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REVIEW ARTICLE THEMED ISSUE

Relaxin family peptides: Structure-activity relationship studies

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Abstract:

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. Considering the vital roles these peptides play in physiology and 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 utilised for design of antagonists and minimised agonist variants. This review summarizes the current knowledge of the SAR of human relaxin 2 (H2 relaxin), human relaxin 3 (H3 relaxin), human INSL3 and human INSL5.

Abbreviation:

H2 relaxin, human relaxin-2; H3 relaxin, human relaxin-3; INSL3, insulin-like peptide 3; INSL4, insulin-like peptide 4; INSL5, insulin-like peptide 5; INSL6, insulin-like peptide 6; IGF, insulin-like growth factor, GPCR, G-protein coupled receptor; RXFP1-4, relaxin-family peptide receptor 1-4; ERK, extracellular-signalregulated kinase; cAMP, 3′,5′-cyclic adenosine monophosphate; SAR, structure-activity relationship; LRR, leucine-rich repeat; ECD, extracellular domain; TM, transmembrane; LDL, low density lipoprotein;

Introduction

In the late 1970s, relaxin and insulin, two functionally-diverse peptide hormones, were shown to be identical with respect to its three disulfide bond distribution which gave rise to the concept of the insulin superfamily (Schwabe & McDonald, 1977). Evolutionary studies revealed that insulin/relaxin-like peptides are ubiquitously found in vertebrates as well as non-vertebrates (Hsu, 2003; Wilkinson & Bathgate, 2007; Wilkinson et al., 2005) In humans, the insulin superfamily consists of insulin, insulin-like growth factors (IGF-I and IGF-II) 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) (Table of Links) (Adham et al., 1993; Bathgate et al., 2002; Chang et al., 2000; Chassin et al., 1995; Conklin et al., 1999; Crawford et al., 1984; Hudson et al., 1983; Alexander et al., 2015) (Figure 1).

While in higher primates (e.g. humans, great apes etc) 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, rat etc) 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 (Haley et al., 1982; Hudson et al.,1981). Like relaxin (equivalent to relaxin 2 in humans), relaxin 3 and INSL3 peptides are expressed as pre-prohormones that after oxidative folding and proteolytic processing result in mature hormones with two chains (A and B) linked by two inter-chain disulfide bond and one intra-A-chain disulfide bond (Büllesbach & Schwabe, 2002; Liu et al., 2003).

The relaxin peptides act upon G protein-coupled receptors that are now known as relaxin family peptide (RXFP) receptors (Bathgate et al., 2006a; Alexander et al., 2015). 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), respectively (Table of Links; 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 RXFP1. All the family members of relaxin peptides and their target receptors (RXFPs) have wide physiological roles and clinical significance (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.

While all relaxin family peptides share little sequence homology apart from six cysteines that form three disulfide bonds (Figure 1A), 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-function relationship studies (SAR) 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 vasodilatory actions (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., 2013; Teerlink et al., 2009). These effects of H2 relaxin are mediated through the G protein-coupled receptor, RXFP1 (Hsu et al., 2002). H2 relaxin also cross-reacts with RXFP2 (Kumagai et al., 2002) which is the cognate receptor for related peptide, INSL3. Most of the biological actions of H2 relaxin are thought to be mediated through RXFP1 (Bathgate et al., 2013b) and there is no evidence in humans that H2 relaxin mediates any of its actions through RXFP2 or that H2 relaxin is

associated with the biological actions of INSL3. Rodent models do not assist in this regard as rodent relaxin does not activate RXFP2 (Bathgate et al., 2006b).

SAR studies

Mutation and truncation studies: 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 & Schwabe, 1988). A detailed SAR study revealed the importance of R^{B13} and R^{B17} (Figure 3) for the biological function of H2 relaxin for RXFP1 activity (Büllesbach & 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 & Schwabe, 2000; Büllesbach & Schwabe, 2005b).

The importance of the key residues was further highlighted by the fact that a 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 (Hossain et al., 2010; Silvertown et al., 2007). Interestingly, this peptide showed antagonistic property in cells endogenously expressing RXFP1 and *in vivo* (Hossain et al., 2010; Silvertown et al., 2007). 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 (ECD) of RXFP1 (Büllesbach & Schwabe, 2005b; Scott, Tregear & Bathgate, 2009).

A secondary interaction involving the A-chain of H2 relaxin and the transmembrane (TM) exoloops of RXFP1 has been suggested (Diepenhorst et al., 2014; Hossain et al., 2008b; Hossain, Wade & Bathgate, 2012; Sethi et al., 2016; Sudo et al., 2003). Recent mutation and structural studies on RXFP1 demonstrate that H2 relaxin binding to the linker between the low density lipoprotein (LDL)a 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 RXFP1 (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 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 contribute to the H2–RXFP2 but not the H2-RXFP1 interaction (Chan et al., 2013).

