta R3/I5$ . This result was initially encouraging as it suggested some



RXFP4-selectivity. However, when tested in RXFP4 cells, all analogues except two (**17** and **19**) showed less antagonistic potency compared with  $\Delta R3/I5$  (Fig. 5D–F, Table 2). Interestingly, analogues **17** and **19** showed high antagonistic potency (**17**:  $IC_{50}$  of 34.67 nM, **19**:  $IC_{50}$  of 79.43 nM; Table 2; Fig. 5E,F) with analogue **19** showing higher efficacy ( $E_{max}$  of 71.3%) than  $\Delta R3/I5$ . Importantly, while analogue **17** has  $\Delta R3/I5$ -like antagonist potency for RXFP4, it was found to be more selective for RXFP4 over RXFP3 (Fig. 6, Table 2) compared with both  $\Delta R3/I5$  and analogue **19**. However, all of the chimeric  $\Delta R3/I5$  analogues still demonstrated partial agonist activity like the parent  $\Delta R3/I5$  peptide and importantly were only able to partially antagonize RXFP4 agonist activation. Nonetheless, analogue **17** demonstrates improved selectivity for RXFP4 and has a simpler structure compared with  $\Delta R3/I5$  which enables its preparation in higher yield (yield of  $\Delta R3/I5$  = 3.3% and yield of analogue **17** = 17.4%; Supplementary Information)

**Structural study of  $\Delta R3/I5$  and analogue **17**.** While the structure of the  $\Delta R3/I5$  peptide has not been determined, the NMR solution structure of the agonist peptide R3/I5 has been solved<sup>28</sup>. The NMR solution structure of R3/I5 revealed that the INSL5 A-chain adopts a conformation similar to the H3 relaxin A-chain, and thus can structurally support a native-like conformation of the H3 relaxin B-chain through which it interacts with both RXFP3 and RXFP4. Truncations of the A-chain and/or removal of the intra-A-chain disulfide bond have been previously used to successfully simplify the structure while maintaining affinity and efficacy of several relaxin peptides<sup>11,22,29,30</sup>. However, extensive truncation or removal of the disulfide bond often leads to a compromised overall structure<sup>29,30</sup>. Such minimized peptides are generally challenging to study by NMR due to the increased structural flexibility resulting in spectral line broadening<sup>31</sup> and tendencies of aggregation<sup>32</sup>. To investigate the conformation of analogue **17** in solution, and to compare it to that of  $\Delta R3/I5$ , we therefore decided to carry out circular dichroism (CD) studies. Consistent with the solution NMR structure of R3/I5, our synthetic  $\Delta R3/I5$  demonstrated clear  $\alpha$ -helical structure in aqueous buffer with characteristic double minima in the spectrum at 208 and 222 nm (Fig. 7A). In contrast, analogue **17** showed spectra representing principally random coil structure (Fig. 7A). As analogue **17** has strong RXFP4 binding affinity ( $IC_{50}$  of 34.67 nM), we postulate that analogue **17** adopted an appropriate conformation upon binding to the receptor. To confirm this hypothesis, we have studied CD spectroscopy of these peptides in 20% trifluoroethanol (TFE) in phosphate buffer. Trifluoroethanol (TFE) is known to stabilize the  $\alpha$ -helical structure of peptide and proteins by improving hydrogen bonding. While 20% TFE did not significantly affect the secondary structure of  $\Delta R3/I5$  (Fig. 7B), it induced helical structure in analogue **17**, suggesting that analogue **17** has a tendency to form a helix. This finding is consistent with our previous studies where the truncated INSL5 analogues **6**<sup>30</sup> and **13**<sup>11</sup> exhibited compromised fold but showed very high RXFP4 affinity<sup>30</sup>. A similar trend was also observed for a H3 relaxin analogue where the A-chain was truncated up to 9 residues at the N-terminus, and the resulting analogue with compromised structure was found not to significantly affect binding or activation of RXFP3<sup>29</sup>.

## Conclusion

In conclusion, we have demonstrated for the first time that the RXFP3/RXFP4 antagonist  $\Delta R3/I5$  exhibits partial agonism at RXFP4 when expressed in CHO cells which is associated with only partial antagonism of INSL5 analogue activation. In an attempt to develop an RXFP4-specific antagonist we have rationally designed and chemically synthesized a series of simplified analogues of  $\Delta R3/I5$ . All of the chimeric analogues still demonstrated partial agonism at RXFP4, however analogue **17** exhibited improved RXFP4 specificity. Moreover, analogue **17** has a simplified structure which enables its synthesis in higher yields than  $\Delta R3/I5$ . Therefore, analogue **17** is a potential template for further SAR to improve RXFP4 antagonistic efficacy and facilitate pre-clinical studies to probe the physiological role of RXFP4/INSL5 axis.

## Methods

**Peptide synthesis.** All the polypeptide chains were assembled on preloaded resins using Fmoc solid phase synthesis, and our optimized synthesis protocols<sup>11</sup> on the microwave assisted liberty peptide synthesizer (CEM liberty, Mathew, USA). The side chain-protecting groups of trifunctional amino acids were TFA-labile, except for tert-butyl (tBu)-protected and acetamidomethyl (Acm)-protected cysteine Cys in the A-chain and Cys (Acm) in the B-chain. The peptides were synthesized on a either 0.1 mmol scale using instrument default protocols with either a 4-fold molar excess of Fmoc-protected amino acids (0.4 mmol for a 0.1 mmol scale; 1.0 mmol for a 0.25 mmol scale) that were activated by using 3.8-fold excess of HCTU in the presence of excess of diisopropylethylene amine (DIEA). N<sup>α</sup>-Fmoc protecting groups were removed by treating the resin-attached peptide with piperidine (20% v/v) in DMF. Using the microwave synthesizer, the coupling and deprotection were carried out at 75 °C using 25 W microwave power for 5 min and 60 W microwave power for 5 or 3 min respectively. For manual synthesis the coupling and deprotection were carried out for 60 min and 5 min 3 times respectively.

**Peptide purification and characterization.** We have analyzed, purified and characterized the synthetic peptides by using reversed-phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Supplementary Information).

**Peptide characterization by amino acid analysis.** The peptide content was determined using Direct Detect assay-free sample cards and the Direct Detect spectrometer. Each card contains hydrophilic spots surrounded by a hydrophobic ring to retain the analyzed sample within the IR beam for convenient sample application and analysis. All measurements were performed using 2  $\mu$ L of sample solution.

**Peptide analogues prepared and characterized.** Human INSL5, H3 relaxin (R3),  $\Delta R3/I5$  and analogue **14**: These peptides have two chains (A and B) with three disulfide bonds (Table 1). Both the individual A- and B-chains were obtained in high yields and purity. We have used orthogonal protecting groups for cysteine

residues. Each of the three disulfide bond was formed as per our previously reported protocol<sup>8,9</sup>. The analogues were identified by MALDI-TOF MS. Previously reported control peptide  $\Delta R3/I5$  was made for this study and others controls **H3 relaxin** and **INSL5** were previously made and used for this study.  $\Delta R3/I5$ : m/z 4847.747 [M + H]<sup>+</sup>, calcd. 4851.71; analogue **14** m/z 4807.084 [M + H]<sup>+</sup>, calcd. 4808.69 (Supplementary Information).

Minimized analogues **2**, **13**, **15**, **16**, **17**, **18**, **19** and **20**: We have used orthogonal protecting groups for cysteine residues and made two disulfide bridges between the A- and B-chain using our recently developed protocol<sup>11,30</sup> that resulted in **15**, **16**, **17**, **18**, **19** and **20**. The analogues were identified by MALDI-TOF MS; **2**: m/z 4545.262 [M + H]<sup>+</sup>, calcd. 4537; **13**: 4263.929 [M + H]<sup>+</sup>, calcd. 4257; **15**: m/z 4064.187 [M + H]<sup>+</sup>, calcd. 4064.8; **16**: m/z 4084.635 [M + H]<sup>+</sup>, calcd. 4081; **17**: m/z 4102.015 [M + H]<sup>+</sup>, calcd. 4099; **18**: m/z 4020.433 [M + H]<sup>+</sup>, calcd. 4021.77; **19**: m/z 4102.603 [M + H]<sup>+</sup>, calcd. 4100; **20**: m/z 4066.955 [M + H]<sup>+</sup>, calcd. 4065. Previously reported analogues **2** and **13** were chemically prepared for this study (Supplementary Information). The purity of each of the peptides ( $\geq 95\%$ ) was determined by using analytical RP-HPLC peak area integration (Supplementary Information).

**Functional assays.** *Binding assays.* Chinese hamster ovary CHO-K1 cells (sourced from American Type Culture Collection (ATCC), Maryland, USA) stably transfected with RXFP3<sup>33</sup> or RXFP4<sup>9</sup> were plated out onto pre-coated poly-L-lysine 96-well view plates at a density of 50 000 cells per well. Medium was aspirated off and the cells were washed with phosphate-buffered saline (PBS) before competition binding assays were performed with 5 nM Eu-H3B1–22R<sup>34</sup> (RXFP3 ligand) or 5 nM Eu-DTPA-mINSL5<sup>9</sup> (RXFP4 ligand) as previously described. Competition binding curves were performed in triplicate and each experiment was performed independently at least three times. Fluorescent measurements were carried out on a BMG POLARstar plate reader (BMG Labtech, Melbourne, Australia). Pooled data are presented as mean  $\pm$  S.E of specific binding and are fitted using one-site binding curve in GraphPad Prism version 8 (GraphPad Inc., San Diego, USA). Statistical analyses were conducted using one-way analysis of variance with uncorrected Fisher's least significant difference (LSD) post-hoc analysis in GraphPad Prism version 8.

*cAMP (agonism or antagonism) assays.* The peptides were tested for their ability to inhibit forskolin induced cAMP activity in CHO-K1-RXFP3 or CHO-K1-RXFP4 cells transfected with a pCRE (cAMP Response Element)  $\beta$ -galactosidase reporter plasmid as previously described<sup>9,34</sup>. For agonist assays cells were stimulated with forskolin (5  $\mu$ M for RXFP3, 1  $\mu$ M for RXFP4) plus or minus increasing concentrations of each peptide for 6 hours. For antagonist assays cells were stimulated with forskolin as in agonist assays plus increasing concentrations of each peptide for 10 minutes followed by stimulation with agonist at an  $\sim$ EC80 concentration for a further 6 hours (10 nM Analogue 2 for RXFP3 and 16.6 nM Analogue 13 for RXFP4). Each peptide was tested in triplicate and each experiment was performed independently at least three times. Agonist data was expressed as the % Forskolin activity whereby 100% was defined as Forskolin alone and 0% as maximum agonist stimulation (1  $\mu$ M Analogue 2 for RXFP3 and 1  $\mu$ M Analogue 13 for RXFP4). Antagonist data was expressed as % inhibition whereby 0% was defined as agonist alone and 100% Forskolin alone. Data were analysed and plotted using GraphPad PRISM 8 and are expressed as the mean  $\pm$  SEM of the pooled data. Statistical analysis was conducted using one-way ANOVA with Uncorrected Fisher's LSD post-hoc analysis in GraphPad Prism version 8.

**Circular dichroism (CD) spectroscopy.** CD spectra data of all peptides were recorded using a JASCO J-800 spectrophotometer at 25 °C using 1 mm path length cell. The peptides were dissolved in 10 mM phosphate buffer at pH 7.5. The parameters used to obtain the spectra were wavelengths 190 to 260 nm with a data pitch of 0.1 nm, continuous scanning mode at a speed of 50 nm per minute and the number of accumulations taken per peptide was 3. The concentration of peptide used was 0.2  $\mu$ g/ $\mu$ l.

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## Author contributions

P.P. synthesized the peptides and conducted the experiments. P. P. analyzed data. R.A.D.B. and M.A.H. conceived experiments and analyzed the data. P.P., R.A.D.B. and M.A.H. wrote the manuscript. All authors have given approval to the final version of the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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## Total Chemical Synthesis of a Nonfibrillating Human Glycoinsulin

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## Supporting Information

**ABSTRACT:** Glycosylation is an accepted strategy to improve the therapeutic value of peptide and protein drugs. Insulin and its analogues are life-saving drugs for all type I and 30% of type II diabetic patients. However, they can readily form fibrils which is a significant problem especially for their use in insulin pumps. Because of the solubilizing and hydration effects of sugars, it was thought that glycosylation of insulin could inhibit fibril formation and lead to a more stable formulation. Since enzymatic glycosylation results in heterogeneous products, we developed a novel chemical strategy to produce a homogeneous glycoinsulin (disialo-glycoinsulin) in excellent yield (~60%). It showed a near-native binding affinity for insulin receptors A and B *in vitro* and high glucose-lowering effects *in vivo*, irrespective of the route of administration (*s.c.* vs *i.p.*). The glycoinsulin retained insulin-like helical structure and exhibited improved stability in human serum. Importantly, our disialo-glycoinsulin analogue does not form fibrils at both high concentration and temperature. Therefore, it is an excellent candidate for clinical use in insulin pumps.

Significant recent advances have been made in the development of insulin analogues for the management of diabetes mellitus.<sup>1,2</sup> However, insulin analogues form oligomers and amyloid-type fibrils both *in vitro*<sup>3</sup> and *in vivo*<sup>4</sup> which reduce the shelf life of pharmaceutical preparations. This is especially problematic for patients that use external insulin pumps whereby fibrils can block the insulin delivery catheter potentially leading to underdosing. The scale of the problem with fibrillation of insulin is enormous in terms of wastage of “expired” drug. For example in the US, savings of more than US\$1 billion per year could be realized if insulin expiry dates were increased from just 2 days to 6 days for insulin pump users alone.<sup>5</sup> Therefore, the development of nonfibrillogenic insulin analogues is of paramount importance. We hypothesize that the improved solubility and hydration effects due to glycosylation will prevent fibril formation of insulin analogues.

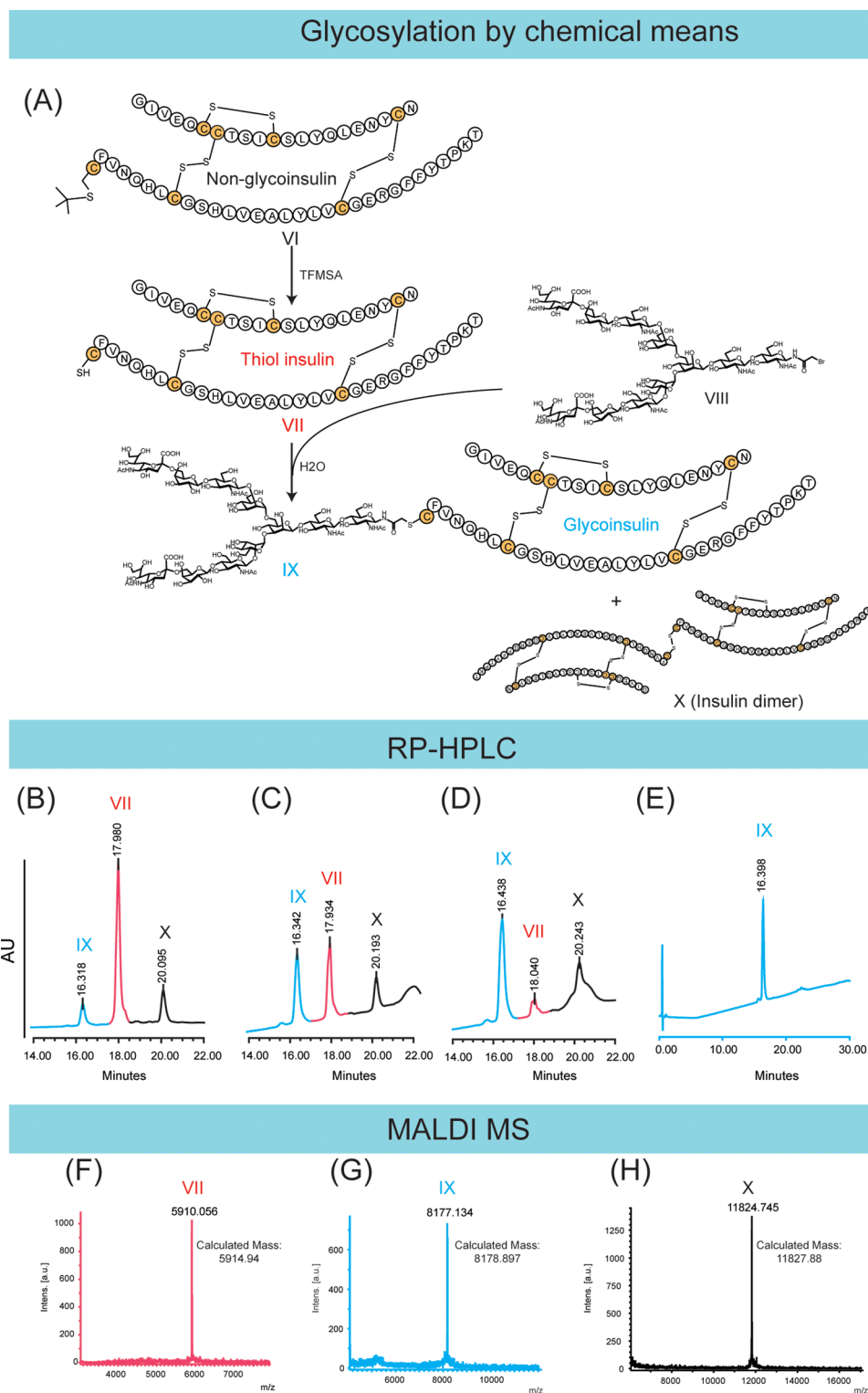
Glycosylation of peptide therapeutics has revolutionized the treatment of many diseases over the past decade.<sup>6–8</sup> Sialylglycosylation (glycosylation with a sialic acid-containing sugar) engineering is particularly attractive, as it was shown to improve the pharmacokinetics of blockbuster biologics including darbepoietin alpha.<sup>7,9</sup> Therefore, we chose to synthesize a sialo-glycoinsulin that is potentially resistant to fibril formation. Improved *in vivo* half-life of such an analogue will be an added advantage. Recently, a low yielding chemical method (yield 0.5%–6.5%) for the synthesis of a nonsialo-glycoinsulin was reported.<sup>10</sup> High yielding enzymatic sialo-glycosylation of insulin, on the other hand, was found to be problematic, as it is difficult to control the sites of glycosylation, resulting in a heterogeneous product that is difficult to characterize and possesses significantly reduced

activity.<sup>11,12</sup> Here, we aimed to overcome this limitation by total chemical synthesis of a designed insulin analogue that can be conjugated with disialo-oligosaccharide with high yield by chemical means.

Insulin is a heterodimeric peptide (A- and B-chain) with six cysteine residues that form three disulfide bridges (three cysteines).<sup>13</sup> The N-terminus of the B-chain of insulin was selected as the glycosylation site for two reasons: (i) it tolerates modification without a significant loss of activity;<sup>14,15</sup> and (ii) N-terminal functionalization of recombinant proinsulin (the single-chain precursor to mature insulin analogues) is amenable to scale-up. Therefore, we incorporated an additional cysteine residue at the N-terminus of the B-chain with temporary *tert*-butyl (tBu) side chain protection to avoid unwanted disulfide shuffling during stepwise folding. The rationale behind using an extra natural amino acid over other functionalities (e.g., alkyne or azido amino acid) is to potentially scale up the modified insulin peptide, if necessary, by a recombinant DNA method. As detailed in the Supporting Information (Figures S1 and S2), the A-chain and modified B-chain with the extra cysteine residue were prepared via continuous flow Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid phase peptide synthesis.<sup>16</sup> Each disulfide bond was formed successively utilizing appropriate S-protecting groups which were subsequently removed in a stepwise fashion<sup>17</sup> resulting in an insulin analogue with one cysteine and three cysteines (VI, nonglycoinsulin, yield ~7% calculated from the B chain; Figure 1A).

