

REVIEW ARTICLE

Relaxin family peptides: structure–activity relationship studies

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The human relaxin peptide family consists of seven cystine-rich peptides, four of which are known to signal through relaxin family peptide receptors, RXFP1–4. As these peptides play a vital role physiologically and in various diseases, they are of considerable importance for drug discovery and development. Detailed structure–activity relationship (SAR) studies towards understanding the role of important residues in each of these peptides have been reported over the years and utilized for the design of antagonists and minimized agonist variants. This review summarizes the current knowledge of the SAR of human relaxin 2 (H2 relaxin), human relaxin 3 (H3 relaxin), human insulin-like peptide 3 (INSL3) and human insulin-like peptide 5 (INSL5).

LINKED ARTICLES

This article is part of a themed section on Recent Progress in the Understanding of Relaxin Family Peptides and their Receptors. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.10/issuetoc>

Abbreviations

H2 relaxin, human relaxin-2; H3 relaxin, human relaxin-3; IGF, insulin-like growth factor; INSL, insulin-like peptide; LRR, leucine-rich repeat; RXFP1–4, relaxin-family peptide receptor 1–4; SAR, structure–activity relationship; TM, transmembrane

Tables of Links

TARGETS	
GPCRs ^a	Enzymes ^b
RXFP1 receptor	ERK1
RXFP2 receptor	ERK2
RXFP3 receptor	
RXFP4 receptor	

LIGANDS	
Relaxin-1	A(4-24)(B7-24)H2
Relaxin-2	B-R1 3/17K H2 relaxin
Relaxin-3	minimised relaxin-3 analogue 2
INSL3	R3-B1-22R
INSL5 (mouse)	R3/I5
INSL5 (human)	R3(BΔ23-27)R/I5 chimeric peptide
Insulin	INSL3 B chain dimer analogue 8
IGF-1	minimised INSL5 analogue 7
IGF-2	

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^aAlexander *et al.*, 2015a,b).

Introduction

In the late 1970s, relaxin and insulin, two functionally diverse peptide hormones, were shown to be identical with respect to the distribution of its three disulfide bonds, which gave rise to the concept of the insulin superfamily (Schwabe and McDonald, 1977). Evolutionary studies revealed that insulin/relaxin-like peptides are ubiquitously found in vertebrates and non-vertebrates (Hsu, 2003; Wilkinson *et al.*, 2005; Wilkinson and Bathgate, 2007). In humans, the insulin superfamily consists of insulin, insulin-like growth factors (IGF-1 and IGF-2) and seven relaxin family peptides. The latter consists of 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 or placentin), insulin-like peptide 5 (INSL5) and insulin-like

peptide 6 (INSL6) (Hudson *et al.*, 1983; Crawford *et al.*, 1984; Adham *et al.*, 1993; Chassin *et al.*, 1995; Conklin *et al.*, 1999; Chang *et al.*, 2000; Bathgate *et al.*, 2002; Alexander *et al.*, 2015a) (Figure 1).

While in higher primates (e.g. humans and great apes) there are two forms of relaxin (relaxin 1 and relaxin 2), which are thought to be a consequence of gene duplication during primate evolution, other mammals (e.g. mouse and rat) have only one relaxin gene that produces a relaxin hormone, equivalent to relaxin 2 in humans (H2 relaxin). Early studies confirmed that relaxin (equivalent to relaxin 2 in humans) is synthesized as a pre-prohormone with four distinct regions: a signal peptide, a B-chain, a C-chain and a COOH-terminal A-chain (Hudson *et al.*, 1981; Haley *et al.*, 1982). Like relaxin (equivalent to relaxin 2 in humans), relaxin 3 and INSL3 peptides are expressed as pre-prohormones that after oxidative folding and

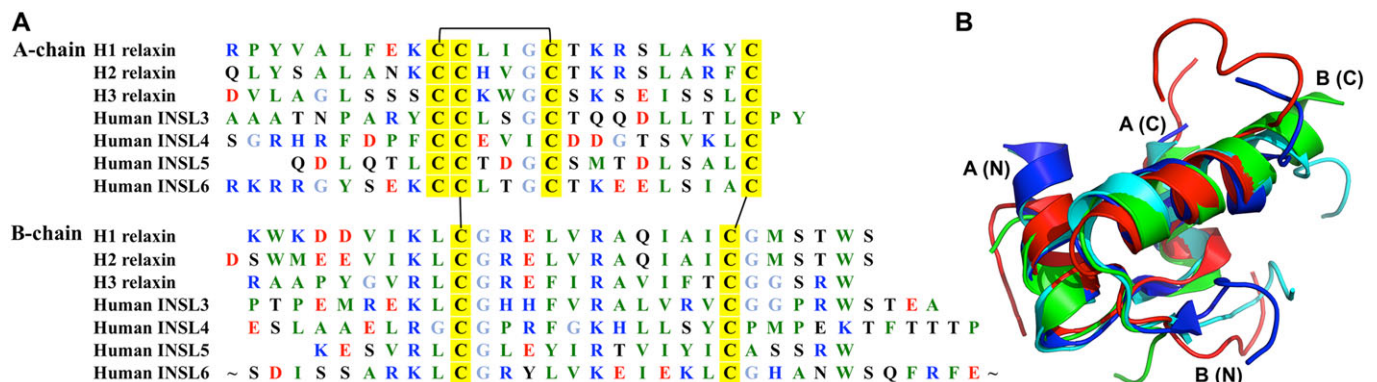


Figure 1

Comparison of relaxin family peptides. (A) Primary sequences of relaxin family peptide in humans. To illustrate the conserved features, the amino acid types are colour coded according to their nature with basic residues in blue (Arg, Lys and His), acidic residues in red (Glu and Asp), hydrophobic residues in green (Ala, Val, Ile, Leu, Phe, Trp, Tyr, Pro and Met), hydrophilic in black (Ser, Thr, Asn and Gln) and glycine in light blue. The cysteine residues are highlighted in yellow, and the disulfide bonds shown by solid lines. (B) Superposition of the NMR structures of four relaxin family peptides. H2 relaxin is shown in cyan (PDB: 2MV1), H3 relaxin is shown in blue (PDB: 2FHW), INSL3 is shown in red (PDB: 2H8B) and INSL5 is shown in green (PDB: 2KBC). The A- and B-chain N- and C-termini are labelled A (N), A (C), B (N) and B (C) respectively.