Role of free C-terminus: The effect of modification of the C-terminus of both 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 to 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 (Patil et al., 2016a; Shabanpoor, Bathgate, Wade & Hossain, 2013), a free C-terminus is not required for RXFP1 activity (Haugaard-Kedström et al., 2015).

Minimum active structure: Extensive truncation studies on H2 relaxin were undertaken to understand the minimum length required for RXFP1 activity. N-terminal truncation of the A-chain up to 4 residues did not have a prominent effect on binding and activity. Further truncation caused reduced activity, but this was significantly rescued 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 & Schwabe, 1986; Büllesbach & Schwabe, 1987; Hossain et al., 2008b). Truncation of the B-chain was tolerated by 6 residues from the N-terminus and 5 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 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 overexpressing RXFP1 and THP1 cells natively expressing RXFP1 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, 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 central nervous system, particularly in a specific nucleus in the hindbrain, 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; Ryan et al., 2013b; Smith et al., 2014). Although H3 relaxin can activate RXFP1 (Hsu et al., 2002) and RXFP4 (Liu et al., 2005b), it is believed that the actions of H3 relaxin in the brain are mediated through its cognate G protein-coupled receptor, 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 selective agonists. Evidence from studies using such specific agonists suggest that 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; Ryan et al., 2013b; 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, 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 using similar residues to H2 relaxin; hence H3 relaxin contains an R*XXX*R*XX*I 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 high affinity RXFP1 binding (Büllesbach & Schwabe, 2000). In contrast, the binding of H3 relaxin to RXFP3 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 RXFP3 activation (Kuei et al., 2007; Liu et al., 2009). The same residues, except R^{B12}, are important for RXFP4 interaction. 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 RXFP3. Specifically, R^{B12}, R^{B16}, R^{B26} and W^{B27} residues of H3 relaxin interact with E^{244}, D^{145}, D^{141} and W^{138} residues in RXFP3, 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 by substantially reducing affinity for RXFP1 (Liu et al., 2005a) Additionally, by truncating the C-terminus of the B-chain of R3/I5 by 5

residues and adding a C-terminal arginine, an RXFP3/4 antagonist, R3(BΔ23-27)R/I5 chimeric peptide (Table of Links) was achieved (Kuei et al., 2007).

Role of free C-terminus: The effect of modification of the *C*-terminus of the 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 significant drop in the binding and activity of the peptide at both RXFP3 and RXFP4. However, modification of the C-terminus of the A-chain did not have any effect on activity (Shabanpoor, Bathgate, Wade & Hossain, 2013). It is clear that the key W^{B27} must be free acid and that these residues likely insert into the orthosteric binding pocket of RXFP3 and RXFP4 for activation. More specifically, molecular modelling and receptor mutations studies suggest that C-terminal W^{B27} and R^{B26} residues of H3 relaxin interact with the W^{138} and E^{141} residues of RXFP3, 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 minimised analogue of H3 relaxin, minimised 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, and its activation of RXFP3 *in vivo* led to increased feeding and significantly reduced anxiety-like behaviour in adult rats (Shabanpoor et al., 2012).

Based on current SAR knowledge it is evident that the B-chain of H3 relaxin contains all the residues important for both binding and activation for RXFP3. This is further supported by the fact that B-chain alone of H3 relaxin is capable of binding to and activating RXFP3 (Liu et al., 2003) albeit with low affinity and potency. Thus it is possible to engineer B-chain only agonist and antagonist 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 high affinity agonist and antagonist, respectively. The stapled R3 B10-27 agonist was demonstrated to be a full agonist of 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). R3-B1-22R, on the other hand, antagonized relaxin-3/RXFP-induced increase in feeding (Haugaard-Kedström et al., 2011). Thus, stapled R3 B10-27 and B1-22R represent excellent tools for probing H3 relaxin-RXFP3 pharmacology and 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 & 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 (Glister et al., 2013; Spanel-Borowski et al., 2001). The effects of INSL3 are mediated through the G protein-coupled receptor, RXFP2 (Kumagai et al., 2002).

SAR studies

Mutation and truncation studies: INSL3 like H2 relaxin binds to the LRR domain of RXFP2 using B-chain residues although these residues are different to those used by H2 relaxin to bind to RXFP1. More specifically, the residues H^{B12}, R^{B16}, V^{B19}, R^{B20} and W^{B27} present are responsible for RXFP2 binding (Büllesbach & Schwabe, 2006; Rosengren et al., 2006b). The corresponding residues in the LLR domain of RXFP2 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 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 & 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 RXFP2 activation (Büllesbach & Schwabe, 2012). Truncations at the N-terminus of INSL3 produced a high affinity

RXFP2 antagonist (Büllesbach & Schwabe, 2005a). Figure 5 shows the solution NMR structure of INSL3 (Rosengren et al., 2006b).