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**Figure 1.** Scheme of the synthesis protocol for glycoinsulin: The two chains (A and B) were assembled via Fmoc-SPPS using orthogonal protecting groups for cysteines [Cys(tBu), Cys(Acm), and Cys(Trt)] to enable regioselective sequential three disulfide bond formation in an insulin analogue, nonglycoinsulin (I–VI; Supporting Information Figures S1, S2, S3). (A) The Cys(tBu) residue in the B-chain of nonglycoinsulin (VI) was cleaved by TFMSA that resulted in thiol insulin (VII). Then Br-undecasaccharide derivative (VIII) was reacted with the thiol insulin (VII) in Milli-Q water for 24 h that resulted in glycoinsulin (IX) as the major product and insulin dimer (X) as the side product. We initially tried different buffers at different pH's and with different concentrations of peptides and/or sugars, and the major product in these conditions was found to be an insulin dimer (X; Supporting Information). RP-HPLC: Monitoring of reaction progress at 2 h (B), 8 h (C), and 24 h (D). RP-HPLC of purified glycoinsulin (E). Conditions: Instrument: Semipreparative RP-HPLC Waters 600 coupled with a Waters 996 detector system; Column: Phenomenex C18 column (4.6 mm × 250 mm, pore size 300 Å, particle size 5 μm); Buffer A, 0.1% aq. TFA; Buffer B, 0.1% TFA in CH<sub>3</sub>CN. Gradient = 20–50% B over 30 min. MALDI-TOF MS of thiol insulin (VII), glycoinsulin (IX), and insulin dimer (X).

The S-*t*Bu group of nonglycoinsulin (VI) was cleaved with 10% trifluoromethanesulfonic acid (TFMSA) in trifluoroacetic acid (TFA, Figure 1A). Pleasingly, the resulting thiol insulin analogue (VII) did not scramble and was stable in Milli-Q water (pH  $\approx$  6) and purified as a single species.

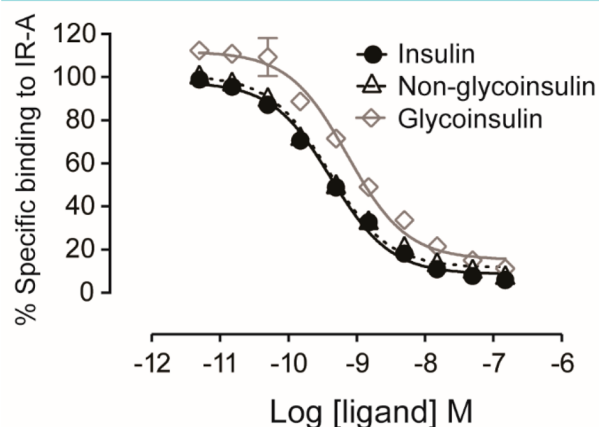
After stepwise formation of the three disulfide bonds (VI) and deprotection of the seventh cysteine residue (VII), we carried out the glycosylation reaction. We used human type sialyloligosaccharide that was isolated from egg yolks<sup>18</sup> and derivatized it to form bromo-acetamidyl undecasaccharide (VIII, Figure 1A) as previously reported.<sup>19</sup> Monolabeled disialo-insulin, glycoinsulin (IX, Figure 1A), was then chemoselectively obtained by reacting bromo-acetamidyl undecasaccharide with the thiol insulin (VII, Figure 1A) via S<sub>N</sub>2 chemistry. To have the maximum yield of glycoinsulin, we optimized the reaction conditions extensively (Supporting Information). The reaction was monitored via analytical reversed phase (RP)-HPLC (Figure 1B–D). The yield of the glycosylation reaction was excellent ( $\sim$ 60%), with the insulin dimer (X, Figure 1A, Figure 1H) being a minor side product. Glycoinsulin, all intermediates, and side products (I–X) were purified using preparative RP-HPLC, and their identity, yield, and purity were confirmed by analytical RP-HPLC and MALDI-TOF mass spectroscopy (Figure 1 and Supporting Information Figure S2). The synthetic glycoinsulin was isolated at high purity (Figure 1E) and with the expected mass (measured MH<sup>+</sup> 8177.13; calcd MH<sup>+</sup> 8178.89) (Figure 1G).

Competition binding assays (Figure 2, as the method in Supporting Information<sup>20</sup>) revealed that the binding affinities of the nonglycoinsulin (VI) for insulin receptor isoform A (Figure 2A) and isoform B (Figure 2B) (IR-A and IR-B) are equal to those of native insulin (Actrapid). The data confirm that modification at the N-terminus of the B-chain is tolerable. There is a 2-fold poorer affinity of glycoinsulin (IX) for both receptors compared to insulin (Figure 2C) which suggests that the disialo modification interferes to a modest extent in the interaction with the IR.

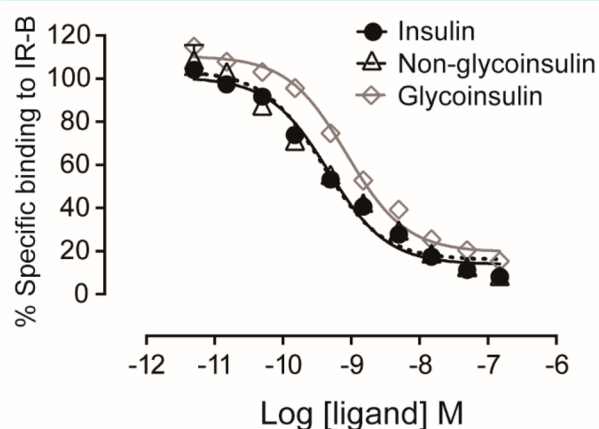
Glycoinsulin (IX) was then injected (intraperitoneally, *i.p.*; Figure 3A and subcutaneously, *s.c.*; Figure 3B) into mice (as the method described in the Supporting Information<sup>21</sup>). The data clearly show that glycoinsulin is active *in vivo* and lowered blood glucose over a 3 h insulin tolerance test (Figure 3). Consistent with *in vitro* binding affinity results, the glucose-lowering efficacy of glycoinsulin seems to be slightly less, although not statistically significant, compared with native insulin (Actrapid). Intriguingly, the glucose-lowering effects of glycoinsulin are shown to be comparable to the long-acting insulin drug (synthetic glargine<sup>22</sup>) as demonstrated by *i.p.* injection data (Figure 3A). However, from this experiment it is difficult to predict if glycoinsulin has a protracted glucose lowering effect compared with nonglycoinsulin or insulin (Actrapid), which also exhibits glargine-like activity.

As hypothesized, time-lapse atomic force microscopy (AFM) demonstrated that insulin forms fibrils at all time points tested at low and high concentrations (50  $\mu$ M and 200  $\mu$ M, Figure 4A and Figure 4B), but no fibrils were detected with glycoinsulin over the same time period (even at 200  $\mu$ M, Figure 4C). Furthermore, glycoinsulin does not form fibrils even at 60  $^{\circ}$ C (Figure 4). There is a clear market for such thermostable analogues,<sup>23,24</sup> particularly for people living in poor tropical regions where reliable refrigeration is often not ubiquitous.

### (A) Eu-Insulin competition binding to IR-A



### (B) Eu-Insulin competition binding to IR-B

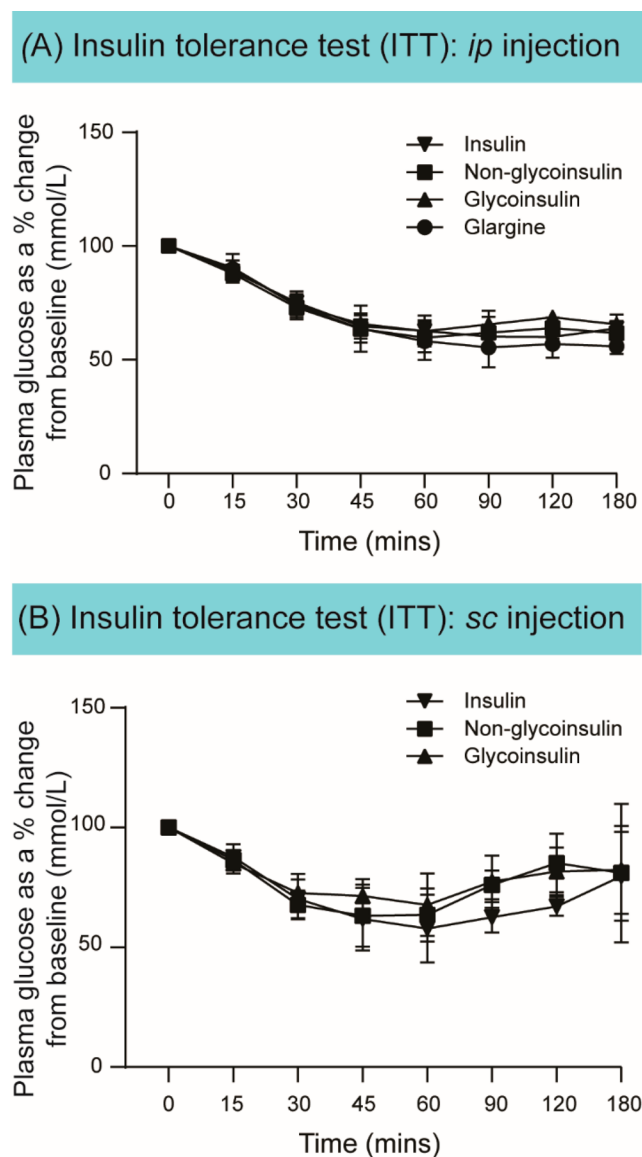


| (C)              | IR-A<br>IC <sub>50</sub> (nM) | IR-B<br>IC <sub>50</sub> (nM) |
|------------------|-------------------------------|-------------------------------|
| Insulin          | 0.52 $\pm$ 0.09               | 0.43 $\pm$ 0.01               |
| Non-glycoinsulin | 0.60 $\pm$ 0.11               | 0.40 $\pm$ 0.06               |
| Glycoinsulin     | 1.09 $\pm$ 0.34               | 0.89 $\pm$ 0.33               |

**Figure 2.** Glycoinsulin exhibits strong binding to insulin receptor (IR) isomers. Competition binding of nonglycoinsulin and glycoinsulin to (A) the IR-A and (B) IR-B. Immunocaptured solubilized IR-A and IR-B were incubated with europium labeled-insulin in the presence or absence of increasing concentrations of insulin (Actrapid), nonglycoinsulin, and glycoinsulin. Results are expressed as a percentage of binding in the absence of competing ligand (%B/Bo), and the data points are the mean  $\pm$  SEM. IC<sub>50</sub> values are shown in the table below. Nonglycoinsulin and glycoinsulin curves are from four separate experiments with each point performed in triplicate. Error bars are shown when greater than the size of the symbols.

Modifications can cause conformational changes to the peptides that can expose sensitive amide linkages to proteases.<sup>25</sup> Therefore, we first analyzed the secondary structure of glycoinsulin by circular dichroism (CD) spectrometry (Supporting Information). The CD spectra

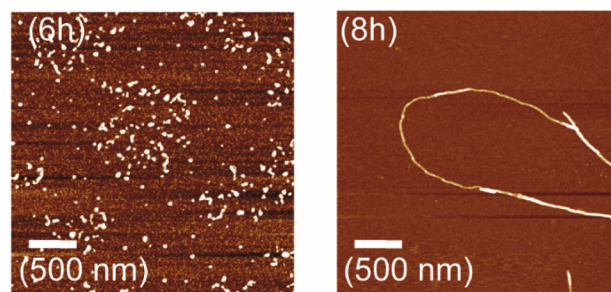




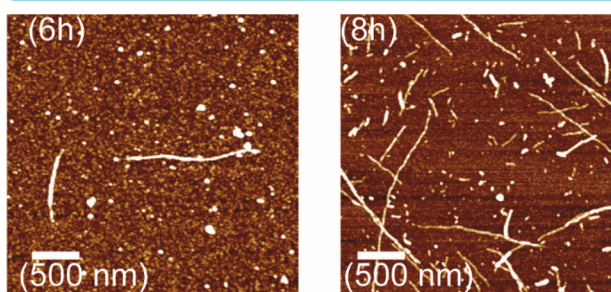
**Figure 3.** Glucose lowering effects of glycoinsulin *in vivo* are comparable to those of native insulin as measured by the insulin tolerance test. Synthetic or commercially obtained insulin (Actrapid) was injected (A; *i.p.*) or (B; *s.c.*) in 8–10-week-old male C57BL/6 mice at 0.75 unit/kg body weight, and blood glucose levels were determined over 180 min. Results are presented as mean  $\pm$  SEM ( $n = 4–5$ ). There was no significant difference between the peptides at any time point in terms of glucose lowering activity. Data analyzed by two-way ANOVA, followed by Tukey's multiple comparison test (GraphPad Prism8.2.1).

showed a typical  $\alpha$ -helical pattern with double minima at 208 and 222 nm (Figure 5A). The mean residual ellipticity at 222 nm,  $[\theta]_{222}$ , was used to calculate the helix content.<sup>26</sup> The  $[\theta]_{222}$  values for glycoinsulin and human insulin were found to be  $-10564.677$  and  $-11115.7$  which correspond to an  $\alpha$ -helix content of 30% and 31%, respectively. Clearly, there was no observable perturbation in the secondary structure of glycoinsulin compared with native human insulin. Finally, the *in vitro* serum stability of glycoinsulin was examined (Supporting Information) and compared with native human insulin for 72 h at 37 °C (Figure 5B). The preliminary data show that disialo-glycosylation confers increased serum stability which may be due to either or both direct shielding

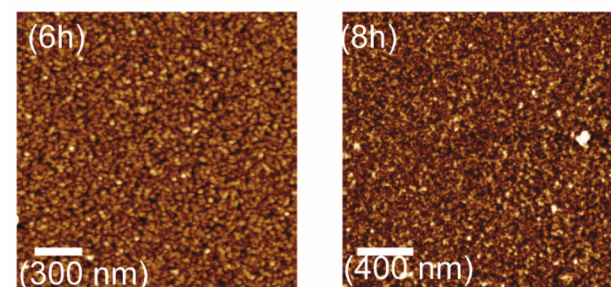
(A) AFM images of 50  $\mu$ M insulin solution



(B) AFM images of 200  $\mu$ M insulin solution



(C) AFM images of 200  $\mu$ M glycoinsulin solution

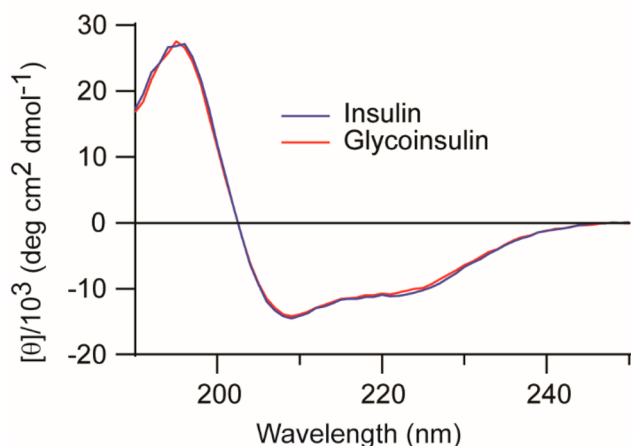
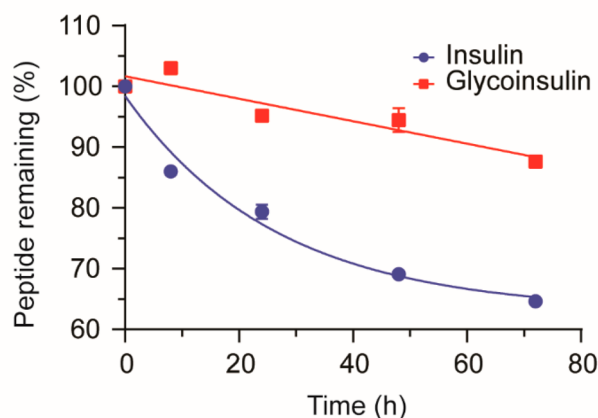


**Figure 4.** Glycoinsulin does not form fibrils. Representative AFM height images of (A) 50  $\mu$ M (Actrapid) and (B) 200  $\mu$ M insulin solution incubated at 60 °C for 6 and 8 h. (C) AFM height images of 200  $\mu$ M glycoinsulin incubated at 60 °C for 6 and 8 h. The vertical color scale is 10 nm. Scan size:  $3 \times 3 \mu\text{m}^2$ . Each of the above images are representative of 3–5 scans at different locations with varying scan sizes from 1 to 10  $\mu\text{m}$ .

from proteolytic enzymes by the glycan or increased binding to serum albumin. Our structural and serum stability data fit well with high affinity IR binding *in vitro* and glucose lowering effects *in vivo*. The slight decrease in the binding affinity is likely due to steric effects from the bulky glycan that is close to the binding site.

In conclusion, we have designed and successfully synthesized a homogeneous, monoglycosylated insulin analogue that exhibits high activity both *in vitro* and *in vivo*. Importantly, we show, for the first time, that disialo-glycoinsulin (IX) does not form fibrils at both high concentration and temperature. The disialo-glycoinsulin has a similar insulin-like fold and exhibits improved stability in human serum. This analogue is

## (A) Circular dichroism spectroscopy study

(B) Serum stability study *in vitro*

**Figure 5.** Glycoinsulin retains native structural fold and is more stable in human serum *in vitro*. (A) Circular dichroism spectra of insulin and glycoinsulin in phosphate buffer (pH 7.5) at 25 °C. (B) Serum stability assay of insulin and glycoinsulin at 37 °C. There were significant differences between the peptides at any time point ( $P$  value = 0.0216,  $t$  test). One-phase exponential decay and paired  $t$  test analyses were performed with GraphPad Prism 8.0.2.

thus an attractive lead candidate for potential use in insulin pumps. Regarding fibril formation for an extended time period (up to 7 days), structural (e.g., NMR) and *in vivo* stability studies (e.g., PK) will be carried out in due course as will alternative placement of the monodisialo moiety elsewhere within the insulin molecule. Given that scalable production of sialo-glycoinsulin will be achieved by continuing improvements in solid-phase peptide synthesis, new ligation or conjugation methods, and chemical means of oligosaccharide assembly, chemical synthesis is likely to be of practical value. However, our discovery that thiol insulin (VII) with a single cysteine and three cysteines is stable indicates that our nonfibrillating glycoinsulin can be produced at large scale via recombinant DNA methods and chemically directed glycosylation. Moreover, the discovery of the stable and active thiol insulin analogue (VII) also opens up a new area of insulin drug development such that scientists will be able to attach pharmacophores (e.g., fatty acid or polyethylene glycol) through thiol-maleimide or thioether chemistry to improve

the *in vivo* half-life of insulin and analogues for better and efficient treatment of diabetes.

## ■ ASSOCIATED CONTENT

## S Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.9b11424>.

Description of peptide synthesis, regioselective disulfide bond formation, optimized conditions for glycosylation and summary of peptide characterization by MALDI TOF MS, RP-HPLC, amino acid analysis, methods for insulin tolerance test insulin receptor binding assay, insulin tolerance test, atomic force microscopy fibrillation assay, circular dichroism and serum stability studies (PDF)

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## Notes

The authors declare no competing financial interest.

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# Coatings Releasing the Relaxin Peptide Analogue B7-33 Reduce Fibrotic Encapsulation

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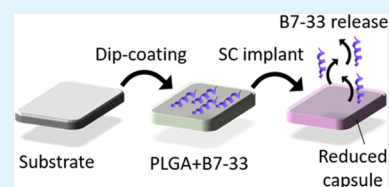
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## Supporting Information

**ABSTRACT:** The development of antifibrotic materials and coatings that can resist the foreign body response (FBR) continues to present a major hurdle in the advancement of current and next-generation implantable medical devices, biosensors, and cell therapies. From an implant perspective, the most important issue associated with the FBR is the prolonged inflammatory response leading to a collagenous capsule that ultimately blocks mass transport and communication between the implant and the surrounding tissue. Up to now, most attempts to reduce the capsule thickness have focused on providing surface coatings that reduce protein fouling and cell attachment. Here, we present an approach that is based on the sustained release of a peptide drug interfering with the FBR. In this study, the biodegradable polymer poly(lactic-co-glycolic) acid (PLGA) was used as a coating releasing the relaxin peptide analogue B7-33, which has been demonstrated to reduce organ fibrosis in animal models. While in vitro protein quantification was used to demonstrate controlled release of the antifibrotic peptide B7-33 from PLGA coatings, an in vitro reporter cell assay was used to demonstrate that B7-33 retains activity against the relaxin family peptide receptor 1 (RXFP1). Subcutaneous implantation of PLGA-coated polypropylene samples in mice with and without the peptide demonstrated a marked reduction in capsule thickness (49.2%) over a 6 week period. It is expected that this novel approach will open the door to a range of new and improved implantable medical devices.