proteolytic processing result in mature hormones with two chains (A and B) linked by two inter-chain disulfide bonds and one intra-A-chain disulfide bond (Büllesbach and Schwabe, 2002; Liu *et al.*, 2003).

The relaxin peptides act upon GPCRs that are now known as relaxin family peptide (RXFP) receptors (Bathgate *et al.*, 2006a; Alexander *et al.*, 2015a). H2 relaxin, INSL3, H3 relaxin and INSL5 signal through RXFP1 (Hsu *et al.*, 2002), RXFP2 (Kumagai *et al.*, 2002), RXFP3 (Liu *et al.*, 2003) and RXFP4 (Liu *et al.*, 2005b) receptors respectively (Figure 2). The native receptors for INSL4 and INSL6 are yet to be identified (Halls *et al.*, 2007). H1 relaxin is the most recently evolved human peptide and is a pseudogene in other primate species. Notably, H2 relaxin is the ortholog of the relaxin-1 gene found with relaxin-3 in most other mammalian species (Wilkinson *et al.*, 2005), and it is presumed that H1 relaxin is also a ligand of the RXFP1 receptor. All the family members of relaxin peptides and their target receptors (RXFPs) have broad physiological roles and are important clinically [reviewed in: (Bathgate *et al.*, 2013b)]. Considering the vital roles of relaxin peptides (Table 1) in various diseases, they are of considerable importance for drug discovery and development.

All the relaxin family peptides share little sequence homology apart from the six cysteines that form three disulfide bonds (Figure 1A), and the overall fold of these peptides is similar (Figure 1B). However, the differences in their amino acid sequences have resulted in subtle changes in the length of the conserved helices and their functional selectivity towards their receptors. Detailed structure–activity relationship (SAR) studies towards understanding the role of residues in each of these chains have been reported over the years. This review summarizes the current knowledge of the SAR of H2 relaxin, H3 relaxin, INSL3 and INSL5 and how this

information has been used to develop smaller peptide agonists and antagonists of RXFP receptors.

H2 relaxin

H2 relaxin is a pleiotropic hormone with key roles in reproductive and non-reproductive processes including anti-fibrotic (Samuel *et al.*, 2011) and vasodilator effects (Du *et al.*, 2010) (Table 1). Based on its cardio-protective effects, the recombinant form of H2 relaxin is currently in Phase III clinical trials for the treatment of acute heart failure (Teerlink *et al.*, 2009; 2013). These effects of H2 relaxin are mediated through the GPCR, RXFP1 (Hsu *et al.*, 2002). H2 relaxin also cross-reacts with the RXFP2 receptor (Kumagai *et al.*, 2002), which is the cognate receptor for its related peptide, INSL3. However, most of the biological actions of H2 relaxin are thought to be mediated through RXFP1 receptors (Bathgate *et al.*, 2013b), and there is no evidence in humans that H2 relaxin mediates any of its actions through RXFP2 receptors or that H2 relaxin is associated with the biological actions of INSL3. Rodent models are of no help in this regard as rodent relaxin does not activate the RXFP2 receptor (Bathgate *et al.*, 2006b).

SAR studies

Mutation and truncation studies. A comparison of amino acid sequences of relaxin from different species reveals that there are three similar residues, R^{B13}, R^{B17} and I/V^{B20}, in the mid-region of the B-chain (Büllesbach and Schwabe, 1988). A detailed SAR study revealed the importance of R^{B13} and R^{B17} (Figure 3) for the biological effects of H2 relaxin mediated through activation of the RXFP1 receptor (Büllesbach and