**KEYWORDS:** antifibrotic, relaxin, B7-33, medical devices, fibrotic encapsulation, coatings



## INTRODUCTION

The effectiveness of current indwelling biomedical devices and sensors is often influenced by the inherent foreign body response (FBR).<sup>1,2</sup> The FBR involves a complex series of cellular cascades initialized upon implantation of a material commencing with protein adsorption and complement activation, which leads to cell adhesion, inflammation, macrophage fusion, fibrosis, and finally encapsulation.<sup>3</sup> The resulting collagenous capsule serves to physically and chemically isolate the foreign material, severely limiting communication and diffusion of molecules and gases.<sup>4</sup> While this is a very useful response considering the many foreign objects that are accidentally implanted, this natural response can have detrimental effects to implantable devices. Accumulation of the fibrotic capsule leads to poor analyte diffusion and inaccurate sensing in implantable continuous blood glucose monitors, increased impedance and poor stimulation in muscle stimulation devices, and lack of essential nutrients in implanted

islet cell therapies, leading to cell death.<sup>5</sup> Thus, there is a pressing need to develop materials and coatings that can resist or significantly reduce fibrotic encapsulation and mitigate the FBR with the aim of extending the lifetime of indwelling sensors and devices. Progress in this field will advance the feasibility of existing biosensor and implant technology and open the door to an entirely new class of medical devices including the next generation of real-time continuous health monitoring sensors and electrostimulation devices such as neural implants.

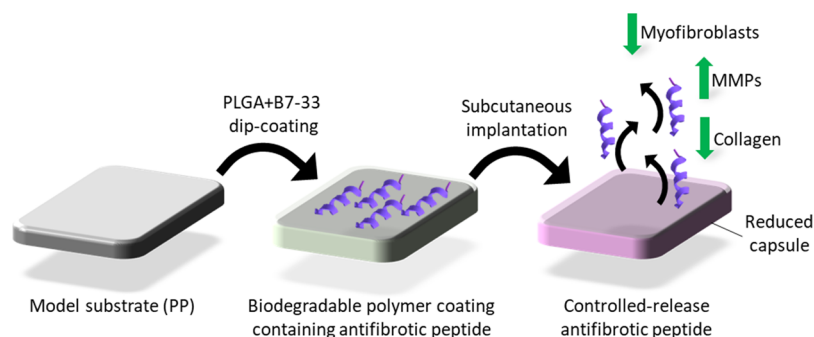
Up to this point in time, the majority of studies in this field have focused on material-based approaches to mitigate the FBR. In particular, nonfouling polymer coatings have been studied as they enable the reduction or prevention of protein

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**Figure 1.** Polypropylene substrates dip-coated with PLGA and the antifibrotic peptide B7-33 reduce fibrotic encapsulation in vivo.

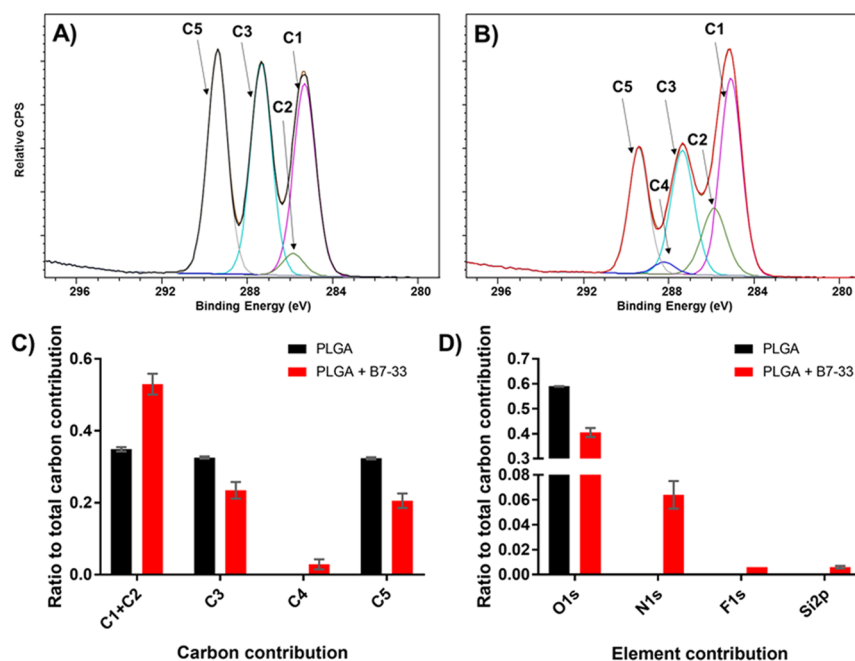
adsorption that is considered to be the first step in the FBR cascade. These polymers act by stabilizing a water layer at the interface,<sup>6</sup> with poly(ethylene glycol),<sup>7</sup> polymer brushes,<sup>8</sup> and zwitterionic polymers<sup>9–11</sup> being the most popular examples. Additionally, high-throughput screening methods have revealed novel polymer constructs such as cationic poly( $\beta$ -amino alcohol)<sup>12</sup> and alginate biopolymer derivatives<sup>13–15</sup> that have had some success in mitigating the FBR. The zwitterionic hydrogels<sup>10</sup> and the triazole-functionalized alginate hydrogels<sup>14</sup> are the most successful materials to date, and although they demonstrate a substantially reduced FBR over 3–6 months, these materials are not sufficiently robust to act as medical device coatings. Subsequently, material approaches alone are insufficient to overcome medical device-related FBR, and a reliable material-based approach has not been described. Drug-eluting stents provide an example for drug delivery coatings in biomedical devices, and this strategy has been employed for biochemical modulation of the FBR to address implant-associated fibrotic encapsulation. Poly(lactic-co-glycolic acid) (PLGA) microspheres have been used for drug delivery of a mast cell-directed tyrosine kinase inhibitor, masitinib, suspended in a poly(ethylene glycol)/poly(ethylene oxide) gel carrier coating. This method offered a 70% reduction of the capsule thickness around a subcutaneous mouse model implant after 4 weeks in initial experiments.<sup>16</sup> However, subsequent work revealed that the FBR persisted in mast cell-deficient mice<sup>17</sup> and that this method offered negligible improvement in an implantable glucose sensor performance evaluation.<sup>18</sup> In another study, PLGA microspheres containing corticosteroid dexamethasone dispersed in a poly(vinyl alcohol) (PVA) hydrogel were used to prevent the fibrotic capsule around a dummy sensor implant in rats over a 1 month period.<sup>19</sup> Later, dexamethasone was used in combination with vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) to improve angiogenesis for blood glucose sensor applications.<sup>20</sup> A recent study<sup>5</sup> employed a novel method of incorporating the crystallized colony-stimulating factor 1 receptor (CSF1R) inhibitor GW2580 into alginate spheres. This method effectively eliminated fibrotic encapsulation when implanted in the intraperitoneal space or subcutaneously for 6 months in nonhuman primates. This method was also used to prevent fibrotic encapsulation of a muscle stimulation device and a blood glucose sensor; however, the function of these devices was not improved significantly. Subsequently, it may be necessary to identify more suitable drug candidates that more faithfully remodel the surrounding tissue following device implantation.

In a related field, reducing organ fibrosis remains one of the greatest current clinical needs.<sup>21–23</sup> Approximately 150

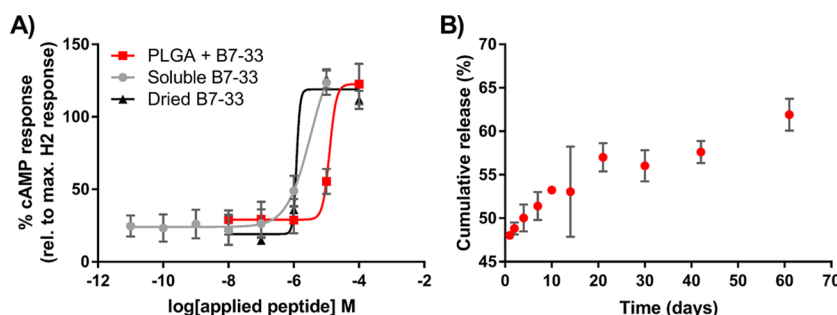
antifibrotic small molecules that target a range of receptors and signaling cascades have been identified.<sup>24</sup> In addition to small molecule drug candidates, the human peptide hormone relaxin is known to regulate tissue remodeling during pregnancy.<sup>25</sup> Additionally, relaxin has demonstrated ability to resolve tissue fibrosis in multiple organ models in mice.<sup>26,27</sup> Relaxin exerts its effect by upregulating matrix metalloproteinases (MMPs) and decreasing fibroblast proliferation thereby increasing collagen degradation and reducing collagen deposition.<sup>26</sup> Importantly, relaxin attenuates profibrotic stimuli and promotes the dedifferentiation of fibrotic myofibroblasts back to fibroblasts and has antioxidant, anti-inflammatory, and angiogenic properties that aid wound healing.<sup>26</sup> Additionally, recombinant relaxin has demonstrated an excellent safety profile in phases 1 and 2 clinical trials for the treatment of acute heart failure.<sup>28,29</sup> However, the relaxin peptide is difficult to access due to the fact that it is composed of two peptide chains that are joined by disulfide linkages. Subsequently, single-chain peptide mimics of relaxin have recently been developed, which can be prepared using solid-phase synthesis at a much lower cost.<sup>30,31</sup> In particular, a truncated B-chain analogue of relaxin known as B7-33 has demonstrated antifibrotic activity similar to the native relaxin in multiple animal models of organ fibrosis.<sup>32</sup> Due to solubility issues with truncated B-chains, the B7-33 single-chain peptide sequence was optimized to improve solubility by the addition of the KRSL sequence to the C-terminus, resulting in a peptide with a +5 positive charge.<sup>32</sup> Given that electrostatic interactions between PLGA and positively charged peptides are known to generate useful materials,<sup>33</sup> coatings prepared from PLGA and B7-33 may have attractive release properties for extending coating efficacy and longevity in vivo.

Despite the successes of relaxin and B7-33, this family of RXFP1 agonists has not been investigated in a materials context for reducing the FBR at an implant surface. This may be due to the fact that systemic administration of the peptide results in rapid renal clearance, with a half-life of only minutes.<sup>34</sup> In the context of organ fibrosis, this can be overcome by continuous infusion with a minipump.<sup>32</sup> We reasoned that the reduced rate of diffusion in the tissue surrounding a subcutaneous implant, combined with the controlled release of the peptide from a polymer coating, may provide biologically relevant peptide concentrations that generate a useful antifibrotic effect at the implant site.

In this study, we demonstrate that B7-33 incorporated into a PLGA coating retains activity against the relaxin family peptide receptor 1 (RXFP1) in vitro. Further, we demonstrate that this coating reduces fibrotic encapsulation around a model implant



**Figure 2.** XPS of PLGA and PLGA+B7-33 coatings. High-resolution C 1s spectra of (A) PLGA and (B) PLGA+B7-33. Carbon contributions are shown with C1 (C–C, C–H), C2 (secondary chemical shifts), C3 (C–O), C4 (C=O, N–C=O), and C5 (O–C=O). Spectra are normalized to the C1 intensity at 285.0 eV. Quantification of (C) the carbon contributions and (D) the elemental composition of the coatings relative to the total carbon contribution. Trace contributions from Na, Cl, and S were observed.



**Figure 3.** Peptide receptor activity and release rate. (A) An in vitro reporter gene assay for cyclic adenosine monophosphate (cAMP) activity in human embryonic kidney cells stably expressing the relaxin family peptide receptor 1 (HEK-RXFP1) demonstrates the dose-dependent response of the applied peptide for PLGA+B7-33 surface coatings (red), PLGA-free B7-33 controls (black), and B7-33 solution controls (gray). cAMP response relative to maximum H2 relaxin response (0.1 nM). Samples were prepared in triplicate, and the data shown is the mean  $\pm$  standard deviation. Data is fit with a sigmoidal four-parameter logistic regression. (B) Cumulative peptide release from PLGA+B7-33 surface coatings over 61 days as measured using a BCA protein quantification kit. The optical density for each value was normalized to the B7-33 peptide control (i.e., maximum release). Samples were prepared in duplicate, and the data shown is the mean  $\pm$  standard deviation.

in a subcutaneous in vivo mouse model by approximately 50% over a period of 6 weeks.

## RESULTS

**PLGA+B7-33 Surface Coating Preparation and XPS Characterization.** We developed a simple method for the incorporation and controlled release of an antifibrotic peptide, B7-33, from a polymer coating. PLGA is a widely used and well-characterized biodegradable polymer with tunable release characteristics,<sup>16</sup> and we reasoned that a PLGA coating would offer a mechanism to deliver the B7-33 peptide locally to the implant site at biologically relevant concentrations. We hypothesized that the peptide would mediate the deposition and degradation of collagen around an implant and would therefore offer the ability to reduce fibrotic capsule formation in vivo (Figure 1).

X-ray photoelectron spectroscopy (XPS) was used to characterize the surface composition of silicon wafers coated with PLGA in the absence and presence of B7-33. The high-resolution C 1s spectra (Figure 2A) confirmed the presence of the PLGA coating with the expected, approximately equal contributions from C1 + C2 (C–C, C–H, and secondary shifts, 285.0 and 285.5 eV), C3 (C–O, 287.3 eV), and C5 (O–C=O, 289.5 eV). For the PLGA+B7-33 coatings (Figure 2B), the ratio of C1 + C2 to both C3 and C5 was increased relative to the PLGA coatings as would be expected with an increase in C–H contributions from the peptide. Quantification of the carbon contributions is shown in Figure 2C.

As expected, when B7-33 was incorporated in the PLGA coating, a reduction in the oxygen-to-carbon (O/C) ratio and an increase in the nitrogen-to-carbon ratio (N/C) for the PLGA+B7-33 relative to PLGA coatings alone were observed, consistent with the presence of the peptide (Figure 2D). A

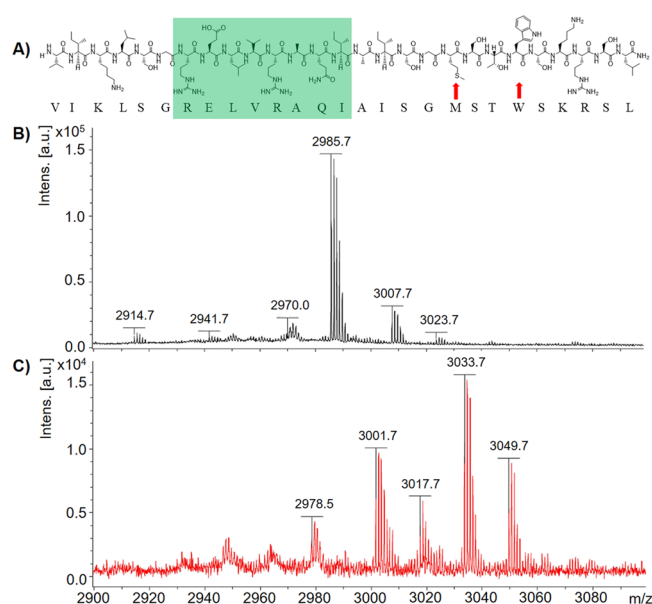
small amount of fluorine was noted, residual from the preparation of the peptide with trifluoroacetic acid (TFA) salt.

**PLGA+B7-33 Cell Assay Assessment.** Satisfied that our method could be used to coat substrates with PLGA+B7-33 coatings, we investigated whether the B7-33 peptide retained its activity against the native relaxin RXFP1 receptor after the coating process. For ease of handling, PLGA+B7-33 coatings were prepared into polypropylene 96-well plates. The coatings were interrogated with an in vitro reporter gene assay for cyclic adenosine monophosphate (cAMP) activity in human embryonic kidney cells stably expressing the relaxin family peptide receptor 1 (HEK-RXFP1) (Figure 3A). The B7-33 peptide solution control (gray) and the PLGA-free dried peptide control (black) exhibited equivalent  $EC_{50}$  at a concentration of 1  $\mu$ M, consistent with our earlier work.<sup>32</sup> Importantly, this indicated that the process used to prepare coatings (i.e., drying the peptide in the wells) did not adversely affect the activity of the peptide against the RXFP1 receptor. Equally importantly, a 10-fold reduction in  $EC_{50}$  for the PLGA+B7-33 coatings was observed as compared to B7-33 alone indicating that the PLGA could be used to retain a portion of the peptide without compromising the biological activity of the peptide.

**Peptide Release Quantification.** The peptide release kinetics of the PLGA+B7-33 surface coatings were assessed in the 96-well plate format using a colorimetric bicinchoninic acid (BCA) protein quantification kit (Figure 3B). During the first day of the in vitro peptide release study, an initial burst release of 48% of the peptide was observed relative to the B7-33 peptide control sample (i.e., in the absence of the polymer). Over the course of 61 days, a total of 62% of the peptide was released from the PLGA+B7-33 surface coating corresponding to a peptide release rate of approximately 2.4 ng/day. The PLGA polymer control in the absence of the peptide gave a negligible signal.

**Peptide Characterization with HPLC-MS and MALDI MS.** To investigate the properties of the B7-33 peptide released from the PLGA+B7-33 surface coatings, the coatings were incubated with Milli-Q water at 37 °C for 6 h and analyzed by high-performance liquid chromatography–mass spectrometry (HPLC-MS) operating in positive-ion electrospray ionization mode. The B7-33 peptide control sample (10  $\mu$ M in water) returned the molecular ion at 2985.7 Da and associated molecular ions at 996.25, 747.44, and 598.15  $m/z$  corresponding to the  $[M + 3H]^{3+}$ ,  $[M + 4H]^{4+}$ , and the dominant  $[M + 5H]^{5+}$  fragments, respectively (Figure S1).

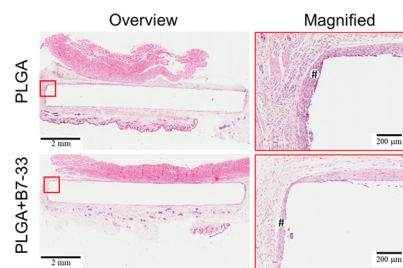
Interestingly, for the peptide samples released from the PLGA+B7-33 coatings and from the dried B7-33 only controls, the expected dominant  $[M + 5H]^{5+}$  molecular ion from B7-33 at 598.15  $m/z$  was not observed and instead the major peak was observed at 607.7  $m/z$ . With the reasonable assumption that this peak was the  $[M + 5H]^{5+}$  molecular ion, this corresponded to a species with a molecular weight of 3033.7 Da. To confirm this finding, the matrix-assisted laser desorption ionization (MALDI) mass spectra of the B7-33 control in water and B7-33 resuspended in water after drying in the well plate (i.e., dried B7-33 only control) were compared (Figure 4). It was observed that the peptide prepared in water had the dominant molecular ion at 2985.7 Da as expected; however, MALDI MS of the resuspended dried B7-33 control exhibited four major species and the absence of the molecular ion at 2985.7 Da. The four species are interpreted as corresponding to oxidized species of B7-33 at  $[M + H]^+$



**Figure 4.** Peptide characteristics. (A) B7-33 peptide structure relaxin-binding cassette in green and potential oxidation points (red arrows). (B) MALDI characterization of B7-33 peptide in water shows the B7-33 molecular ion at 2985.7 Da. (C) By comparison, peptide recovered (in water) from dried B7-33 samples shows oxidation peaks at 3001.7, 3017.7, 3033.7, and 3049.7 Da.

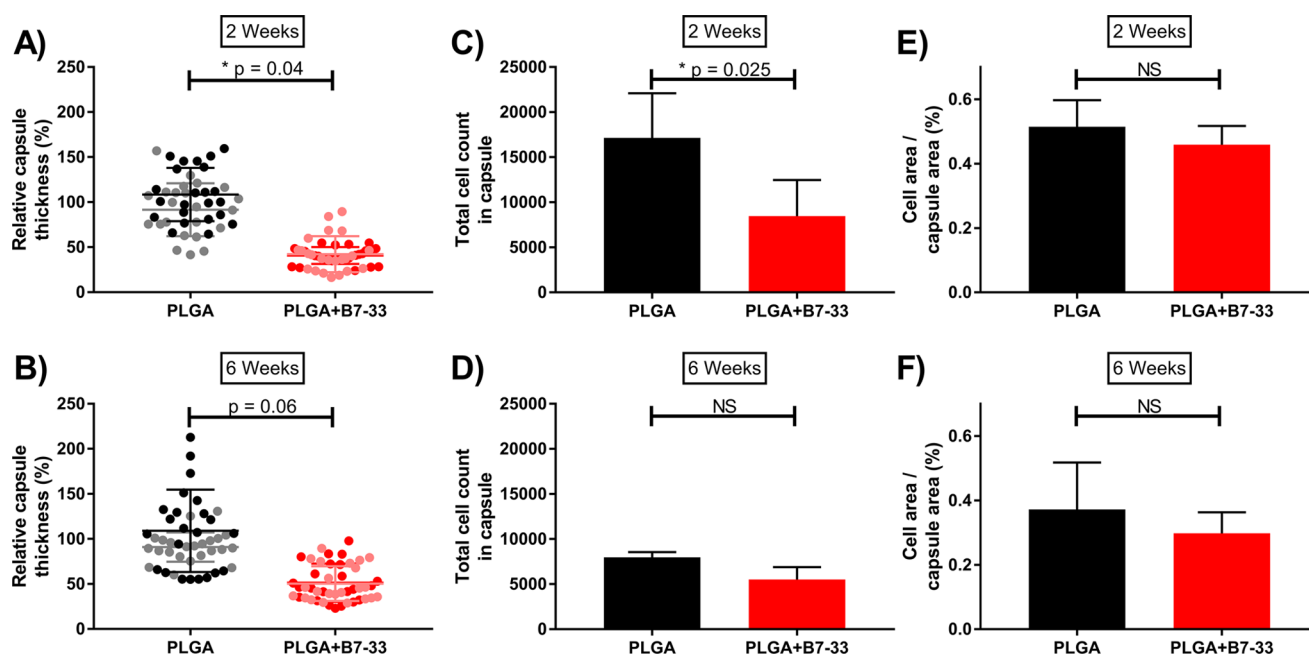
+16, 32, 48, and 64 (see Figure S2 for details), which was not unexpected given the presence of methionine and tryptophan.<sup>35</sup> Oxidation of the native H2 relaxin has also been documented with supporting evidence that it retains receptor recognition,<sup>36</sup> and importantly, as demonstrated by the earlier in vitro HEK-RXFP1 cell assay (Figure 3A), the B7-33 indeed retains activity against the RXFP1 receptor. This is not surprising as the relaxin-binding cassette responsible for receptor recognition (RXXXXXXI, shown in green) is distanced from the methionine and tryptophan susceptible to oxidation (red arrows) (Figure 4A).

**In Vivo Subcutaneous Assessment of PLGA+B7-33-Coated Samples.** Polypropylene (PP) model implants coated with PLGA or PLGA+B7-33 were implanted subcutaneously in mice for periods of 2 and 6 weeks (within the time frame of continuous release identified from our in vitro studies). The implants were then excised and sectioned to determine the thickness of the resulting fibrotic capsule. Histology images of sectioned explants are shown in Figure 5. Remarkably, the PLGA+B7-33 coating demonstrated a significant reduction in the capsule thickness relative to the PLGA coating alone



**Figure 5.** Subcutaneous explants of polypropylene model implants coated with PLGA or PLGA+B7-33 at the 6 week time point. Fibrotic capsule formation is shown by a number sign.





**Figure 6.** (A, B) Quantification of the fibrotic capsule thickness. Data were normalized to PLGA control mean. Twenty-three thickness measurements were taken for each of the biological duplicates (shown as light or dark) of each sample type, and the mean  $\pm$  standard deviation was shown. Quantification of (C, D) total cell count in the implant capsule and (E, F) cell area normalized to total capsule area. Single large-area analysis conducted for biological duplicate samples with the mean  $\pm$  standard deviation is shown. NS means not significant.

(Figure 6A,B). After 2 weeks, the capsule thickness was reduced by 58.5%, from  $250.7 \pm 11.2 \mu\text{m}$  for the PLGA control to  $104.0 \pm 5.7 \mu\text{m}$  for the PLGA+B7-33 coating. After 6 weeks, the capsule thickness was reduced by 49.2%, from  $107.8 \pm 5.6 \mu\text{m}$  for the PLGA control to  $54.8 \pm 3.2 \mu\text{m}$  for the PLGA+B7-33 coating. Interestingly, the total cell count within the capsule also exhibited a significant reduction for the PLGA+B7-33 coatings of 50.5% after 2 weeks and 30.8% after 6 weeks, compared to the control PLGA coatings (Figure 6C,D, respectively). Automated image analysis was used to quantify the total fibrotic capsule area and revealed that the proportion of the cell area to the capsule area was equivalent for all samples at both time points (Figure 6E,F). This is not an unexpected result as a higher cell number will produce more extracellular matrix leading to an increase in the capsule area.

## DISCUSSION

Here, we have demonstrated a robust technique to generate antifibrotic and biodegradable coatings from PLGA and the B7-33 peptide. After coating, the peptide retains activity against the RXFP1 receptor, as exemplified in an *in vitro* reporter gene assay for cAMP in a HEK-RXFP1 cell line, although it was observed that this process leads to oxidation of the peptide, as confirmed with MALDI. Importantly, when the coating was applied to a model PP substrate and implanted subcutaneously *in vivo* for up to 6 weeks, the capsule thickness was reduced by 49% compared to the PLGA coating control.

The B7-33 peptide exerts the antifibrotic effect observed for our peptide-incorporated coating in-part by the upregulation of MMPs that actively degrade collagen. Additionally, B7-33 is known to attenuate profibrotic stimuli, promote differentiation of fibrotic myofibroblasts back to fibroblasts, and reduce proliferation of fibroblasts, which may explain the decreased cell count at the PLGA+B7-33 interface.

Our observations from the *in vitro* peptide release experiment indicate that approximately 50% of the peptide was released from the coating after the first day. The burst release was then followed by peptide release from the coating for a period of at least 60 days. Systemic administration of the peptide results in rapid renal clearance with a half-life of just minutes.

Additionally, 0.5 mg/kg/day of relaxin (corresponding to circulating concentrations in the serum of 20–40 ng/mL<sup>37</sup>) or the molar equivalent of 0.25 mg/kg/day of B7-33 has been used to exemplify their antifibrotic effect in organ diseases.<sup>32</sup> Clearance from tissue is significantly reduced as compared to clearance from blood; however, the initial burst release of the peptide is unlikely to sustain a biologically relevant peptide concentration for up to 6 weeks. Subsequently, the sustained release of 2 ng/day from the PLGA+B7-33 coatings appears to meet the threshold for a biologically relevant concentration in the small volume around the capsule where the myofibroblast target cells reside. Additionally, it is unclear to what extent the FBR will return after the peptide reserve is depleted. On the one hand, regulating the tissue-remodeling in the initial stages of injury may serve to prevent or mitigate further inflammation stimulated by a mismatch in physicochemical material properties (i.e., stiffness, nanotopography, and surface chemistry). On the other hand, external influences that promote injury may not be avoidable. For example, implant-associated micromotion is recognized to promote injury and fibrotic response<sup>18</sup> and is likely to occur independent of the material type but will depend on the implant size, shape, and location.

Nevertheless, this work provides proof-of-concept that the incorporation of an antifibrotic relaxin peptide analogue into a PLGA surface coating can be used to reduce the fibrotic encapsulation of an implanted biomaterial over a clinically relevant time frame. The authors note that while this is a small

study (with few biological replicates), this work provides compelling evidence to suggest that the B7-33 peptide can be employed in a materials context to help control the FBR at the site of an implant. This study provides a starting point for further investigations exploring the use of relaxin peptide analogues to further reduce and ultimately prevent capsule formation over a clinically meaningful time frame. Additional studies to determine both the duration of peptide release and the concentration of peptide required to achieve optimal FBR reduction will follow. We will also investigate the half-life of the peptide in the implant environment, given the short half-life of the peptide when administered systemically.

Unlike many small molecules investigated in this space, we believe that RXFP1 agonists, including the B7-33 peptide studied here, have the potential to offer an improved wound healing response in the context of medical devices. Relaxin has demonstrated antifibrotic, antioxidant, anti-inflammatory, and angiogenic properties *in vivo* that aid its tissue remodeling and wound healing efficacy.<sup>26</sup> This is likely to be particularly useful for implantable devices where, for example, promoting angiogenesis during healing is necessary to facilitate and maximize blood analyte sensing.<sup>18,20</sup>

## CONCLUSIONS

We demonstrate for the first time that B7-33, a peptide agonist of the RXFP1 receptor, can be incorporated into a simple PLGA coating to reduce fibrotic encapsulation *in vivo*. Our PLGA+B7-33 coating demonstrated continuous peptide release for at least 60 days *in vitro* and in a subcutaneous mouse model reduced the fibrotic capsule surrounding a model implant by 49% after 6 weeks *in vivo*.

Fibrotic encapsulation of implanted biomaterials and sensor devices is a significant hurdle that must be overcome to facilitate advances in current and next-generation therapies. Controlled release of antifibrotic compounds can alter the foreign body response to aid in tissue regeneration, wound healing, and reduction of the fibrotic capsule. This simple and effective method is amenable to scale up for industry processing, which will aid in the translation of the technology. Overall, we expect this work to have significant impact in the field of implantable biomedical sensors and neural interface technologies, with immediate applications in subcutaneous indwelling devices.

## MATERIALS AND METHODS

**Materials.** Greiner Bio-One polypropylene microplates (655201), Resomer RG 756 S poly(D,L-lactide-co-glycolide) (PLGA), and the QuantiPro BCA Assay Kit (QPBCA-1KT) were purchased from Sigma (Australia). Microscope slide tray plates (1345-40) were purchased from MoBiTec GmbH (Germany). Dimethylformamide (DMF) was purchased from Merck (Australia).

**B7-33 Peptide Synthesis.** B7-33 was synthesized as reported previously.<sup>30</sup> Briefly, the peptide was manually assembled on solid phase as C-terminal amide, cleaved by TFA after synthesis, and analyzed and purified using reversed-phase high-performance liquid chromatography via an analytical and preparative columns respectively. The molecular mass of B7-33 was determined by electrospray ionization mass spectroscopy (ESI MS), B7-33:  $m/z$  2986.4  $[M + H]^+$ , calculated 2986.59. The peptide content was quantified using a Direct Detect spectrometer, an infrared-based protein quantitation system. The purity of B7-33 was determined to be 97%.

**PLGA+B7-33 Coatings.** PLGA solutions (5 mg/mL) were prepared in DMF with the desired concentration of the B7-33 antifibrotic peptide. Peptide solutions in DMF (in the absence of

PLGA) were also prepared as controls. The desired solution was dispensed (50  $\mu$ L) into polypropylene well plates and dried under vacuum to <0.01 mbar to remove the residual solvent. Wells were then sealed with an adhesive film seal and stored at 4 °C until use. XPS samples were prepared by cleaning silicon wafers (1  $\times$  1 cm) in a sonication bath (30 min) with 2% v/v RBS detergent (Sigma) and 2% v/v ethanol in Milli-Q water (18.2 M $\Omega$ ·cm). Wafers were then rinsed with excess ethanol and water before being dried with a stream of nitrogen. A PLGA (5 mg/mL) solution containing B7-33 (100  $\mu$ M) was then dispensed (50  $\mu$ L) onto each wafer before drying under vacuum.

**Peptide Quantification.** PLGA+B7-33 coatings were prepared in well plates as above incorporating 10  $\mu$ M B7-33. Peptide solutions in DMF (in the absence of PLGA) were also prepared as controls. Phosphate-buffered saline (PBS, 200  $\mu$ L) was added to each well and incubated at 37 °C. At the desired time points (1–61 days), 150  $\mu$ L was sampled from the appropriate wells in triplicate and dispensed into a storage well plate sealed with an adhesive microplate film seal and stored at 4 °C until the completion of the experiment. The peptide control (in the absence of PLGA) was sampled on the final day, that is, day 61. The QuantiPro BCA Assay Kit was then used as per the manufacturer's instructions in triplicate. The plate was analyzed at 562 nm in a BioTek ELISA plate reader using KC4 software, and the mean and standard deviation of the optical density (OD) value for each triplicate were calculated in Microsoft Excel. Each sample value was then normalized relative to the peptide control sample value.

**HEK-RXFP1 cAMP Reporter Gene Assay.** B7-33 activity at RXFP1 was assessed using a cAMP reporter gene assay as previously described<sup>38</sup> with a slight modification. PLGA B7-33 well plate coatings and peptide controls in the absence of PLGA were compared to solubilized B7-33 solution standards (10  $\mu$ M to 10 pM) and H2 relaxin (0.1 nM). Wells were seeded (25,000 cells per well, 100  $\mu$ L) with dispersed HEK-RXFP1-pCRE reporter cells and incubated for 6 h. Cells were then lysed and the reporter gene stimulated  $\beta$ -galactosidase activity was measured using a colorimetric substrate to evaluate the relative cAMP responses. Ligand-induced cAMP stimulation was expressed as a % of maximal response to H2 relaxin. Each data point was measured in triplicate, and each experiment conducted independently at least two separate times.

**X-ray Photoelectron Spectroscopy.** Surface analysis was conducted using an AXIS Ultra DLD X-ray photoelectron spectrometer (Kratos Analytical, Inc., Manchester, U.K.) fitted with a monochromated Al K $\alpha$  source supplying an X-ray power of 180 W (15 kV  $\times$  12 mA). The hemispherical analyzer operated in the fixed transmission mode and the aperture limited the analysis area to 0.3  $\times$  0.7 mm. The total pressure inside the analysis chamber was maintained below 10<sup>−8</sup> mbar during analysis. All survey spectra were acquired at a pass energy of 160 eV. High-resolution C 1s spectra were acquired at a pass energy of 40 eV. All samples were analyzed at an emission angle of 0° relative to the surface normal. X-ray photoelectron spectroscopy (XPS) spectra quantification and data processing were performed using CASAXPS software version 2.3.16 (CASA Software, Ltd., Teignmouth, U.K.). All elements present were identified from the survey spectra, the atomic concentrations were calculated using integral peak intensities, and the relative sensitivity factors were supplied by the manufacturer. Binding energies are referenced to the aliphatic hydrocarbon peak at 285.0 eV.

**HPLC-MS and MALDI-TOF MS Measurement Methods.** PLGA B7-33 well plate coatings (prepared at 10  $\mu$ M per 96 well as above) were incubated with 100  $\mu$ L of Milli-Q water at 37 °C for 6 h. Aliquots (50  $\mu$ L) were removed from wells and characterized by HPLC-MS and MALDI-TOF MS. First, mass spectrometric analyses were performed on a Thermo Scientific Q Exactive mass spectrometer fitted with an HESI-II ion source. Positive ion electrospray mass spectra were recorded in an appropriate mass range at 140,000 mass resolution. The probe was used with 0.3 mL/min flow of solvent. The nitrogen nebulizing/desolvation gas used for vaporization was heated to 100 °C in these experiments. The sheath gas flow rate was set to 25

and the auxiliary gas flow rate was set to 7 (both arbitrary units). The spray voltage was 3.8 kV and the capillary temperature was 300 °C.

In UHPLC conditions, chromatographic analysis was performed using a Dionex 3000 UHPLC system. The chromatographic conditions were as follows: column: Thermo Hypersil Gold C18 (50 × 2.1 mm, 1.9 μm particle size); mobile phase A: 98% water and 2% methanol with 0.1% formic acid; mobile phase B: methanol with 0.1% formic acid; gradient: 100% mobile phase A to 100% mobile phase B in 8 min and then held for 5 min; flow rate: 300 μL/min; column temperature: 30 °C; sample injection volume: 10 μL.

MALDI measurements were performed on a Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer. For MALDI analysis, the 6 h time point for the peptide control (i.e., no PLGA) was compared to the freshly prepared peptide in Milli-Q water. Buffer and media solutions were avoided to reduce the complexity and maintain the integrity of the peptide mass spectrum.

In sample preparation, from the extracted water solutions, the B7-33 peptide samples were then dissolved in 85% acetonitrile, 15% water, and 0.1% trifluoroacetic acid (TA85) solvent at a concentration of 1 mg/mL. A stock solution of the matrix was prepared by dissolving 10 mg of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) matrix name in 1 mL TA85. The sample and matrix solutions were combined in the ratio of 100 to 10 μL, respectively. A 1 μL aliquot of the mixture was then applied to the PAC (prespotted anchor chip) MALDI plate and air-dried at ambient temperature (20 °C). Measurements were performed at an acceleration voltage of 20 kV, in a positive ion reflectron mode. Suitable values for laser power, gain, and laser shots were determined for each sample to produce the best quality data (best resolution, reduced fragmentation, and ion statistics).

**In Vivo PLGA+B7-33 Sample Preparation.** Polypropylene (PP) samples (1 × 1 cm, 1 mm-thick) were cut from microscope slide tray lids and sonicated in 2% RBS detergent, 2% ethanol, and water solution for 1 h and then rinsed with excess ethanol and Milli-Q water before drying with a stream of nitrogen. To improve wettability, PP samples were subjected to air plasma in a custom built reactor<sup>39</sup> for 30 s each side (frequency, 200 kHz; load power, 20 W; and initial chamber pressure, 0.6 mbar). Plasma-treated PP samples were then dip-coated in a DMF solution of PLGA (5 mg/mL) with or without B7-33 peptide (100 μM) before being dried under vacuum to <0.01 mbar. Samples were then placed in well plates, vacuum sealed, and gamma sterilized at 15 kGy (Steritech, Australia). Samples were then stored at 4 °C until implantation.

**In Vivo Assessment.** All animal husbandry, housing, and experimental procedure were performed as approved by Monash Medical Centre Animal Ethics Committee A (2017/05). Female C57BL/6 mice aged 8–16 weeks were randomly divided into two experimental groups, namely, (i) PLGA and (ii) PLGA+B7-33. The mice were anaesthetized with 3% w/v isoflurane and carprofen (5 mg/kg body weight) was used as analgesia. A longitudinal skin incision was performed in the lower abdomen, and scaffold of 1 × 1 cm size each was implanted into each animal using Ethicon 6-0 suture. All animals were housed in the animal house at Monash Medical Centre according to the National Health and Medical Research Council of Australia guidelines for the care and use of laboratory animals. Mice were housed individually for 1 week followed by four mice per cage until endpoint. Animals were euthanized in a CO<sub>2</sub> chamber, and scaffold tissue areas were harvested at 2 or 6 weeks (2 mice/group/time point) for histological analysis. The harvested tissues were fixed using formalin and embedded in paraffin blocks followed by cutting into 8 μm sections on poly(L-lysine) slides. The sections were stained using hematoxylin and eosin stains by Monash Histology Platform as per established protocols.<sup>40</sup>

**Statistics.** GraphPad Prism (version 7.04) was used to calculate sigmoidal and linear regressions and to calculate statistical significance using the *t*-test analysis function. Statistics were calculated on technical replicates with biological duplicates for each group and time point.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.9b17859>.