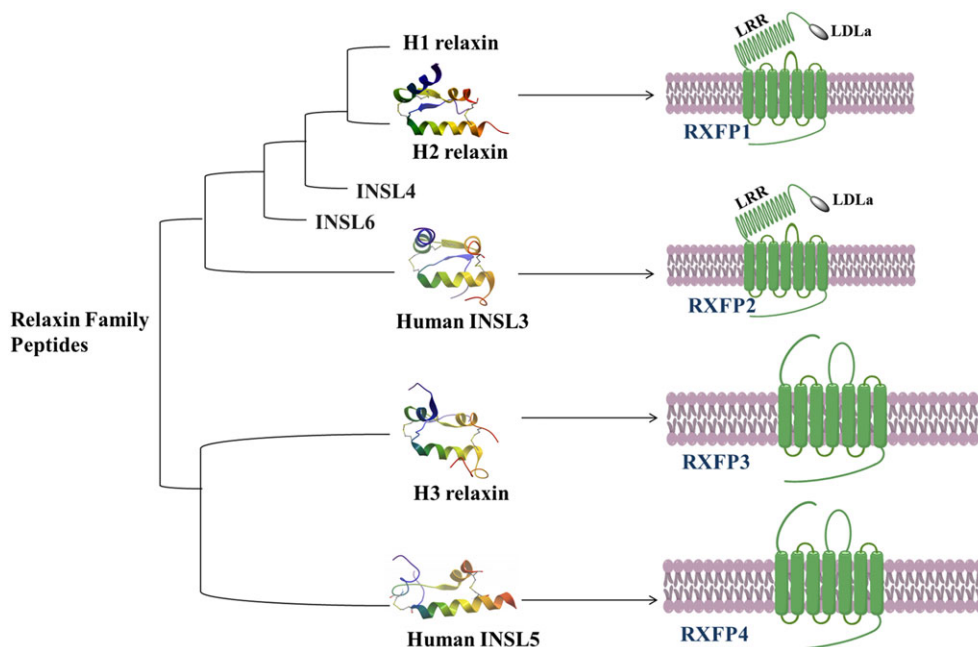


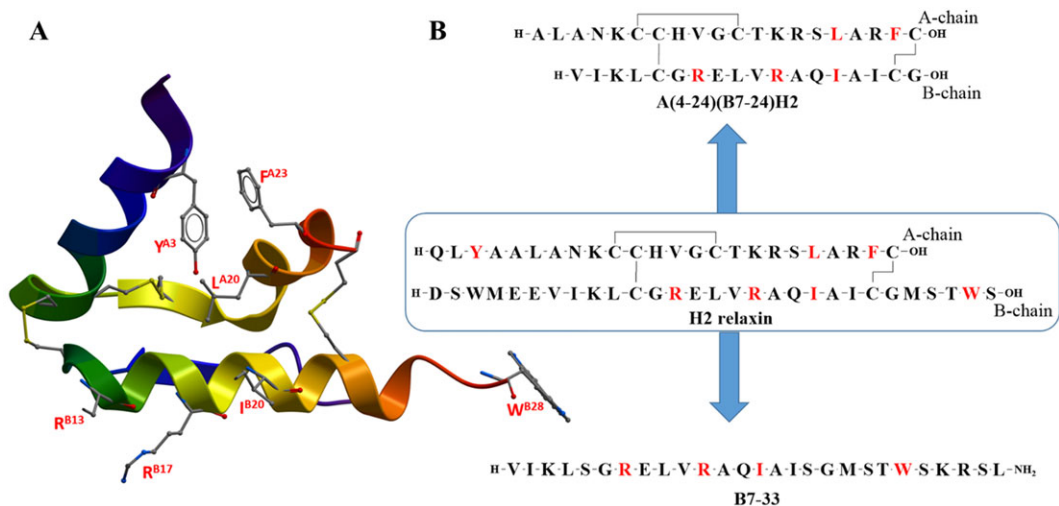
Figure 2

Peptide-receptor pairing within the relaxin family peptides. H2 relaxin (PDB: 6RLX), H3 relaxin (PDB: 2FHW), INSL3 (PDB: 2H8B) and INSL5 (PDB: 2KBC). The receptors are models (generated with ChemDraw v15.0).

Table 1

Physiological roles of relaxin family peptides in humans and other mammals and potential clinical significance

Peptide–Receptor pair	Physiological role	Clinical significance
Relaxin 1	Unknown	Unknown
Relaxin 2-RXFP1	Pregnancy roles 1. Remodelling of the uterus throughout pregnancy 2. Development of mammary nipple Cardiovascular adaptations	Heart disease Fibrosis
INSL3-RXFP2	1. Growth and development of the gubernaculum 2. Male and female germ cell survival 3. Ovarian follicle function	Infertility Birth control
Relaxin 3-RXFP3	Modulatory role in 1. Arousal 2. Feeding 3. Stress responses 4. Cognition and drug addiction	Anxiety and depression Drug addiction
INSL4	Possible role in bone development	Unknown
INSL5-RXFP4	1. Regulating appetite 2. Glucose metabolism	Obesity Anorexia Diabetes
INSL6	Possible role in male fertility	Unknown

**Figure 3**

Summary of SAR of H2 relaxin. (A) X-ray crystal structure of H2 relaxin (PDB: 6RLX) with important residue side chains (residue number labelled red). (B) Primary sequence of H2 relaxin, A(4-24)(B7-24)H2 and B7-33 (important residues are coloured red).

Schwabe, 1991; Büllesbach *et al.*, 1992). In addition, a third amino acid, I^{B20}, located one further helical turn away was identified, suggesting a three-point interaction site named a 'relaxin binding cassette' whereby B20 can be either I (Ile) or V (Val) (R^{B13}XXX R^{B17}XXI/V^{B20}) (Büllesbach and Schwabe, 2000; 2005b).

The importance of the key residues was further highlighted by the finding that an H2 analogue, B-R13/17K

H2 relaxin (Table of Links) in which two arginine residues are substituted by lysine, showed very low affinity for RXFP1 receptors (Silvertown *et al.*, 2007; Hossain *et al.*, 2010). Interestingly, this peptide showed antagonistic properties in cells endogenously expressing RXFP1 receptors and *in vivo* (Silvertown *et al.*, 2007; Hossain *et al.*, 2010). This receptor-binding cassette (R^{B13}XXX R^{B17}XXI/V^{B20}) present within the mid-region of the B-chain is considered to be responsible

for the primary binding interaction between H2 relaxin and the leucine-rich repeat (LRR) region of the large extracellular domain of the RXFP1 receptor (Büllesbach and Schwabe, 2005b; Scott *et al.*, 2009).