HPLC-MS analysis of B7-33 peptide and proposed oxidized forms of B7-33 (PDF)

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N.G.W., M.A.H., J.D.W., R.A.D.B., D.A.W., H.T. and conceived and designed the experiments. P.P. and M.A.H. prepared the B7-33 peptide. S.M. and J.A.W. performed the in vivo experiments and processed the data. N.G.W. performed experiments, interpreted the data and wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

cAMP, cyclic adenosine monophosphate  
CSF1R, colony-stimulating factor 1 receptor  
DMF, dimethylformamide  
FBR, foreign body response  
HEK, human embryonic kidney cells  
HPLC-MS, high-performance liquid chromatography–mass spectrometry  
MALDI, matrix-assisted laser desorption/ionization  
MMP, matrix metalloproteinase  
PDGF, platelet-derived growth factor  
PLGA, poly(lactic-co-glycolic acid)  
PVA, poly(vinyl alcohol)  
RXFP1, relaxin family peptide receptor 1  
TFA, trifluoroacetic acid;



VEGF, vascular endothelial growth factor  
XPS, X-ray photoelectron spectroscopy

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ORIGINAL RESEARCH

# B7-33, a Functionally Selective Relaxin Receptor 1 Agonist, Attenuates Myocardial Infarction–Related Adverse Cardiac Remodeling in Mice

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**BACKGROUND:** Human relaxin-2 is a peptide hormone capable of pleiotropic effects in several organ systems. Its recombinant formulation (serelaxin) has been demonstrated to reduce infarct size and prevent excessive scar formation in animal models of cardiac ischemia-reperfusion injury. B7-33, a synthetically designed peptide analogous to B-chain of relaxin-2, invokes signaling at relaxin family peptide receptor 1 (cognate receptor for relaxin-2) by preferentially phosphorylating the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2. We sought to investigate the effects of B7-33 treatment post ischemia-reperfusion injury in mice.

**METHODS AND RESULTS:** Adult male CD1 mice were subjected to ischemia-reperfusion via ligation of left anterior descending artery for 30 minutes, followed by 24 hours or 7 days of reperfusion. Echocardiography was performed to assess cardiac function, and cardiac tissue was stained to determine infarct size at 24 hours. B7-33 significantly reduced infarct size (21.99% versus 45.32%;  $P=0.02$ ) and preserved fractional shortening (29% versus 23%;  $P=0.02$ ) compared with vehicle. The difference in fractional shortening further increased at 7 days post myocardial infarction (29% versus 20% for B7-33 and vehicle groups, respectively). In vitro, primary cardiomyocytes were isolated from adult hearts and subjected to simulated ischemia-reperfusion injury (simulated ischemia reoxygenation). B7-33 (50 and 100 nmol/L) improved cell survival and reduced the expression of GRP78 (glucose regulated protein), an endoplasmic reticulum stress marker. Subsequently, B7-33 (100 nmol/L) reduced tunicamycin (2.5  $\mu\text{g/mL}$ ) induced upregulation of GRP78 in an extracellular signal-regulated kinase 1/2–dependent manner.

**CONCLUSIONS:** B7-33 confers acute cardioprotection and limits myocardial infarction–related adverse remodeling in mice by attenuating cardiomyocyte death and endoplasmic reticulum stress as well as preserving cardiac function.

**Key Words:** adverse cardiac remodeling ■ echocardiography ■ endoplasmic reticulum stress ■ myocardial infarction ■ relaxin

**M**yocardial infarction (MI) is characterized by cessation of blood supply to the cardiac tissue, leading to damage and death of cardiomyocytes within the affected region. Despite the significant reduction in in-hospital mortality because of advances in revascularization techniques,<sup>1</sup> reintroduction of oxygen initiates a cascade of maladaptive signaling within the ischemic tissue.

The resultant pathologic injury, termed ischemia-reperfusion (IR) injury, facilitates proinflammatory changes within the tissue during early stages of infarct healing.<sup>2</sup>

The pleiotropic hormone relaxin (human relaxin 2) has been shown to confer cardioprotection in several animal models of heart disease.<sup>2</sup> Relaxin is the cognate ligand for relaxin family peptide receptor 1

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## CLINICAL PERSPECTIVE

### What Is New?

- The protective effects of B7-33, a functionally selective relaxin family peptide receptor 1 agonist, against ischemia-reperfusion injury are demonstrated in the heart post infarction.
- A novel association between the mitogen-activated protein kinase signaling elicited by B7-33 and the unfolded protein response in the postinfarcted heart is identified.

### What Are the Clinical Implications?

- Despite the benefits of timely reperfusion, there is a paucity of therapeutics that are directly indicated for thwarting adverse remodeling following ischemia-reperfusion injury to the heart.
- The hormone relaxin has been identified to play a role in mitigating the complex pathophysiological characteristics of the postinfarcted heart, and the signaling diversity elicited by alternative ligands at the relaxin receptor 1 (relaxin family peptide receptor 1) represents the potential of unexplored therapeutic strategies.
- In this article, we outline our findings involving B7-33, a relaxin family peptide receptor 1 ligand, in conferring cardioprotection post myocardial infarction.

## Nonstandard Abbreviations and Acronyms

|              |                                                                                 |
|--------------|---------------------------------------------------------------------------------|
| <b>ASC</b>   | apoptosis-associated speck-like protein containing a caspase recruitment domain |
| <b>FS</b>    | fractional shortening                                                           |
| <b>LVID</b>  | left ventricular internal diameter                                              |
| <b>RXFP1</b> | relaxin family peptide receptor 1                                               |
| <b>SIRO</b>  | simulated ischemia reoxygenation                                                |
| <b>TUN</b>   | tunicamycin                                                                     |

(RXFP1), a G-protein-coupled receptor. In most cell types, it generates a biphasic cAMP signal, along with activation of the extracellular signal-regulated kinase (Erk) 1/2 pathway.<sup>3</sup> Interestingly, RXFP1 has been shown to exhibit ligand-specific biased signaling; the allosteric agonist ML290 biases the receptor toward a cGMP (as opposed to the canonical cAMP) response in cardiac fibroblasts.<sup>4</sup> Recently, a synthetic variant of the B-chain of relaxin (B7-33) has been shown to cause RXFP1-mediated Erk 1/2 activation, without generating cAMP in fibroblasts.<sup>5</sup> This signaling paradigm has potential benefits because cAMP signaling during the progression of heart

failure may cause deleterious consequences. To this end,  $\beta$ -blocker therapy has been shown to thwart ventricular hypertrophy, dysfunction, and fibrosis through inhibition of  $\beta_2$ -adrenergic receptor-induced cAMP activity.<sup>6</sup> Furthermore, the activation of Erk 1/2 triggered by several protective strategies, including ischemic preconditioning, has been shown to reduce infarct size and prevent cell death during the reperfusion period.<sup>7</sup> Among several forms of injury mediated by ischemia, endoplasmic reticulum (ER) stress contributes to the pathophysiological characteristics because of cell death pathways initiated by unfolded protein response (UPR). Mitogen-activated protein kinase signaling, including that transduced via Erk 1/2 activation, mitigates the inflammatory response downstream of UPR in in vitro systems.<sup>8,9</sup> We sought to investigate the benefits of short- and long-term B7-33 administration in the context of MI, while focusing on the potential intersection between B7-33-induced Erk 1/2 signaling and UPR.

## METHODS

In adherence to Transparency and Openness Promotion Guidelines, raw data supporting the findings of this study can be made available on reasonable request to corresponding author.

## Animals

Adult male (aged 6–8 weeks) CD1 mice were purchased from Charles River, and were allowed to acclimate in a temperature-controlled vivarium for up to a week before any intervention, with access to water and food. All experiments were performed with strict adherence to *Guidelines for the Care and Use of Laboratory Animals*, as updated by the National Institute of Health (eighth edition, 2011). All of our animal techniques are approved by Virginia Commonwealth University Institutional Animal Care and Use Committee.

## Drugs and Chemicals

Triphenyltetrazolium chloride and tunicamycin were purchased from Sigma-Aldrich. Trypan blue solution (0.4%) was obtained from Thermo Fisher Scientific. PD98059 (Erk 1/2 inhibitor) was purchased from Cell Signaling Technologies. Phthalo blue dye was purchased from Quantum Ink (Louisville, KY). B7-33 was kindly provided by our collaborators from University of Melbourne (Victoria, Australia). Reagents used in myocyte perfusion and digestion buffers (NaCl, KCl, MgSO<sub>4</sub>, CaCl<sub>2</sub>, NaHCO<sub>3</sub>, 2,3-butanedione monoxime, glucose, protease XIV from streptomyces griseus, and taurine) were purchased from

Sigma-Aldrich. Collagenase II was obtained from Worthington Biochemical Corporation (Lakewood, NJ).

## Experimental Protocol

### *In Vivo*

#### *Myocardial IR protocol*

Adult male CD1 mice were anesthetized with pentobarbital IP (70 mg/kg). Sedation was confirmed on lack of responsiveness to toe pinch. After intubation, thoracotomy was performed to visualize the left anterior descending coronary artery. A 7.0 silk ligature was tied around the artery, and a polyethylene tube (PE10) was used to occlude blood flow for the 30-minute ischemic period. Animals were assigned to sham, vehicle, or treatment (B7-33) groups, and the surgeon was blinded to the assignment throughout the entire procedure. Five minutes before the onset of reperfusion, vehicle (0.9% saline) (34 mice) or B7-33 (38 mice) (0.25 mg/kg) IP was administered. Sample size was estimated on the basis of previous studies that followed a similar experimental design and evaluated comparable functional and molecular parameters. After 30 minutes, the polyethylene tube was removed, and the color change in ventricular tissue (from pale to pink) was recorded to ensure proper induction of reperfusion. When reperfusion was not successfully achieved, animals were excluded from the study after confirming the presence of significant wall thinning and global dilatation via echocardiography at 24 hours (6 mice total: 4 from B7-33, and 2 from vehicle). In mice assigned to sham group (9 mice), thoracotomy was performed without induction of the left anterior descending ligature. Postoperatively, analgesia (buprenorphine SR LAB, 0.5 mg/kg; SC) and antibiotic (gentamicin, 0.7 mg/kg) were administered, in compliance with our animal surgery protocol. Mice

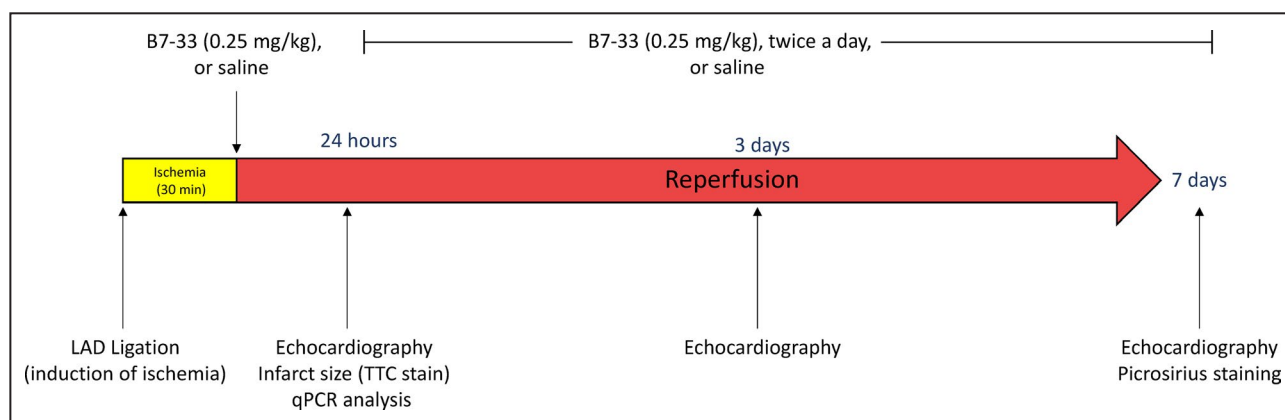
were monitored for up to 7 days, and were given either daily doses of B7-33 (twice a day, 0.25 mg/kg per dose, once every 12 hours) or saline until the time of euthanasia. The protocol for general in vivo method is outlined in Figure 1.

#### *Echocardiography*

Cardiac function was assessed via echocardiography before surgery, and at 24 hours, 3 days, and 7 days postoperatively using the Vevo2100 imaging system (VisualSonics Inc, Toronto, Canada). The general procedure for acquiring short-axis images, estimating fractional shortening (FS), and obtaining left ventricular (LV) internal diameter (systolic and diastolic) was detailed previously.<sup>1</sup> The epicardium and endocardium were traced, and the VevoStrain software was used to measure radial strain, radial strain rate (as a function of time), and Max dV/dT (maximum instantaneous rate of change in volume, during relaxation). Once the radial strain of the anterior apical, anterior mid, anterior basal, posterior apical, posterior mid, and posterior basal segments was assessed, the SD from average was calculated to estimate dyssynchrony of radial strain.

#### *Infarct size assessment*

After 24 hours of reperfusion, mice were sedated with a terminal dose of pentobarbital (100 mg/kg). The heart was subsequently excised along with the aortic root and cannulated for subsequent perfusion in Langendorff mode. Briefly, the heart was perfused with Krebs-Henseleit buffer, followed by 10% triphenyltetrazolium chloride. Then, the ligature around coronary artery was retightened, and aorta was perfused with phthalo blue dye to delineate the nonrisk area. Hearts were then removed from the apparatus and frozen before slicing them into several apical and basal



**Figure 1. Schematic illustrating the experimental protocol for in vivo studies.**

Left anterior descending coronary (LAD) artery in mice was occluded for 30 minutes, followed by either 24 hours or 7 days of reperfusion. Mice were treated at the onset of reperfusion with either B7-33 or saline. In the longer-term group of mice (7 days), mice received regular (twice a day) administration of B7-33 or saline until time of euthanasia. qPCR indicates quantitative polymerase chain reaction; and TTC, triphenyltetrazolium chloride.

sections. After fixing the sections in 10% formaldehyde for 6 hours, the area of infarct size as a percentage of at-risk area was calculated using ImageJ software (W.S. Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, <https://imagej.nih.gov/ij/index.html>), as we described previously.<sup>1</sup>

### Quantification of LV scar

After 7 days of reperfusion, in the long-term vehicle or treatment groups, hearts were excised from anesthetized mice and stored in 10% formaldehyde for at least 48 hours. LV cross-sections were then paraffin embedded, and sections were subsequently analyzed for estimation of scar size via picrosirius red staining, as we previously reported.<sup>10</sup> Multiple sections from each sample were analyzed for infarct and peri-infarct associated scar and expressed as a percentage of total LV area per section, and the outputs were averaged to estimate mean scar size per sample. Image analysis was performed using Image Pro Plus software (Media Cybernetics, Rockville, MD) by an investigator blinded to treatment allocation.

## In Vitro

### Cardiomyocyte and fibroblast isolation

Our protocol for isolating primary cardiomyocytes from adult mice, and performing simulated ischemia reoxygenation (SIRO) in isolated cells was followed as outlined previously.<sup>11</sup> Hearts were isolated from anesthetized mice, followed by perfusion with  $\text{Ca}^{2+}$  free myocyte isolation buffer via Langendorff apparatus. Then, collagenase II and protease XIV were used to digest the extracellular matrix. The digested tissue was centrifuged at 300g for 2 minutes to collect the myocyte pellet. The supernatant was saved to culture fibroblasts. The myocyte pellet was allowed to reconstitute in calcium reintroduction buffers before plating in laminin (Thermo Fisher Scientific) coated dishes. Myocytes were subsequently incubated in myocyte medium (MEM-NEAA and 10% fetal bovine serum, 1% penicillin/streptomycin). Cardiac fibroblasts were isolated from the supernatant, as described above. The pellet was then left to adhere for 2 hours at 37°C with 5%  $\text{CO}_2$  using DMEM/F-12 with 10% fetal bovine serum and plated on 1% porcine type-B gelatin (Sigma Aldrich) precoated 30-mm dishes.<sup>12</sup> Before experimentation, fibroblasts were cultured in DMEM/F-12 with 10% fetal bovine serum for at least 2 passages while monitoring for morphological changes until reaching 70% of cellular confluency.

### SIRO protocol

Myocyte medium was aspirated from freshly plated myocytes, and the cells were allowed to equilibrate in an ischemia buffer (consisting of NaCl,  $\text{NaHCO}_3$ ,

$\text{NaH}_2\text{PO}_4$ ,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , sodium lactate, KCl, and deoxyglucose). Plated myocytes were placed in a 1%  $\text{O}_2$  hypoxia chamber to induce simulated ischemia for 40 minutes. After the ischemic period, cells were placed back in normoxia and reperfused with fresh myocyte media (with or without B7-33) until sample collection/analysis. For cardiac fibroblasts, the protocol was modified to allow for 4 hours of hypoxia, followed by 12 hours of reperfusion with control or B7-33 infused media. Fibroblast viability was assessed with the MTT cell proliferation assay kit (ab211091; Abcam, Cambridge, UK) at the end of the reperfusion period.

### Western blotting

Western blotting to quantify protein expression was done as explained previously in our literature.<sup>1</sup> Briefly, frozen tissue samples or live cells were incubated in a radioimmunoprecipitation assay buffer (Cell Signaling Technologies) infused with protease and phosphatase inhibitors. Lysates were ultrasonicated and centrifuged at 12 000g for 10 minutes at 4°C. Total protein was quantified via Bradford assay using the Quick Start Bradford Protein Assay (Bio-Rad). Subsequently, 50  $\mu\text{g}$  per sample was separated via SDS-PAGE on 4% to 20% acrylamide gel, and then transferred onto nitrocellulose membranes. The membranes were blocked by incubation with 5% nonfat dry milk dissolved in Tris-buffered saline. Primary antibodies were dissolved in 5% BSA in Tris-buffered saline for overnight incubation to probe for phosphorylated (Thr202/Tyr204) and total Erk 1/2 (Cell Signaling Technologies), GRP78 (Cell Signaling Technologies), and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain; Sigma Aldrich).

### Real-time polymerase chain reaction

mRNA was extracted from frozen tissue samples using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany), and the concentration was estimated via nanodrop analyzer (Thermo Fisher Scientific). Genomic DNA was digested, and reverse transcription was performed with iScript gDNA clear cDNA synthesis kit (Bio-Rad). Real-time polymerase chain reaction was performed using SSoAdvanced Universal SYBR Green Supermix (Bio-Rad), with the following sequences for forward and reverse primers ( $\beta$ -actin: CTAAGGCCAACCGTGAAAAG [forward] and ACCAGAGGCATACAGGGACA [reverse]; CCAAT/enhancer-binding protein-homologous protein (Chop): CCCAGGAAACGAAGAGGAAGAA [forward] and ATGTGCGTGTGACCTCTGTT [reverse]; Grp78: CTATTCCTGCGTCGGTGTGT [forward] and GCCCTGATCGTTGGCTATGA [reverse]; toll-like receptor 4: TGGTTGCAGAAAATGCCAGG [forward] and ATTAGGAACCTCTATGCAGGG [reverse]; tissue



inhibitor of metalloproteases [Timp] 1: CTCGGA CCTGGTCATAAGGG [forward] and ACGCTGGTATAA GGTGGTCTC [reverse]; Timp2: CACGCTTAGCAT CACCCAGA [forward] and GAGTGATCTTGCCTCA CAGC [reverse]. Data were recorded and analyzed on Bio-Rad CFX96 to quantify gene expression.