A secondary interaction involving the A-chain of H2 relaxin and the transmembrane (TM) exoloops of the RXFP1 receptor has been suggested (Sudo *et al.*, 2003; Hossain *et al.*, 2008b; 2012; Diepenhorst *et al.*, 2014; Sethi *et al.*, 2016). Recent mutation and structural studies on RXFP1 receptors demonstrate that H2 relaxin binding to the linker between the LDLa module and the LRR domain stabilizes the α -helical conformation of this linker and positions residues of the linker and the LDLa module to bind the TM domain to activate the RXFP1 receptor (Sethi *et al.*, 2016). The potential A-chain residues involved in this interaction and the potential interaction with the TM domain are unknown. Recent mutation studies of the A-chain of the ligand indicated that binding is not driven by a single amino acid, although residues Y^{A3}, L^{A20} and F^{A23} (Figure 3) appeared to contribute (Chan *et al.*, 2012). Interestingly, these residues are also important drivers of the affinity and activity of H2 relaxin for RXFP2 receptors with additional minor contributions from K^{A9}, H^{A12}, K^{A17}, R^{A18} and R^{A22} (Chan *et al.*, 2012) (Figure 3). A recent study also showed that, in addition to R^{B13}XXX R^{B17}XXI^{B20}, the W^{B28} residue in the B-chain contributes to the H2–RXFP2 but not the H2–RXFP1 interaction (Chan *et al.*, 2013).

Role of free C-terminus. The effect of modifying the C-terminus of both the A- and B-chain of H2 relaxin on receptor binding and activation has been investigated (Haugaard-Kedström *et al.*, 2015). An amidated H2 relaxin (with both the A- and B-chain C-termini amidated) was chemically synthesized and compared with native H2 relaxin (with both the C-termini as free acids). These peptides were found to be structurally very similar and equipotent at the RXFP1 receptor (Haugaard-Kedström *et al.*, 2015). Interestingly, the amide analogue did not self-associate into the characteristic dimers, as seen in the crystal structure of native H2 relaxin (Eigenbrot *et al.*, 1991). This indicates that the monomeric form of the hormone is pharmacologically active and that, unlike several other members of the family (Shabanpoor *et al.*, 2013; Patil *et al.*, 2016a), a free C-terminus is not required for its activity on RXFP1 receptors (Haugaard-Kedström *et al.*, 2015).

Minimum active structure. Extensive truncation studies on H2 relaxin were undertaken to elucidate the minimum length required for its activity on the RXFP1 receptor. N-terminal truncation of the A-chain of up to four residues did not have a prominent effect on binding and activity. Further truncation caused reduced activity, but this was significantly restored by non-native residue substitution (Hossain *et al.*, 2008b). This indicated the importance of the α -helix at the N-termini of the A-chain for structural integrity (Büllesbach and Schwabe, 1986; Büllesbach and Schwabe, 1987; Hossain *et al.*, 2008b). Truncation of the B-chain was tolerated by six residues from the N-terminus and five residues from the C-terminus (Hossain *et al.*, 2011). These truncation studies resulted in the identification of A(4-24)(B7-24)H2 (also known as mini-H2 relaxin) which is one-third smaller in

size (Hossain *et al.*, 2011) compared with native H2 relaxin (Figure 3B). While this minimized peptide is a full agonist at RXFP1 receptors its potency is ~100-fold lower than H2 relaxin (Hossain *et al.*, 2011).

As most of the residues associated with high affinity binding are within the B-chain, it was predicted that B-chain-only analogues could be developed. Preliminary single-chain peptides were found to be notoriously insoluble and inactive (Del Borgo *et al.*, 2005). However, by producing a B-chain with an N-terminal truncation and C-terminal elongation, a soluble peptide B7-33 was recently developed (Hossain *et al.*, 2016) (Figure 3B). Initial testing in HEK-293T cells over-expressing RXFP1 receptors and THP1 cells natively expressing RXFP1 receptors suggested that the peptide was a weak agonist. However, when the peptide was tested in rat renal myofibroblasts (from injured kidneys) and human cardiac fibroblasts (native RXFP1-expressing cells) for its ability to promote matrix metalloproteinase 2 (MMP2), a collagen-degrading enzyme, it was found to exhibit similar potency to H2 relaxin (Hossain *et al.*, 2016). Importantly, like H2 relaxin, B7-33 reversed lung and heart fibrosis *in vivo* in rat and mouse models (Hossain *et al.*, 2016) and was a potent agonist *in vivo* in rodents. B7-33 also potently activated ERK phosphorylation in both rat and human fibroblasts while only showing weak activity in HEK-RXFP1 cells. Thus, B7-33 seems to demonstrate fibroblast-specific actions and may be a lead molecule for further development as a therapeutic for fibrosis and related disorders. This peptide also demonstrates that there is still much to be learnt as to the mechanism of action of relaxin in specific cell types.

H3 relaxin

H3 relaxin is a neuropeptide with its highest level of expression in the CNS, particularly in a specific nucleus in the hind-brain, the nucleus incertus (Bathgate *et al.*, 2002). Recent work suggested that H3 relaxin has a modulatory role in arousal, feeding, stress responses, cognition and drug addiction (Table 1) (Ryan *et al.*, 2013a,b; Smith *et al.*, 2014). Although H3 relaxin can activate RXFP1 (Hsu *et al.*, 2002) and RXFP4 (Liu *et al.*, 2005b) receptors, it is believed that the actions of H3 relaxin in the brain are mediated through its cognate GPCR, RXFP3 (Liu *et al.*, 2003). However, pharmacological studies in rodents must take the receptor cross-reactivity into account; hence, much effort has been put into developing RXFP3 receptor-selective agonists. Evidence from studies using such specific agonists suggest that the H3 relaxin/RXFP3 signalling system is a potential therapeutic target for psychiatric disorders, such as anxiety and depression, and drug addiction (Ryan *et al.*, 2013a,b; Smith *et al.*, 2014).

SAR studies

Mutation and truncation studies. There are distinct differences in the residues and modes of binding and activation of the different receptors – RXFP1, RXFP3 and RXFP4. Recent studies demonstrate that deletion of the N-terminal seven residues of the B-chain does not significantly affect the ligand binding activity for RXFP1, RXFP3 or RXFP4 receptors, thus confirming that the

N-terminus of the peptide does not play a critical role in binding (Kuei *et al.*, 2007; Liu *et al.*, 2009).

H3 relaxin binds to RXFP1 receptors using similar residues to H2 relaxin; hence, H3 relaxin contains an RXXXRXXI motif (R^{B12}, R^{B16} and I^{B19}), and mutation studies (Kuei *et al.*, 2007; Liu *et al.*, 2009) confirm that this is the driver of its high affinity binding to RXFP1 receptors (Büllesbach and Schwabe, 2000). In contrast, the binding of H3 relaxin to RXFP3 receptors requires two distinct domains within the peptide with the core B-chain residues (R^{B8}, R^{B12}, I^{B15}, R^{B16} and F^{B20}) involved in binding and the C-terminal R^{B26} and W^{B27} driving the activation the RXFP3 receptor (Kuei *et al.*, 2007; Liu *et al.*, 2009). The same residues, except R^{B12}, are important for its interaction with the RXFP4 receptor. Figure 4A shows the solution NMR structure of H3 relaxin (Rosengren *et al.*, 2006a) where the side chains of important residues are highlighted in red. Molecular modelling and receptor mutation studies reveal most of the complementary residues in the RXFP3 receptor. Specifically, R^{B12}, R^{B16}, R^{B26} and W^{B27} residues of H3 relaxin interact with E²⁴⁴, D¹⁴⁵, D¹⁴¹ and W¹³⁸ residues in the RXFP3 receptor respectively (Bathgate *et al.*, 2013a; Hu *et al.*, 2016b).

In an attempt to develop a receptor-specific agonist, the A-chain of H3 relaxin was replaced with that from INSL5 and the resulting chimeric R3/I5 analogue exhibited selectivity for RXFP3 and RXFP4 receptors by substantially reducing the affinity for the RXFP1 receptor (Liu *et al.*, 2005a). Additionally, by truncating the C-terminus of the B-chain of R3/I5 by five residues and adding a C-terminal arginine, an RXFP3/4 antagonist, R3(BΔ23-27)R/I5 chimeric peptide was achieved (Kuei *et al.*, 2007).

Role of free C-terminus. The effect of modifying the C-terminus of H3 relaxin on receptor binding and activity has been investigated (Shabanpoor *et al.*, 2013). Amidation of the C-terminus of the B-chain led to a significant drop in the binding and activity of the peptide at both RXFP3 and RXFP4 receptors. However, modification of the C-terminus of the A-chain did not have any effect on activity (Shabanpoor *et al.*, 2013). It is

clear that the key W^{B27} must be free acid and that these residues probably insert into the orthosteric binding pocket of RXFP3 and RXFP4 receptors in order to activate them. More specifically, molecular modelling and receptor mutations studies suggest that C-terminal W^{B27} and R^{B26} residues of H3 relaxin interact with the W¹³⁸ and E¹⁴¹ residues of the RXFP3 receptor respectively (Bathgate *et al.*, 2013a; Hu *et al.*, 2016b).

Minimum active structure. Extensive truncation studies on H3 relaxin were undertaken to understand the minimum length required for receptor activity. A minimized analogue of H3 relaxin, minimized relaxin-3 analogue 2 (Table of Links) was achieved by removal of the intra-molecular disulfide bond within the A-chain and by truncation of the N-terminus of the A-chain (Shabanpoor *et al.*, 2012). Like R3/I5, the resulting peptide also exhibited selectivity for RXFP3 and RXFP4 over RXFP1 receptors, and its activation of RXFP3 receptors *in vivo* led to increased feeding and significantly reduced anxiety-like behaviour in adult rats (Shabanpoor *et al.*, 2012).

Based on the current SAR knowledge, it is evident that the B-chain of H3 relaxin contains all the residues important for both binding and activation of the RXFP3 receptor. This is further supported by the finding that the B-chain alone of H3 relaxin is capable of binding to and activating the RXFP3 receptor (Liu *et al.*, 2003), albeit with low affinity and potency. Thus, it is possible to engineer B-chain-only agonists and antagonists with high affinity and potency. Consequently, single-B-chain-only analogues, stapled R3 B10-27 (Hojo *et al.*, 2016) and R3-B1-22R (Haugaard-Kedström *et al.*, 2011) have been designed and developed as a high affinity agonist and antagonist respectively. The stapled R3 B10-27 agonist was demonstrated to be a full agonist for both cAMP inhibition and pERK activation (Hojo *et al.*, 2016). Additionally, *in vivo* studies demonstrated that the stapled R3 B-chain analogue significantly stimulated food intake to the same level as H3 relaxin (Hojo *et al.*, 2016). In contrast, R3-B1-22R antagonized relaxin-3/RXFP-induced increase in feeding (Haugaard-Kedström *et al.*, 2011). Thus, stapled R3 B10-27 and B1-22R represent