## Statistical Analysis

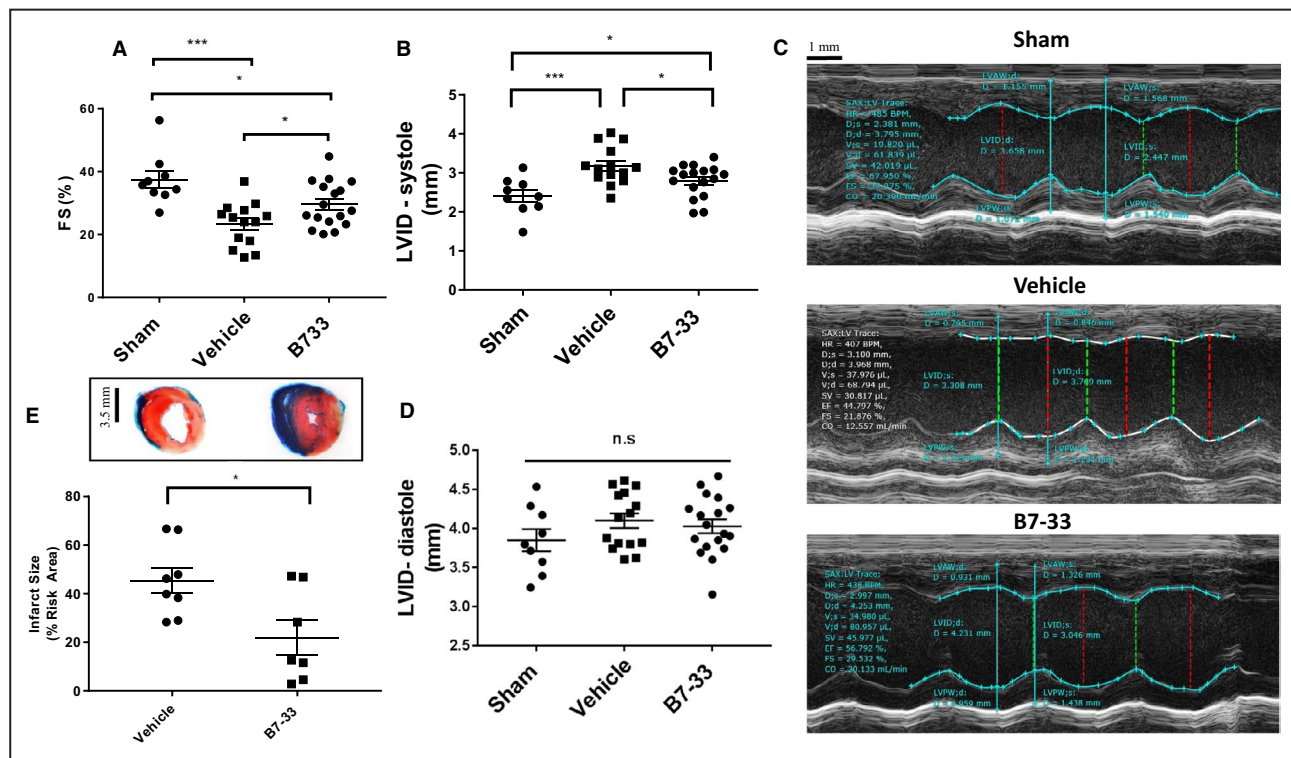
Data for infarct size, LV fibrosis, quantitative polymerase chain reaction, Western blotting, echocardiography parameters, and cell survival experiments were assessed for normality via Shapiro-Wilk normality test. Normally distributed data were summarized as averages $\pm$ SEM, and nonnormally distributed data (LV fibrosis) were summarized as medians with interquartile ranges. Unpaired T test was used for comparing 2 normal distributions, and ANOVA was chosen for  $\geq 3$  groups. Comparisons were deemed significant if  $P$  value for rejecting null hypothesis was  $<0.05$ . For post hoc analysis, Holm-Sidak multiple comparisons test was used for pairwise comparisons. For nonnormally distributed data (LV fibrosis), Mann-Whitney  $U$

test was implemented. GraphPad Software Inc (version 8) was used for generating data and statistical analysis. All authors have supervised and assume responsibility for the progress and presentation of the current work.

## RESULTS

### B7-33 Improves Cardiac Function 24 Hours Post MI

Twenty-four hours post IR surgery, echocardiography was performed to compare several functional parameters, including FS and LV internal diameter (LVID) at systole and diastole through analysis of M-mode images. Before IR, FS and LVID at systole and diastole were measured in healthy CD1 male mice to exclude cardiac abnormalities. Mice that received B7-33 at reperfusion had significantly higher FS (Figure 2A) and lower LVID at systole (Figure 2B) compared with vehicle-treated mice at 24 hours. LVID at diastole did not significantly vary from sham IR group levels compared with B7-33



**Figure 2. B7-33 improves cardiac function 24 hours post MI.**

**A**, Fractional shortening (FS) is preserved in mice treated with B7-33 (0.25 mg/kg) 24 hours post myocardial infarction ( $29.57 \pm 1.74\%$  vs  $23.34 \pm 1.84\%$ ), compared to vehicle. **B**, Left ventricular internal diameter (LVID) is significantly elevated in vehicle-treated mice ( $3.17 \pm 0.13$  mm) vs B7-33-treated mice ( $2.83 \pm 0.11$  mm). **C**, Representative sketches of M-mode acquisitions and subsequent measurements (bar=1 mm). **D**, No significant differences in LVID at diastole among the experimental groups (for **A–D**,  $n=9$  for sham, and  $n=14–18$  for vehicle and B7-33 groups). **E**, Infarct size, expressed as a percentage of risk area, is significantly lower in B7-33 group ( $21.99 \pm 7.17\%$ ;  $n=7$ ) vs vehicle group ( $45.32 \pm 5.28\%$ ;  $n=8$ ) (bar=3.5 mm). For **A**, **B**, and **D**, 1-way ANOVA was used to determine significance, and if  $P < 0.05$ , Holm-Sidak test was used for post hoc analysis. For **E**, unpaired T test was used. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

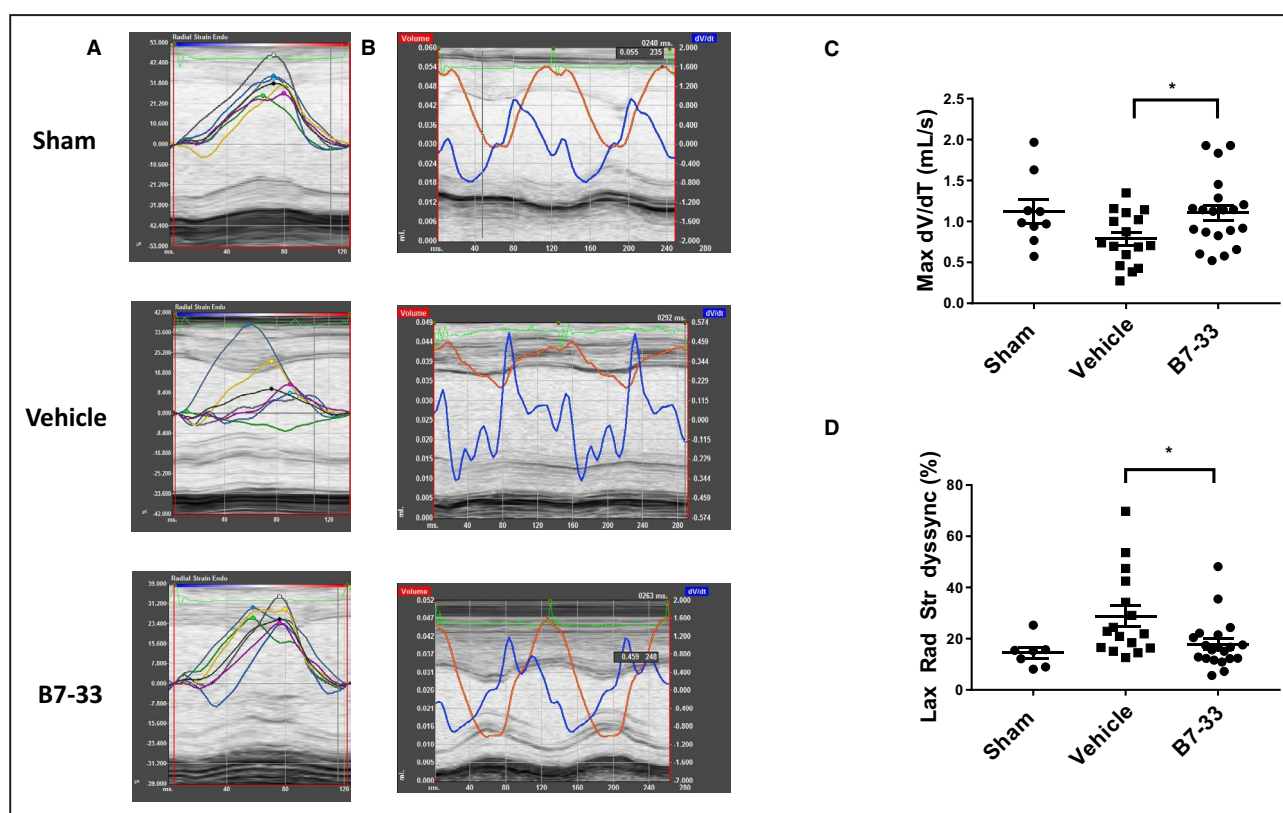
and vehicle-treated groups (Figure 2D). Infarct size was assessed via triphenyltetrazolium chloride staining, and quantified as a percentage of area at risk distal to the left anterior descending ligature. Area at risk did not differ significantly between the groups (not shown). Infarct size was significantly reduced in B7-33 versus vehicle-treated animals 24 hours post MI (Figure 2E). Speckle tracking analysis was performed on long-axis B-mode images acquired 24 hours post MI (Figure 3A and 3B). Baseline radial strain dyssynchrony and Max dV/dT were also measured before IR. Animals treated with B7-33 demonstrated significantly higher Max dV/dT compared with vehicle animals (Figure 3C). Radial dyssynchrony was also significantly lower in the treated group (Figure 3D).

mRNA isolated from cardiac tissue samples 24 hours post MI showed an increase in the expression of Chop (mediates apoptosis during ER stress),<sup>13</sup> ASC and toll like receptor 4 (Figure 4A through 4C). The latter 2 are involved in priming of

the inflammasome, a required event preceding its activation.<sup>14</sup>

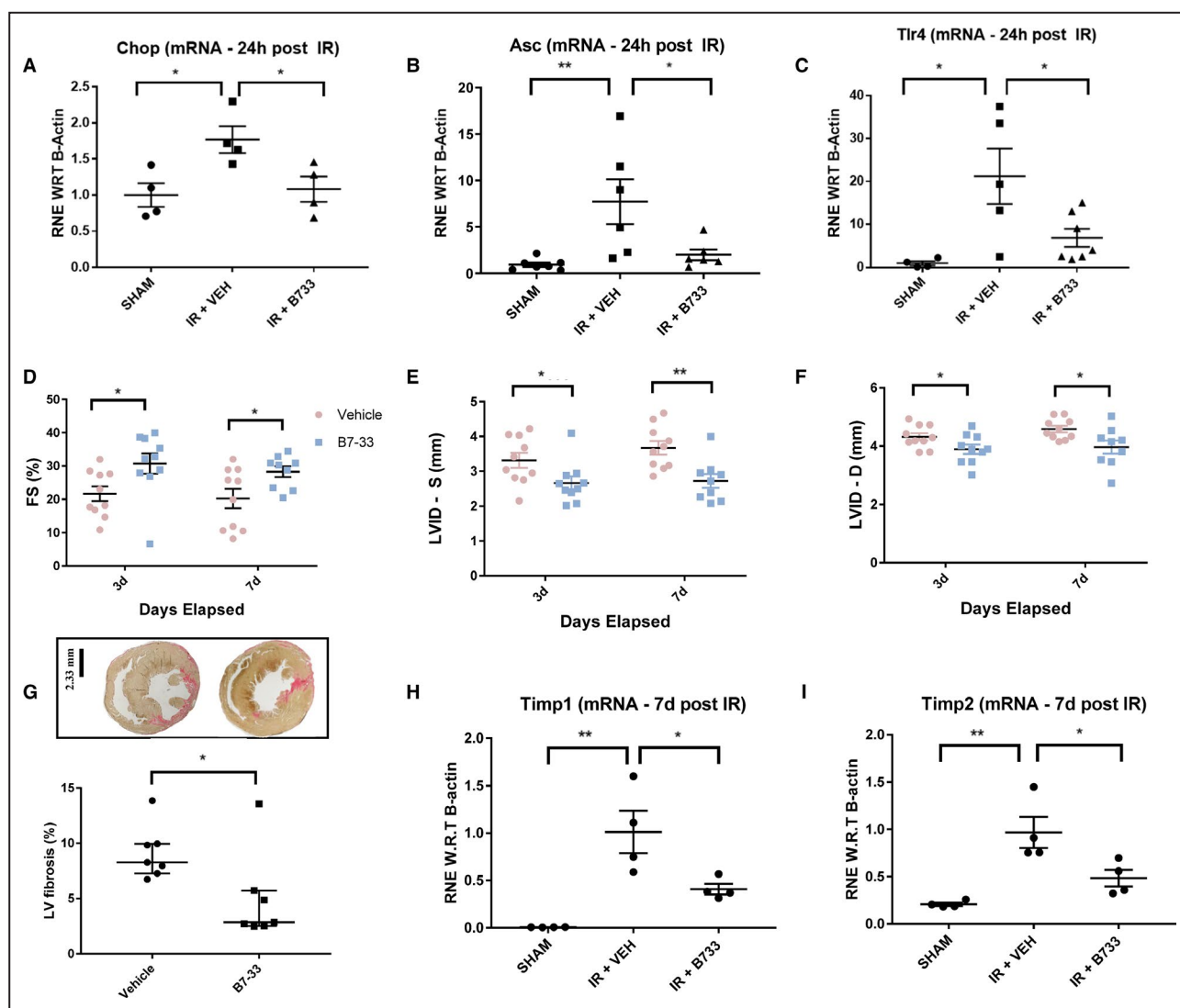
## B7-33 Improves Functional Parameters and Mitigates Adverse Remodeling 7 Days Post MI

At the end of the 7-day period, cardiac function was compared among vehicle and B7-33 treated mice. Functionally, LV FS was preserved at 3 and 7 days post MI (Figure 4D) in B7-33-treated animals in comparison to vehicle group. LVID at systole was significantly lower at 3 and 7 days with treatment (Figure 4E). LVID at diastole demonstrated similar trends at 3 and 7 days (Figure 4F). Paraffin-fixed LV cross-sections from mice at the end of the 7-day period were assessed for fibrosis via picrosirius staining. Animals treated with B7-33 had a significantly lower scar size compared with vehicle-treated animals (Figure 4G). Quantitative polymerase chain reaction analysis of cardiac tissue samples shows significantly decreased expression of



**Figure 3.** Analysis of long-axis echocardiography views 24 hours post MI.

**A**, Representative tracings from experimental groups demonstrating radial velocity vs time, with individual colors representing the 6 long-axis left ventricular segments to calculate radial strain dyssynchrony. **B**, Representative tracings from experimental groups depicting dV/dT as a function of time, with the local maxima corresponding to peak relaxation velocity, estimated via speckle tracking analysis. **C**, Preserved Max dV/dT in B7-33-treated group ( $1.11 \pm 0.09$  mL/s for B7-33 vs  $0.79 \pm 0.08$  mL/s for vehicle) 24 hours post myocardial infarction (MI). **D**, Reduced left ventricular radial strain dyssynchrony in B7-33-treated group 24 hours post MI ( $17.51 \pm 2.17\%$  vs  $28.77 \pm 4.13\%$  for vehicle;  $P < 0.05$ ). Dyssynchrony was calculated from SD of left ventricular segmental radial strains, and expressed as a percentage of average radial strain. For **C** and **D**,  $n = 9$  for sham and  $n = 15$  to 21 for vehicle and B7-33 groups. For **C** and **D**, 1-way ANOVA was used to determine significance, and if  $P < 0.05$ , Holm-Sidak test was used for post hoc analysis.  $*P < 0.05$ .



**Figure 4. Real-time polymerase chain reaction analysis at 24 hours, and assessment of cardiac function at 3 & 7 days post MI.** **A** through **C**, Treatment with B7-33 significantly lowers Chop (CCAAT/enhancer-binding protein-homologous protein), Asc (apoptosis-associated speck-like protein containing a caspase recruitment domain), and toll-like receptor 4 (Tlr4) mRNA in extracts from cardiac tissue, 24 hours post myocardial infarction (MI) (for **A** through **C**,  $n=9-10$ ). **D**, Preserved fractional shortening (FS) 3 and 7 days post MI in B7-33-treated group ( $30.73 \pm 2.20\%$  and  $28.29 \pm 1.63\%$  vs  $21.66 \pm 3.05\%$  and  $20.25 \pm 2.92\%$  for B7-33- and vehicle [VEH]-treated group, respectively). **E**, Left ventricular internal diameter at systole (LVID-S) at 3 and 7 days ( $2.66 \pm 0.18$  and  $2.72 \pm 0.2$  mm vs  $3.31 \pm 0.21$  and  $3.70 \pm 0.19$  mm for B7-33 and VEH, respectively). **F**, LVID at diastole (LVID-D) for 3 and 7 days ( $3.90 \pm 0.15$  and  $3.96 \pm 0.22$  mm vs  $4.32 \pm 0.12$  and  $4.58 \pm 0.12$  mm for B7-33 and VEH groups, respectively) was significantly lower in B7-33-treated groups (for **D** through **F**,  $n=4-7$  per group). **G**, LV fibrosis is significantly lower in B7-33-treated animals ( $n=7$ ;  $2.86\%$  [ $2.52\%-5.71\%$ ]) than in VEH-treated animals ( $n=7$ ;  $8.27\%$  [ $7.27\%-9.95\%$ ]; bar= $2.33$  mm). **H** and **I**, Treatment with B7-33 significantly lowers tissue inhibitor of metalloproteases (Timp) 1 and Timp2 mRNA expression in cardiac tissue 7 days post ischemia-reperfusion (IR) surgery ( $n=4$  for per group). Data expressed as relative normalized expression with respect to (RNE W.R.T) B-actin. For **A** through **C**, **H**, and **I**, 1-way ANOVA was used to determine significance, and if  $P < 0.05$ , Holm-Sidak test was used for post hoc analysis. For **D** through **F**, unpaired T test was used. For **G**, Mann-Whitney U test was used to account for the nonnormal distribution. \* $P < 0.05$ , \*\* $P < 0.01$ .

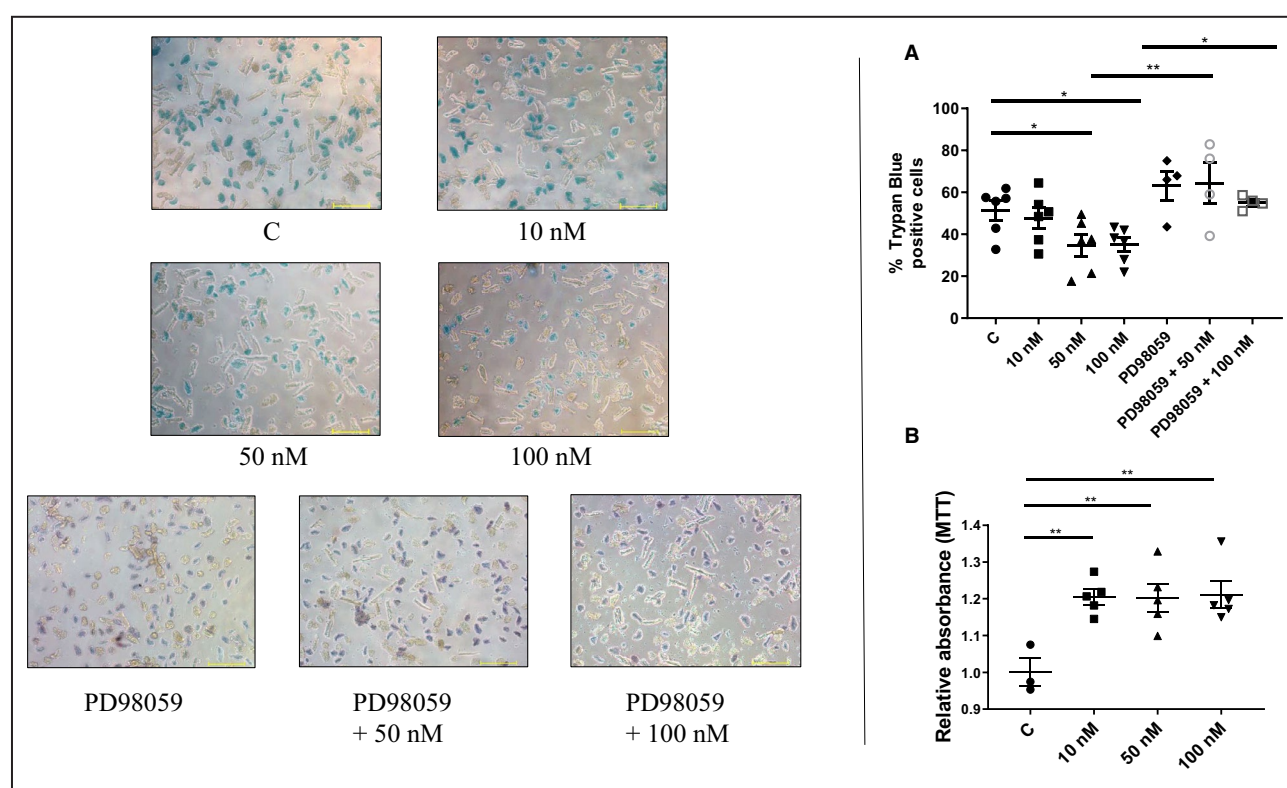
the profibrotic markers Timp1 and Timp2 (Figure 4H and 4I) 7 days post MI in B7-33 group.