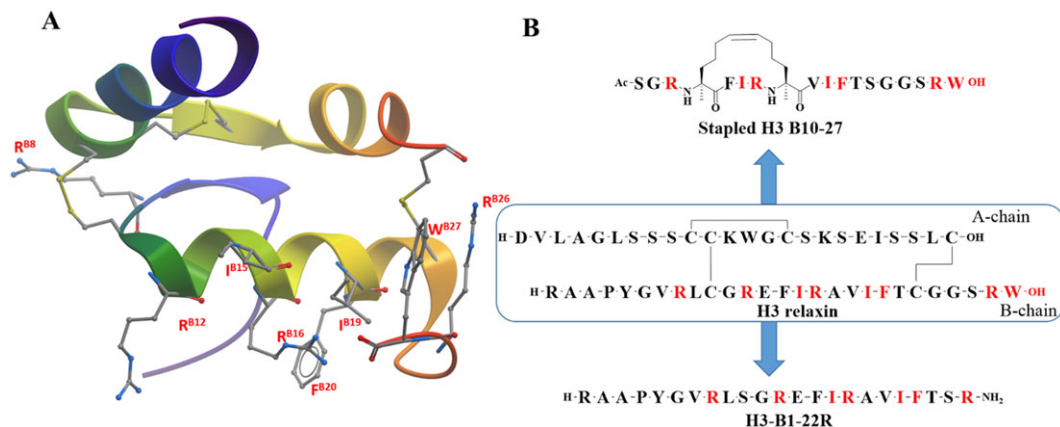


Figure 4

Summary of SAR of H3 relaxin. (A) NMR structure of H3 relaxin (PBD: 2FHW) with important residue side chains (residue number labelled red). (B) Primary sequences of H3 relaxin, stapled H3 B10-27 and H3-B1-22R (important residues are coloured red).

excellent tools for probing the pharmacology of H3 relaxin-RXFP3 and are lead molecules for further development for potential clinical use.

Human INSL3

INSL3 is highly expressed in the Leydig cells of the testis (Adham, Burkhardt, Benahmed & Engel, 1993) and has a critical role in testis descent. INSL3 knockout mice are cryptorchid and as a result infertile (Nef and Parada, 1999). INSL3 plays an important role in the development of the gubernaculum and also seems to have a role in the maintenance of fertility in females (Spanel-Borowski *et al.*, 2001; Glister *et al.*, 2013). The effects of INSL3 are mediated through the GPCR, RXFP2 (Kumagai *et al.*, 2002).

SAR studies

Mutation and truncation studies. INSL3 like H2 relaxin binds to the LRR domain of the RXFP2 receptor using B-chain residues, although these residues are different from those used by H2 relaxin to bind to RXFP1 receptors. More specifically, the residues H^{B12}, R^{B16}, V^{B19}, R^{B20} and W^{B27} present are responsible for RXFP2 receptor binding (Büllesbach and Schwabe, 2006; Rosengren *et al.*, 2006b). The corresponding residues in the LLR domain of the RXFP2 receptor that interact with the B-chain of INSL3 were also determined and characterized by receptor mutation studies (Scott *et al.*, 2007). INSL3 also requires the A-chain for activity although unlike H2 relaxin the A-chain contains a domain that is essential for activation but not binding. Hence, while no single amino acid side chain within the INSL3 A-chain was found to be critical for RXFP2 receptor binding and activity, the potency of the INSL3 peptide was found to be driven predominantly by a cluster of four N-terminal residues (residues A6–A9) in the A-chain (Bathgate *et al.*, 2012; Büllesbach and Schwabe, 2005a). Additionally, the amide bond between residues 8 and 9 (R^{A8} and Y^{A9}) at the N-terminus of the A-chain was reported to be important for activation of the RXFP2 receptor

(Büllesbach and Schwabe, 2012). Truncations at the N-terminus of INSL3 produced a high affinity RXFP2 antagonist (Büllesbach and Schwabe, 2005a). Figure 5 shows the solution of the NMR structure of INSL3 (Rosengren *et al.*, 2006b).

It is intriguing that H2 relaxin binds to and activates both RXFP1 and RXFP2 receptors whereas INSL3 is a selective ligand for RXFP2 receptors. Molecular modelling and receptor mutation studies suggest that H2 relaxin binds to the RXFP2 receptor utilizing a hybrid H2 relaxin/INSL3 binding site comprising some of the INSL3–RXFP2 interactions and also utilizing the partially conserved RXFP1–H2 relaxin binding site in the RXFP2 receptor (Scott *et al.*, 2009).

Minimum active structure. The SAR data suggest that parts of both the A and B-chain are necessary for agonistic activity on the RXFP2 receptor. Consequently, a minimized INSL3 was designed and synthesized, INSL3 A5-26/B7-27 (Figure 5), that retained near native agonistic activity (Bathgate *et al.*, 2012). This peptide contains a truncated A- and B-chain and is almost one-quarter smaller than the native peptide and thus easier and cheaper to make and represents a potential lead peptide for further development as a fertility regulator.

As the B-chain contains the key residues for binding the LRR domain, INSL3 B-chain analogues have been designed and developed as functional antagonists. The lack of structural support from the A-chain resulted in poor affinity of the B-chain-specific antagonist. Thus, a synthetic parallel dimer of the INSL3 B-chain was developed that exhibited high affinity for the RXFP2 receptor (Shabanpoor *et al.*, 2011) and antagonized INSL3-mediated cAMP signalling through this receptor. Further refinement by truncation of 18 residues yielded a minimized analogue, INSL3:B10-24/B1-31, (Figure 5) that retained full binding affinity and INSL3 antagonism. This is an attractive lead for *in vivo* evaluation as an inhibitor of male and female fertility (Del Borgo *et al.*, 2006).