### B7-33 Protects Primary Cardiomyocytes and Fibroblasts Against SIRO

To establish the in vitro dose for the treatment of primary myocytes with B7-33, freshly isolated cells were treated

with 10, 50, and 100 nmol/L doses of B7-33. Trypan blue staining was used to estimate the percentage of dead myocytes after SIRO, in the presence of varying doses of B7-33. Compared with control, cells treated with either 50 or 100 nmol/L of B7-33 had reduced trypan blue positive cells (Figure 5A). To test whether the reduction in cell death was through B7-33 mediated phosphorylation of Erk 1/2, the dual specificity mitogen-activated protein





**Figure 5. Simulated ischemia reoxygenation (SIRO) of primary cardiomyocytes and fibroblasts.**

**A**, Primary cardiomyocytes treated with either 50 or 100 nmol/L of B7-33 in myocyte media following simulated ischemia have a lower trypan blue positive percentage of cells (control [51.33±4.53%] vs 50 nmol/L [34.72±5.20%] or 100 nmol/L [35.03±3.43%] of B7-33) (n=4–6 experiments/group). Pretreating cardiomyocytes with extracellular signal-regulated kinase (Erk) 1/2 inhibitor PD98059 followed by coincubation with B7-33 abolishes the protective effects at both 50 nmol/L (64.28±9.73%) and 100 nmol/L (54.35±2.23%) concentrations of B7-33 (bar=100  $\mu$ m). **B**, Fibroblast viability is higher, compared with control, when treated with 10, 50, or 100 nmol/L of fibroblast media (n=3–5 experiments/group) post SIRO. For **A** and **B**, 1-way ANOVA was used to determine significance, and if  $P<0.05$ , Holm-Sidak test was used for post hoc analysis. \* $P<0.05$ , \*\* $P<0.01$ .

kinase (MEK1 and MEK2) inhibitor PD98059 (50  $\mu$ mol/L) was used to reduce phosphorylated Erk 1/2 levels via pretreatment of primary cardiomyocytes for 1 hour before SIRO. The mitigation of SIRO-induced cell death by B7-33 is significantly abolished when coincubated with PD98059 (Figure 5A). The viability of primary cardiac fibroblasts after SIRO was assessed through the MTT assay, and cells treated with 10, 50, or 100 nmol/L B7-33 showed significantly higher relative absorbance compared with the control group (Figure 5B).

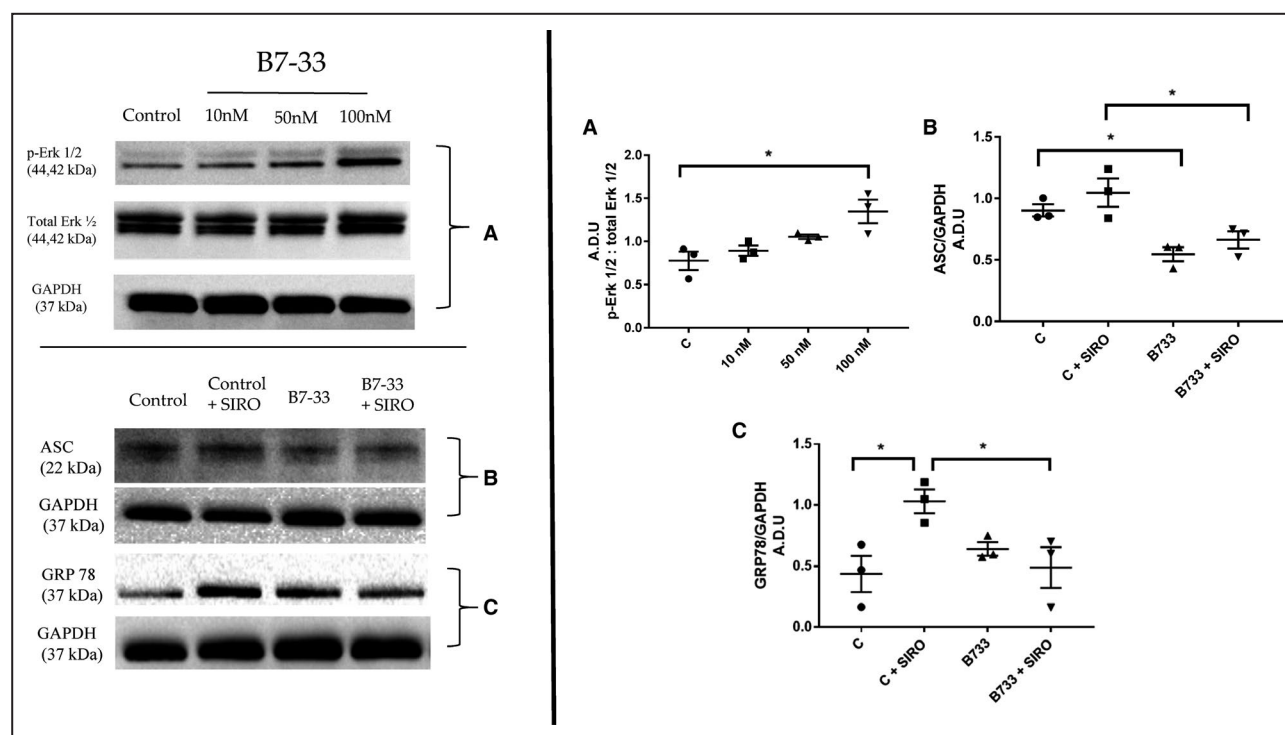
To evaluate the signaling effects of B7-33 on cardiomyocytes, protein was extracted and phosphorylation of Erk 1/2 was assessed via Western blot after 15 minutes of exposure. Phosphorylation ratio increased with higher doses of B7-33, reaching significance at 100 nmol/L dose (Figure 6A). For subsequent myocyte experimentation, 100 nmol/L dose of B7-33 was chosen. Primary myocytes subjected to SIRO were treated with B7-33 for 24 hours during the reoxygenation phase, and protein isolated from cell lysates was analyzed. Treatment with B7-33 reduced expression of ASC 6 hours post SIRO (Figure 6B), and GRP78,

an ER chaperone that functions as a key mediator in several UPR-associated signaling cascades, was reduced in B7-33-treated myocytes 24 hours post SIRO (Figure 6C).

### B7-33 Reduces Tunicamycin-Induced ER Stress in Primary Cardiomyocytes

The reduction in expression of IR injury-related ER stress associated markers in vivo and in vitro on treatment with B7-33 prompted further inquiry into studying the association between ER stress and relaxin signaling. An alternative model of injury was sought because IR induces dysfunction via multiple interlinked causes (sterile inflammation, reactive oxygen species production, mitochondrial disruption), along with UPR. Therefore, we used tunicamycin, a bioactive compound that inhibits N-linked glycosylation of proteins, to study ER stress exclusively.<sup>13</sup> Primary cardiomyocytes were incubated with either 2.5 or 7.5  $\mu$ g/mL tunicamycin for 24 hours. Western blot analysis after the incubation period with





**Figure 6. Western blot analysis of primary cardiomyocytes treated with B7-33.**

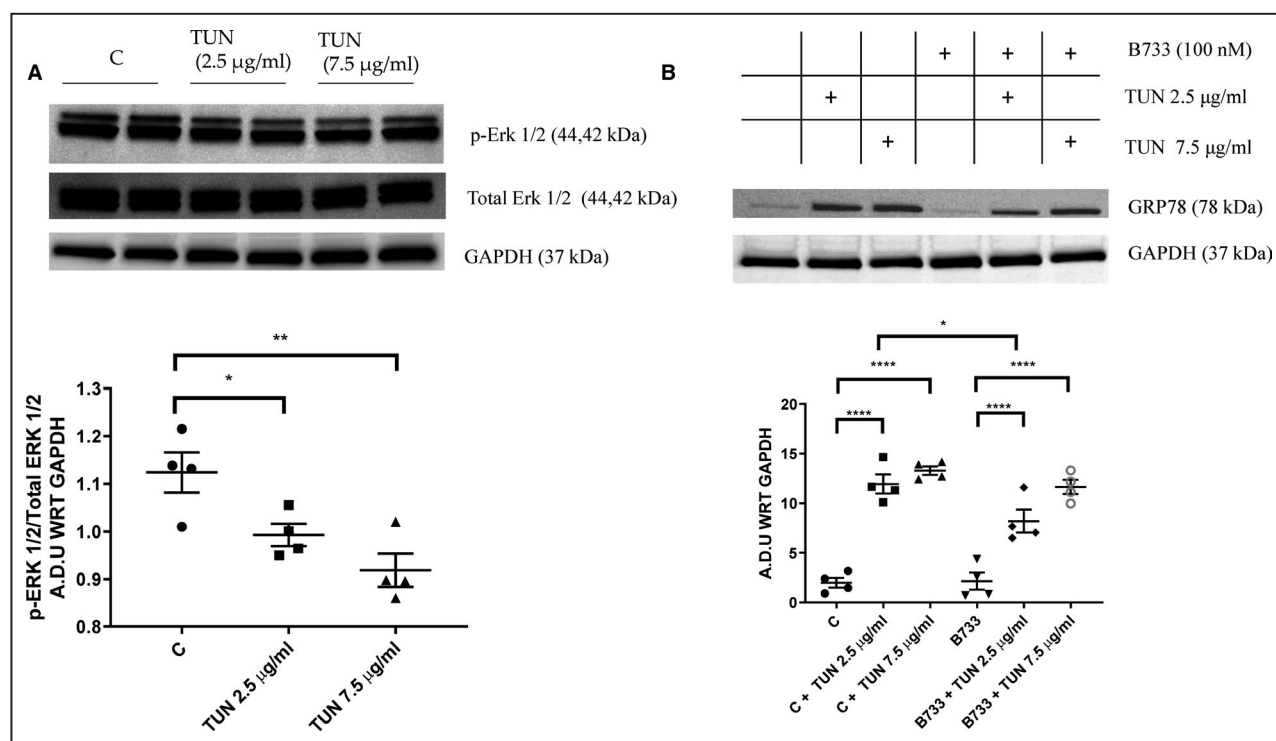
In primary cardiomyocytes, increased phosphorylation of extracellular signal-regulated kinase (Erk) 1/2 (p-Erk 1/2) in cells treated with B7-33, reaching significance at 100 nmol/L (A); reduced expression of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) in cells 6 hours post simulated ischemia reoxygenation (SIRO), on treatment with B7-33 (B); and reduced expression of GRP78 in cells 24 hours post SIRO, on treatment with B7-33 (C) (for A through C, n=3 experiments per group). Western blots are quantified and expressed as arbitrary densitometric units (A.D.U.). For A through C, 1-way ANOVA was used to determine significance, and if  $P < 0.05$ , Holm-Sidak test was used for post hoc analysis. \* $P < 0.05$ . C indicates control.

tunicamycin significantly reduced phosphorylation of Erk 1/2 at 2.5 and 7.5  $\mu\text{g/mL}$  doses (Figure 7A). Furthermore, treatment with tunicamycin showed a significant increase in GRP78 expression in myocytes at both the concentrations (Figure 7B). Coincubation with 100 nmol/L B7-33 significantly reduced GRP78 expression at 2.5  $\mu\text{g/mL}$  dosage of tunicamycin (Figure 7B). To test the hypothesis that B7-33-induced phosphorylation of Erk 1/2 is essential to limit ER stress, primary cardiomyocytes were pretreated with PD98059 for 1 hour before tunicamycin and/or B7-33 exposure for 24 hours. PD98059 significantly reduced Erk 1/2 phosphorylation despite incubation with B7-33 (Figure 8A and 8B). Inhibition of Erk 1/2 phosphorylation through PD98059 abolished the protective effect of B7-33 in reducing tunicamycin-induced GRP78 expression (Figure 8A and 8C).

## DISCUSSION

Human relaxin-2 is extensively involved in mammalian pregnancy because of its modulation of maternal hemodynamics by lowering total peripheral resistance and increasing cardiac output.<sup>15</sup> The vasodilatory effects of the hormone are primarily transduced

through NO signaling in smooth muscle cells. Because of these pharmacologic properties, recombinant relaxin-2 (serelaxin; Novartis Pharmaceuticals) was considered a viable option for the treatment of acute heart failure. The phase 3 trials, RELAX-AHF (Relaxin for the Treatment of Acute Heart Failure)<sup>16</sup> and RELAX-AHF2,<sup>17</sup> used a short-term (48-hour) infusion strategy to assess clinical end points pertinent to acute heart failure. Specifically, cardiovascular-related mortality at 180 days and worsening heart failure at 5 days were considered primary end points in the latter, more recent trial (RELAX-AHF2).<sup>17</sup> Serelaxin failed to achieve these outcomes, in contrast to findings from the former trial.<sup>16</sup> The recently published article on RELAX-AHF2<sup>17</sup> attributes this inconsistency to either chance or variability in patient populations, because RELAX-AHF2 enrolled patients with worse renal function and incorporated a higher threshold for natriuretic peptide levels. Nevertheless, the authors question the efficacy of a 48-hour infusion protocol in addressing long-term end points for acute heart failure. Another plausible explanation could be attributed to recent findings suggesting that the effects of relaxin may be compromised in the context of heart failure in patients who are already



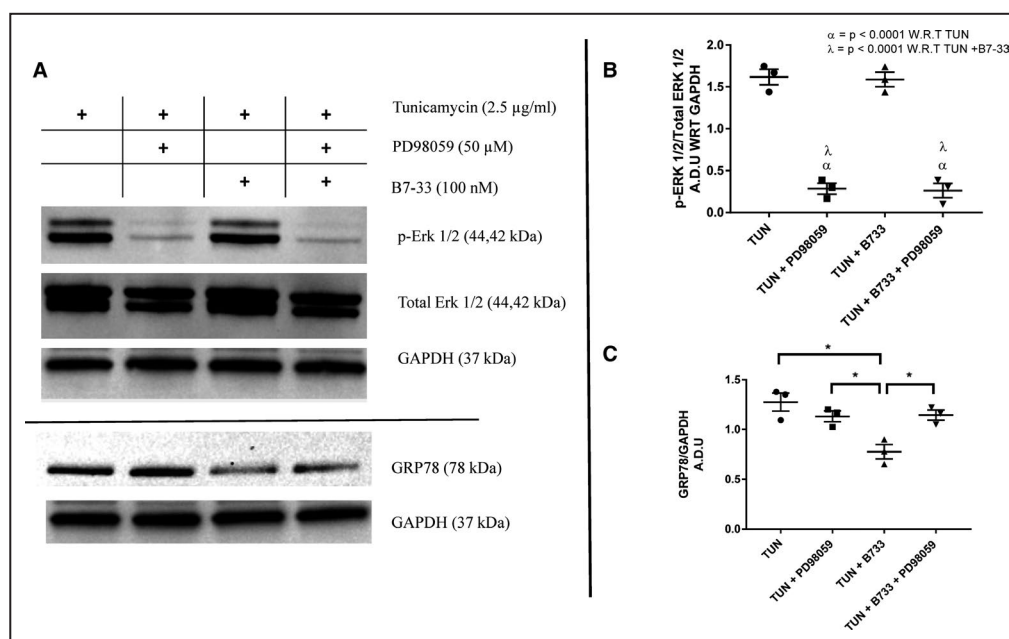
**Figure 7. Western blot analysis of tunicamycin-induced endoplasmic reticulum (ER) stress in protein lysates from primary cardiomyocytes.**

Exposure of primary cardiomyocytes to tunicamycin (TUN) for 24 hours significantly reduces phosphorylation of extracellular signal-regulated kinase (Erk) 1/2 (p-Erk 1/2) at both 2.5 and 7.5 µg/mL concentrations of TUN (**A**); and significantly increases expression of GRP78 at both doses of TUN (**B**). B7-33 (100 nmol/L) significantly lowers GRP78, compared with control (C), on coinubation with 2.5 µg/mL TUN (for **A** and **B**,  $n=4-6$  experiments/group). Western blots are quantified and expressed as arbitrary densitometric units with respect to (A.D.U WRT) the expression of GAPDH. For **A** and **B**, 1-way ANOVA was used for significance testing, and if  $P<0.05$ , Holm-Sidak test was used for post hoc analysis. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\*\* $P<0.0001$ .

receiving angiotensin receptor blockers.<sup>18</sup> The utility of relaxin-associated therapeutics remains potentially unharnessed, given the wealth of preclinical data implicating relaxin signaling in mitigating progression toward heart failure in various preclinical models.<sup>2</sup> Translationally, this potential could be realized in causes where overt damage to the myocardium occurs in a symptomatically or clinically detectable manner, such as in the case of MI. Our laboratory previously investigated the effect of serelaxin in conferring cardioprotection against acute IR injury. We demonstrated that relaxin-induced cardioprotection is dependent on endothelial NO synthase activity, and showed that the hormone suppresses inflammasome activity in cardiac tissue through endothelial NO synthase-dependent mechanism.<sup>1</sup> Sterile inflammation, through the priming and activation of Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing 3 (NLRP3) inflammasome, has been conclusively shown to induce activated interleukin-1 $\beta$  production and cardiomyocyte pyroptosis (through activated caspase-1) in the heart.<sup>14</sup> Other studies show reduced mitochondrial swelling during IR injury on treatment with

relaxin.<sup>19</sup> This is especially relevant, because reactive oxygen species released by dysfunctional mitochondria contribute further to inflammasome activity.<sup>14</sup>

The peptide structure of relaxin resembles that of other insulin-like peptides: two chains (A and B) linked through disulfide bonds. The single B-chain analogue B7-33 was synthesized on the basis of the premise that the lack of disulfide linkages facilitates lower production costs and increased affordability.<sup>5,20</sup> When RXFP1 was expressed in a heterologous HEK-293T system, B7-33 failed to induce a cAMP response when compared with analogous concentrations of relaxin-2.<sup>5</sup> In vitro exposure to B7-33 increased phosphorylated Erk 1/2 levels within 10 minutes in rat renal myofibroblasts.<sup>5</sup> We observed similar results in primary adult cardiomyocytes within 15 minutes of exposure. The preferential bias toward Erk 1/2 signaling, mediated by the  $\beta\gamma$  subunits of RXFP1,<sup>2</sup> has important physiological implications; the presence of excessive cAMP in the ischemic heart could potentially increase myocardial workload, further exacerbating contractile demand on myocytes from remote/viable myocardium.<sup>6</sup> On the other hand, Erk 1/2-mediated activation of NO synthases has potent antifibrotic effects through the involvement



**Figure 8.** Treatment of primary cardiomyocytes with tunicamycin (TUN) with/without PD98059 and/or B7-33.

**A**, Representative Western blots of primary cardiomyocytes treated with tunicamycin (TUN) with/without PD98059 and/or B7-33. **B**, Exposure to PD98059 (50  $\mu$ mol/L) significantly lowers phosphorylation of extracellular signal-regulated kinase (Erk) 1/2 (p-Erk 1/2) 24 hours post incubation in primary cardiomyocytes treated with TUN with/without B7-33. No significant difference between p-Erk 1/2 levels was found between TUN and TUN+B7-33 groups. **C**, Coincubating TUN with PD98059 abrogates the reduction in GRP78 observed when treated with B7-33 (for **B** and **C**,  $n=3$  experiments/group). Western blots are quantified and expressed as arbitrary densitometric units with respect to (A.D.U. WRT) the expression of GAPDH. For **B** and **C**, 1-way ANOVA was used for significance testing, and if  $P<0.05$ , Holm-Sidak test was used for post hoc analysis. \* $P<0.05$ .

of cGMP-induced matrix metalloproteinase (2 and 9) upregulation.<sup>21</sup> Therefore, RXFP1-mediated Erk 1/2 phosphorylation transduces multiple protective mechanisms, and B7-33 is well placed to recapitulate these effects in lieu of relaxin-2 therapy.