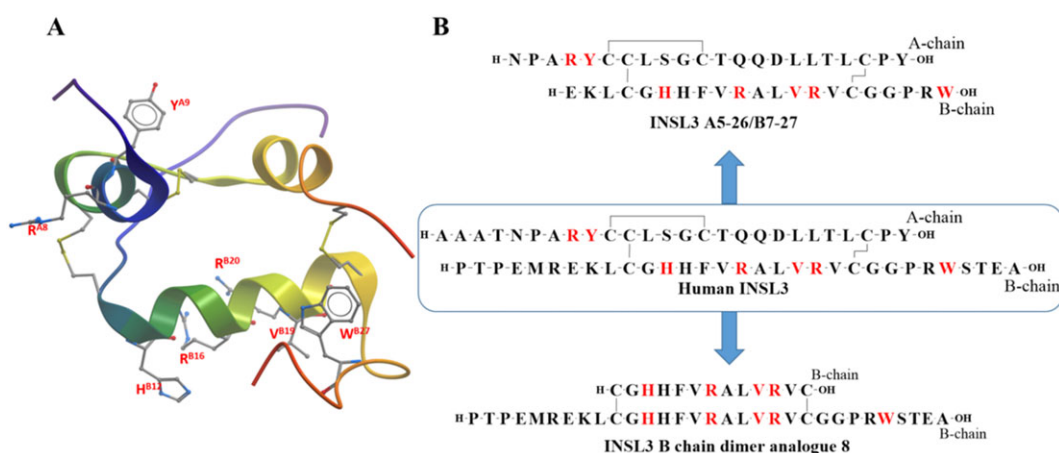


Figure 5

Summary of SAR of human INSL3. (A) NMR structure of human INSL3 (PDB: 2H8B) with important residue side chains (residue number labelled red). (B) Primary sequence of human INSL3, INSL3 A5-26/B7-27 and INSL3 B chain dimer analogue 8 (important residues are coloured red).

Human INSL5

INSL5 was originally identified as a novel peptide hormone that is highly expressed in the gut (Conklin *et al.*, 1999). Subsequent studies identified its cognate receptor as the GPCR, RXFP4 (Liu *et al.*, 2005b). It is a hormonal product of colonic L-cells, and recent data suggest that INSL5 is an orexigenic hormone that plays a physiological role in driving food intake (Table 1) (Grosse *et al.*, 2014). In addition, another recent study suggests that INSL5 augments glucose-stimulated insulin secretion from MIN6 pancreatic beta cells and GLP-1 release from murine enteroendocrine GLUTag cells, indicating that INSL5–RXFP4 interaction may constitute a novel incretin axis involved in the regulation of metabolism and energy balance (Luo *et al.*, 2015).

SAR study

Mutation and truncation studies. Human INSL5 is one of the most difficult peptides to produce chemically as both A and B chains were unusually resistant to standard synthesis protocols and required highly optimized conditions for their production (Hossain *et al.*, 2008a). A preliminary SAR study, therefore, was carried out using a mouse homologue of INSL5, which was thought to be easier to assemble chemically for SAR studies. Interestingly, mouse INSL5 was found to be more potent than human INSL5 on human RXFP4 receptors (Belgi *et al.*, 2011). On the basis of the H3 relaxin–RXFP3 structure–function studies, two analogues of mouse INSL5 were produced (Belgi *et al.*, 2013a,b). The analogue in which R^{B24} and W^{B25} were substituted with alanine completely abolished its interaction with the RXFP4 receptor (Belgi *et al.*, 2013a,b). This observation is somewhat different to the H3 relaxin–RXFP3 interaction where mutation at equivalent residues of the ligand (or deletion of these residues) only partially reduced binding, but abolished activation (Kuei *et al.*, 2007). A second analogue from this study in which three residues K^{B6}, R^{B13} and Y^{B18} were substituted with alanine showed a significant loss in activity, which correlated with binding, suggesting that K^{B6}, R^{B13} and Y^{B18} each play an important role in the binding of INSL5 to the RXFP4 receptor.

To elucidate the mode of interaction of INSL5 with the RXFP4 receptor, a charge-exchange mutagenesis study was performed where the positively charged residues in the INSL5 B-chain were substituted with negatively charged residues (Wang *et al.*, 2014). This study confirmed that R^{B13} and R^{B23} play important roles in INSL5 binding to RXFP4 receptors and its activity. The solution for the NMR structure (Haugaard-Jönsson *et al.*, 2009) indicates that the B-chain of INSL5 has an extended C-terminal α -helix compared with H3 relaxin (Figure 6A). In order to investigate the role of this extended α -helix, an analogue where A^{B20} and S^{B21} were substituted by glycine was studied. The results indicated that a rigid α -helical conformation at the C-terminus of INSL5 B-chain is important for its interaction with the RXFP4 receptor (Wang *et al.*, 2014). While H3 relaxin cross-reacts with other receptors including RXFP4 receptors, INSL5 is very selective (RXFP4) and does not interact with RXFP3 receptors. The mechanism by which INSL5 distinguishes RXFP4 and RXFP3 receptors has recently been identified (Hu *et al.*, 2016a). The authors have identified four determinants (E^{B2}, L^{B9}, Y^{B17} and a rigid B-chain C-terminus) on INSL5 that are responsible for its inactivity at RXFP3 receptors (Hu *et al.*, 2016a).

Role of the C-terminus. The role of the C-terminus of INSL5 in its binding to and activation of the RXFP4 receptor has been investigated (Belgi *et al.*, 2011; Patil *et al.*, 2016a). Modification of the C-terminus of the A-chain did not have any effect on activation. However, the free C-terminus of the B-chain is important for its activation of the RXFP4 receptor (Patil *et al.*, 2016a), similar to what was seen for H3 relaxin at RXFP3 receptors. The loss of binding and activation due to C-terminal W^{B25} mutation (Belgi *et al.*, 2013a,b) or amidation (Patil *et al.*, 2016a) suggest that the W^{B25} residue cannot be replaced or modified.