In this current study, B7-33 was administered after 30 minutes of ischemia, immediately before the onset of reperfusion. A short-term, IP dosage of 0.25 mg/kg was chosen on the basis of a previous in vivo study (Hossain et al<sup>5</sup>). Although this study used 0.5 mg/kg per day for treatment (which we replicated in our 7-day studies), we used 0.25 mg/kg at reperfusion to achieve equimolar levels of relaxin concentration used in Hossain et al, because relaxin has roughly twice the molecular weight of B7-33. B7-33 has been shown to achieve a similar pharmacodynamic profile as relaxin-2 in cells (cardiac fibroblasts) endogenously expressing RXFP1,<sup>5</sup> and is likely to possess similar half-life to relaxin<sup>22</sup> because of common structural features. We were unable to quantify the bioavailability of B7-33 post injection, because the ELISA kit (R&D Systems) for relaxin-2 failed to recognize B7-33 in our preliminary studies. However, as discussed previously,<sup>1</sup> it is likely that a small peptide,

such as B7-33, reaches circulation within minutes of intraperitoneal administration. B7-33 reduced infarct size, preserved FS, and reduced radial strain dyssynchrony 24 hours post MI. The reduction in infarct size (21% versus 45% in saline-treated group) with B7-33 is roughly comparable to the reduction in infarct size on reperfusion therapy with serelaxin (15.2% versus 46.5% in saline-treated group) in our previous study.<sup>1</sup> Similarly, FS 24 hours post MI on B7-33 treatment (29.57%) roughly corresponds to FS in mice treated with serelaxin (28%).<sup>1</sup> Therefore, B7-33 recapitulates the protective effects conferred by serelaxin in mice subjected to the same MI protocol. In the current study, LVID at systole was significantly smaller in the treatment group (because of preserved contractility), but no differences were found in LVID at diastole at 24 hours. However, LVID at diastole diverged between the groups at 3- and 7-day time points, perhaps because of increased dilatation attributable to progressive adverse remodeling in the vehicle group. Therefore, B7-33-induced protection was not solely limited to infarct-sparing effects, as therapeutic benefits from daily administrations were realized over the course of remodeling. Cell type-specific responses

were further studied when cardiomyocytes and fibroblasts were subjected to SIRO. We chose a longer duration of ischemia for fibroblasts because they are more resilient to ischemic damage compared with primary cardiomyocytes.<sup>23</sup> Fibroblast viability was increased, at all concentrations of B7-33 (10, 50, and 100 nmol/L), after SIRO. This is interesting, considering the long-term antifibrotic effects of B7-33. Fibroblast proliferation in the early stages of infarct healing (as it coincides temporally with our *in vitro* experiment) might be vital,<sup>23</sup> but the long-term effect of B7-33 exposure to remodeling fibroblasts is yet to be fully characterized. Relaxin signaling has been shown to inhibit phenotypic differentiation of these cells into myofibroblasts on exposure to transforming growth factor- $\beta$ .<sup>24</sup> Interestingly, a recent study demonstrates that relaxin-induced inhibition of myofibroblast transition on transforming growth factor- $\beta$  stimulation is dependent on Wnt signaling. This recently characterized arm of relaxin signaling occurs independent to cAMP generation.<sup>25</sup> Therefore, the interaction of this pathway with B7-33-induced activation of RXFP1 is of potential interest in future studies. In our study, LV sections stained for scar size show the antifibrotic effects of B7-33, and corroborate previous findings, where reduced interstitial fibrosis was detected in B7-33-treated animals subjected to MI (permanent ligation) and isoproterenol induced heart failure.<sup>5</sup> Analysis of LV samples 7 days post MI shows reduced expression of the profibrotic markers Timp 1 and 2 on treatment with B7-33. The inhibitory effects of relaxin signaling on Timp expression,<sup>26,27</sup> and antifibrotic modulation of matrix metalloproteinase/TIMP ratio by relaxin,<sup>21</sup> have been previously examined.

The complex interplay between mitochondria and ER in the ischemic heart<sup>28,29</sup> led us to investigate alternate, previously unexamined modalities of protection through relaxin therapy. IR injury damages mitochondria, and leads to production of reactive oxygen species through damaged complex I and III within the electron transport chain when oxygen is re-introduced.<sup>30</sup> Mitochondrial reactive oxygen species is central to inflammasome activation,<sup>14</sup> and can contribute to UPR.<sup>28</sup> Given the mitoprotective features of relaxin therapy, we tried to elucidate whether B7-33 impacted ER stress response post IR injury. Relaxin-2 was previously shown to inhibit glucose-induced apoptosis in neonatal rat myocytes by inhibiting ER stress.<sup>31</sup> In our study, B7-33 reduced the expression of GRP78, a chaperone involved in transducing UPR-associated pathways, 24 hours post SIRO in cardiomyocytes. As misfolded proteins accumulate in ER lumen, GRP78 is disengaged from the inner ER membrane as it activates the protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme (IRE1), and activating transcription factor

(ATF6) arms of the response.<sup>28</sup> The role and significance of ER stress signaling in ischemic heart disease is of intense scrutiny. Although the net effect could be restorative and reduce the burden of misfolded proteins, UPR can also trigger proapoptotic and inflammatory cascades, causing cell death.<sup>29</sup> For instance, endothelin-1-induced overexpression of GRP78 increased hypoxia tolerance in neonatal rat myocytes,<sup>32</sup> but use of tauroursodeoxycholic acid reduced apoptosis in rats<sup>33</sup> post MI, and reduced adverse modeling through inhibition of GRP78 in a pressure overload model of heart failure.<sup>34</sup> In our model, B7-33 could potentially be acting upstream of GRP78 recruitment as misfolded proteins accumulate. Nevertheless, this remained unclarified because the data were confounded by the possibility that B7-33-mediated GRP78 reduction was a consequence of overall reduction in cell death through other protective pathways. To circumvent this issue, we studied the effect of induced ER stress in isolation by incubating cardiomyocytes with a nonlethal dose of tunicamycin. B7-33 reduced tunicamycin-induced upregulation of GRP78, indicating a role for RXFP1 in UPR, when activated.

B7-33-mediated reduction of GRP78 expression was abrogated when cardiomyocytes were coincubated with PD98059 and tunicamycin, potentially implicating mitogen-activated protein kinase signaling in the protective effect. As shown elsewhere, activation of Erk 1/2 in NIH3T3 cells was protective against ER stress-induced apoptosis.<sup>9</sup> In MCF-7 breast cancer cells, ER stress induction led to rapid activation of Erk 1/2, an event shown to divert cell fate toward removal of misfolded proteins and survival, while curbing proapoptotic signaling.<sup>35</sup> In our study, tunicamycin led to overall reduction in phosphorylated Erk 1/2 levels at 24 hours. Although B7-33 led to a short-term increase in phosphorylated Erk 1/2, this activation was transient as the levels normalized at 24 hours. This corroborates the findings seen in rat renal myofibroblasts, where the Erk 1/2 response with B7-33 stimulation was muted within minutes of exposure.<sup>5</sup> The transient upregulation observed in our primary cardiomyocytes potentially underscores the importance of downstream mediators in reducing the burden of misfolded proteins.

In summary, B7-33 confers short-term cardioprotection by reducing infarct size and preserving cardiac function 24 hours post IR injury, and prevents further adverse remodeling. *In vitro* assessment using B7-33 demonstrates remarkable protection in cardiomyocytes and fibroblasts, while implicating Erk 1/2 activation in mediating the protection against IR injury-induced ER stress. Therefore, B7-33 could potentially be used as a novel therapy for attenuation of MI and prevention of MI-related adverse cardiac remodeling and failure.



## Limitations

The results from our current study, although potentially impactful, are limited by the nature of our animal model. Although the murine model recapitulates the structural and functional deficits observed in human MI, multifactorial influences (including environment, genetics, and existing pharmacotherapy) underscoring the pathophysiological characteristics of adverse remodeling and heart failure in humans are manifestly absent in mice. Characterizing the therapeutic utility of B7-33 necessitates extensive safety and efficacy trials in humans, and this requires formulation of strategies ensuring large-scale production and distribution of B7-33. Although the prospect of commercializing B7-33 is still in its infancy, the reduced complexity and fewer purification steps (compared with recombinant relaxin) could potentially streamline the process in the long-term. In the current study, CD1 mice were used because of a wider genetic diversity stemming from outbreeding within mice populations.<sup>36</sup> The exclusive use of male mice limits the scope of this current work. However, expression of RXFP1 in female rodents has been shown to be altered over the course of estrus cycle in other tissues (skeletal).<sup>37</sup> To reduce the confounding effect of reproductive hormone-induced changes in RXFP1, male mice were chosen in this study. Analysis in both male and female animal populations would be necessary for evaluating the potential benefits of treatment with B7-33 in the future.

## ARTICLE INFORMATION

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### Disclosures

None.

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

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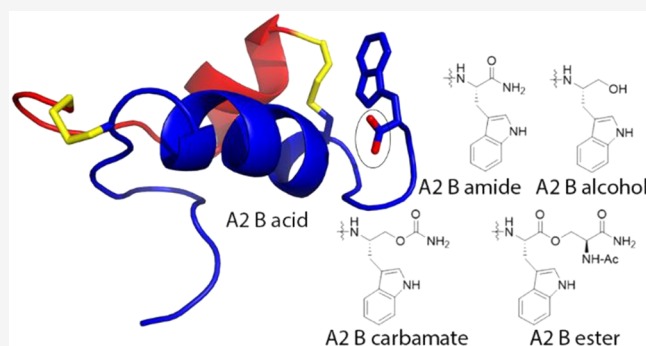
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**ABSTRACT:** The receptor for the neuropeptide relaxin 3, relaxin family peptide 3 (RXFP3) receptor, is an attractive pharmacological target for the control of eating, addictive, and psychiatric behaviors. Several structure–activity relationship studies 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.<sup>1–4</sup> 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.<sup>3</sup> Current literature suggests that RXFP3 is a potential therapeutic target for psychiatric disorders such as anxiety and depression.<sup>5</sup> Central administration of RXFP3 agonists into rats increases feeding behaviors and that of antagonists inhibits food consumption and addictive behaviors.<sup>6</sup> 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.<sup>1,7</sup> H3 relaxin structure–activity relationship (SAR) data suggest that the B-chain of H3 relaxin is the sole determinant of RXFP3 activity.<sup>8,9</sup> Briefly, R8, R12, I15, R16, and F20 are involved in receptor binding and R26 and W27 are involved in receptor activation (Figure 1).<sup>10</sup> A recombinant approach has been developed for efficient preparation of H3 relaxin by the Guo group.<sup>11</sup> 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.<sup>12</sup> This high yielding analogue exhibits H3

relaxin-like activity, both in vitro and in vivo.<sup>12</sup> 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<sup>13</sup> and RXFP4<sup>14,15</sup> 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.<sup>16</sup> 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.<sup>12</sup> 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).

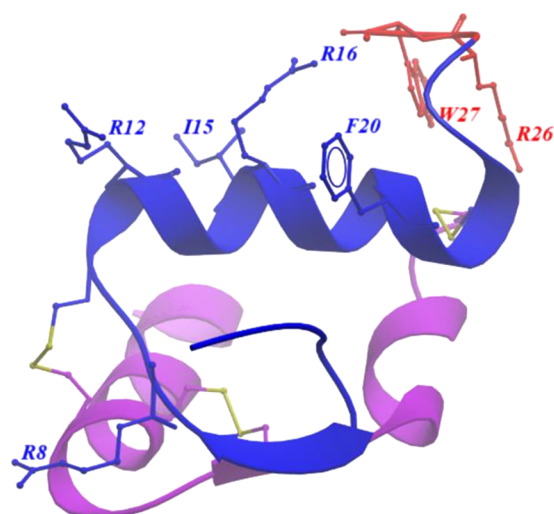
To study the effects of C-terminal modifications of the A2 peptide, we chemically synthesized four novel analogues (A2 B-alcohol, A2 B-ester, A2 B-carbamate, and A2 B-amide)

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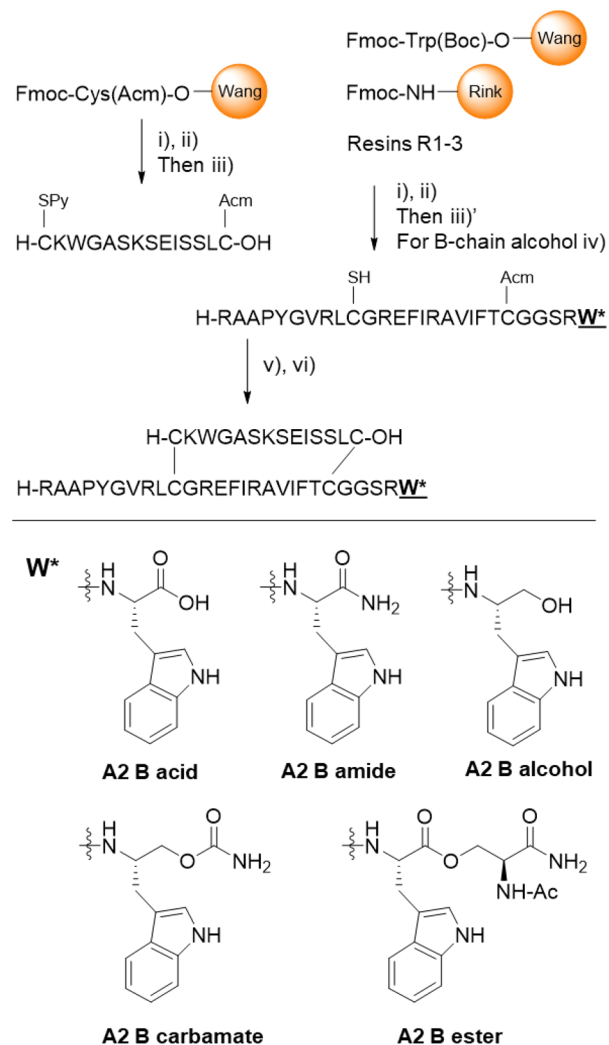


**Figure 1.** Human relaxin-3 representation based on the NMR data highlighting all the key residues. Purple, A-chain; blue, B-chain highlighting the amino acids involved in RXFP3 binding; red, the C-terminal dipeptide important in RXFP3 activation; yellow, disulfide bridges.

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.<sup>12</sup> 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.<sup>17</sup> Then, an intramolecular O-to-N acyl transfer<sup>18</sup> 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.<sup>19</sup> 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<sup>18</sup> 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).<sup>20,21</sup> 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)<sup>a</sup>



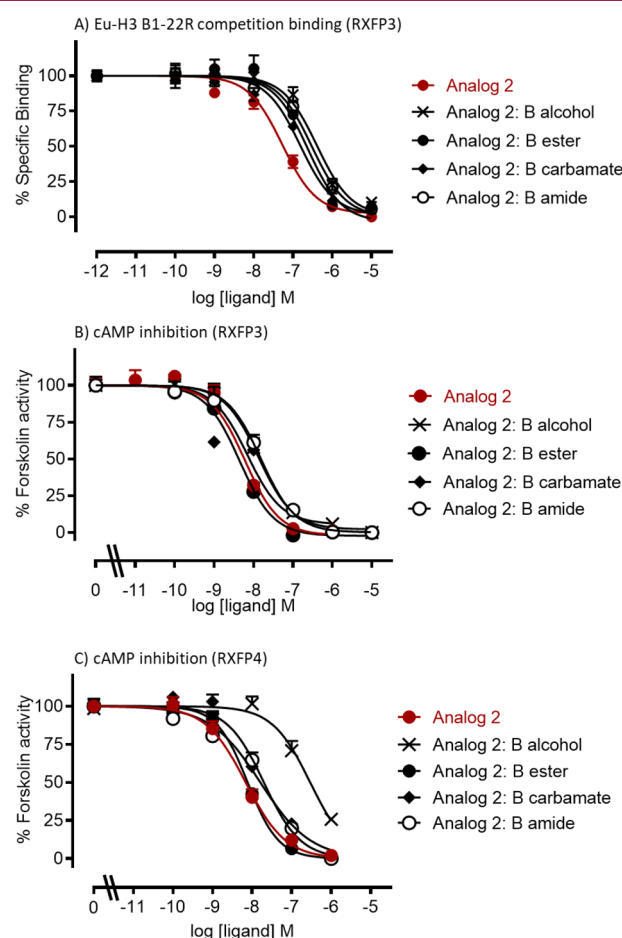
<sup>a</sup>Synthetic conditions: (i) 20% piperidine in DMF, 20 min, RT; (ii) Fmoc-AA-OH, HCTU, DIEA, DMF, 1 h, RT; (iii) TFA/DODT/TIS/H<sub>2</sub>O with 10 equiv of DPDS, 2 h, RT; (iii)' TFA/DODT/TIS/H<sub>2</sub>O, 2 h, RT; (iv) PBS/ACN (80:20) at pH 7.4, 2 h, RT; (v) addition of A-chain in 6 M GnHCl (pH 8.5) to B-chain in 6 M GnHCl (pH 5.5), 15 min, RT; (vi) I<sub>2</sub>, AcOH, 1 h, RT.

disulfide formation was performed by thiolysis 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 [ $M + H$ ]<sup>+</sup>, calcd 4536.312; A2 B-ester  $m/z$  4668.35 [ $M + H$ ]<sup>+</sup>, calcd 4662.249; A2 B-alcohol  $m/z$  4540.4907 [ $M + H$ ]<sup>+</sup>, calcd 4543.249; A2 B-carbamate: 4569.5468 [ $M + H$ ]<sup>+</sup>, calcd 4563.249; A2 B-amide:  $m/z$  4539.4366 [ $M + H$ ]<sup>+</sup>, calcd 4535.312. The purity of these peptides ( $\geq 98\%$ ) 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)



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,<sup>13</sup> 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<sup>13</sup>, 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).

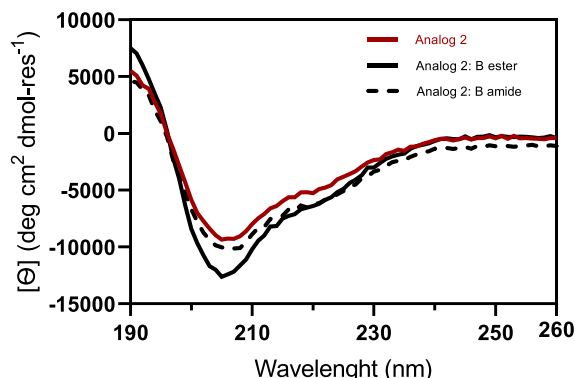
It is possible that the less restrained structure of the truncated peptide, which lacks a large portion of the chain and one disulfide bond, allows A2 to have a more adaptable binding mode. Similar effects are seen for linear and unstructured antagonists of RXFP3.<sup>22</sup> RXFP3 mutagenesis and modeling data<sup>23</sup> 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.<sup>23,24</sup> 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<sup>a</sup>

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

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

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  $\alpha$ -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/acsmchemlett.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<sub>2</sub>O, water; DODT, 3,6-dioxa-1,8-octanedithiol; GnHCl, guanidine

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

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