Minimum active structure. The recent SAR studies on mouse INSL5 led to the design and synthesis of a simplified mouse analogue (mouse INSL5: A8-21) (Belgi *et al.*, 2013a,b). This analogue showed similar affinity and potency to human INSL5. However, the human orthologue of this peptide (minimized INSL5 analogue 7; Table of Links) was found to

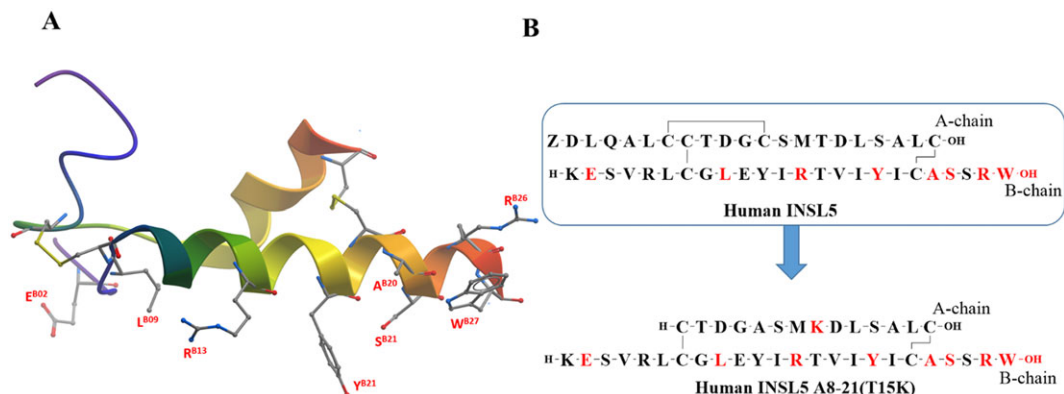


Figure 6

Summary of SAR of human INSL5. (A) NMR structure of human INSL5 (PDB: 2KBC) with important residue side chains (residue number labelled red). (B) Primary sequences of human INSL5 and hINSL5 A8-21(T15K) (important residues are coloured red).

be significantly less potent (Belgi *et al.*, 2013a,b). A specific residue, K^{A15}, that contributed to the high potency of mouse INSL5 was identified, and a minimized human INSL5 orthologue human INSL5:A8-21K15 was engineered (Figure 6B) (Patil *et al.*, 2016b). This analogue exhibited near native hINSL5-like affinity for the RXFP4 receptor and was a potent full agonist of both cAMP inhibition and pERK activation. The minimized analogue is much easier to assemble in large quantities and thus very useful as a readily available tool to analyse the physiological role of RXFP4 receptors.

SAR of H1 relaxin, INSL4 and INSL6

The physiological role of H1 relaxin is unclear. To date, a native human relaxin-1 has not been isolated. However, a synthetic H1 relaxin was shown to have similar biological properties and potency to H2 relaxin at the RXFP1 receptor (Tan *et al.*, 1998). There is little information available for INSL4–6, and also, their target receptors are yet to be identified. INSL4 is highly expressed in the placenta (Chassin *et al.*, 1995) and was shown to play a role in bone development (Laurent *et al.*, 1998). INSL6 is found predominantly in the testis (Lok *et al.*, 2000), and INSL6 knockout mice showed a marked reduction in sperm numbers (Burnicka-Turek *et al.*, 2009) and motility suggesting a potential role of INSL6 in male fertility.

Conclusion

There is a considerable similarity in the tertiary structure between members of the relaxin family of peptides. However, the different peptides clearly utilize distinct residues and modes of interaction with their native receptors. Detailed SAR studies have identified key residues in the A- and B-chain of the peptides involved in both binding and activation. The B-chain of H3 relaxin is the sole determinant for its binding to and activation of RXFP3 receptors, and thus, it was possible to replicate the B-chain and achieve a high affinity single-chain agonist and antagonist. While H3 relaxin binds to and activates both RXFP3 and RXFP4 receptors in a similar manner, INSL5 interacts with the RXFP4 receptor in a different way. Although very little is known about the interaction of INSL5 with the RXFP4 receptor, current SAR data suggest that the B-chain is responsible for both its binding to and activation of the RXFP4 receptor. Although a simplified RXFP4 agonist has been developed based on both the A- and B-chain, a single-chain RXFP4 ligand with high affinity might be not very far from reality. The B-chain of INSL3 is important for its binding to the RXFP2 receptor, and the A-chain is important for activation of this receptor. As a result, high affinity agonists with minimized A- and B-chains and antagonists with B-chain dimers have been developed. The interaction of H2 relaxin with the RXFP1 receptor has proved to be much more complex. Studies over many years have suggested that both the A- and B-chain are essential for activity, which has made it difficult to produce smaller agonists and antagonists. However, the recent discovery that a B-chain only analogue is a potent cell-specific agonist of the RXFP1

receptor highlights that there is still a lot to learn about the interaction of H2 relaxin with the RXFP1 receptor. Further work in this area must take into account the clear cell type-specific actions of relaxin analogues.

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Author contributions

N.A.P, K.J.R., J.D.W., R.A.D.B. and M.A.H. wrote the manuscript. F.S. revised and edited the manuscript. R.A.D.B. and M.A.H. designed the lay out and sequences of the headlines of the manuscript. N.A.P and M.A.H. designed and made the figures.

Conflict of interest

The authors declare no conflicts of interest.

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