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

Minimum active structure: The SAR data suggest that part of both A and B-chain are necessary for RXFP2 agonistic activity. 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 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 INSL3 B-chain was developed that exhibited high RXFP2 affinity (Shabanpoor et al., 2011) and antagonized INSL3-mediated cAMP signalling through RXFP2. 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).

Human INSL5

INSL5 was originally identified as a novel peptide hormone with high expression in the gut (Conklin et al., 1999). Subsequent studies identified its cognate receptor as the G protein coupled receptor, RXFP4 (Liu et al., 2005b). It is a hormonal product of colonic L-cells, and the 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 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 acquisition (Hossain et al., 2008a). Preliminary SAR study, therefore, was carried out using 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 (Belgi et al., 2011). On the basis of H3 relaxin-RXFP3 structure-function studies, two analogues of mouse INSL5 were produced (Belgi et al., 2013). The analogue in which R^{B24} and W^{B25} were substituted with alanine completely abolished the interaction with RXFP4 (Belgi et al., 2013). 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 binding to RXFP4.

To understand the mode of interaction of INSL5 with RXFP4, 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 RXFP4 binding and activity. The solution 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 interaction with RXFP4 (Wang et al., 2014). While H3 relaxin cross-reacts with other receptors including RXFP4, INSL5 is very selective (RXFP4) and does not interact with RXFP3. The mechanism by which INSL5 distinguishes RXFP4 and RXFP3 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 (Hu et al., 2016a).

Role of C-terminus: The role of the *C*-terminus of INSL5 on RXFP4 binding and activity 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 activity. However, the free C-terminus of the B-chain is important for RXFP4 activity (Patil et al., 2016a), similar to what was seen for H3 relaxin at RXFP3. The loss of binding and activation due to C-terminal W^{B25} mutation (Belgi et al., 2013) or amidation (Patil et al., 2016a) suggest that W^{B25} residue cannot be replaced or modified.

Minimum active structure: The recent SAR studies on mouse INSL5 led to design and synthesis of a simplified mouse analogue (mouse INSL5: A8-21) (Belgi et al., 2013). This analogue showed human INSL5-like affinity and potency. However, the human orthologue of this peptide (minimised INSL5 analogue 7; Table of Links)) was found to be significantly less potent (Belgi et al., 2013). 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 RXFP4 affinity and was a potent full agonist of both cAMP inhibition and pERK activation. The minimised analogue is much easier to assemble in large quantities and thus very useful as a readily available tool to dissect physiological role of RXFP4.

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 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 tertiary structure similarity 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 RXFP3 binding and activation, and thus it was possible to truly mimic the B-chain and achieve a high affinity single-chain agonist and antagonist. While H3 relaxin binds to and activates both RXFP3 and RXFP4 in a similar manner, INSL5 interacts with RXFP4 in a different way. Although very little is known about INSL5-RXFP4 interaction, current SAR data suggest that B-chain is responsible for both RXFP4 binding and activation. Although a simplified RXFP4 agonist has been developed based on both 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 RXFP2 binding and the A-chain is important for RXFP2 activation. As a result, high affinity agonists with minimized A- and B-chains, and antagonists with B-chain dimers have been developed. The H2 relaxin RXFP1 interaction 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 RXFP1 highlights that there is still a lot to learn about the H2 relaxin-RXFP1 interaction. Further work in this area must take into account the clear cell type-specific actions of relaxin analogues.

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.

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Conflict of interest

The authors declare no conflict of interest.

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Table of Links

TARGETS	
GPCRs[a]	Enzymes[b]
RXFP1	ERK1
RXFP2	ERK2
RXFP3	
RXFP4	

LIGANDS	
relaxin-1	A(4-24)(B7-24)H2
relaxin-2	B-R13/17K 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 of Links 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 ([a,b]Alexander et al., 2015a,b).*

Table 1

Physiology 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 legends

Figure 1. Comparison of relaxin family peptides. A) Primary sequences of relaxin family peptide in humans. To illustrate conserved features the amino acid types are colour coded according to their nature with basic residues in blue (Arg, Lys, His), acidic residues in red (Glu, Asp), hydrophobic residues in green (Ala, Val, Ile, Leu, Phe, Trp, Tyr, Pro, Met), hydrophilic in black (Ser, Thr, Asn, 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 labeled A (N), A (C), B (N) and B (C), respectively.

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).

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).

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).

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).

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).

Figure 1

Figure 2

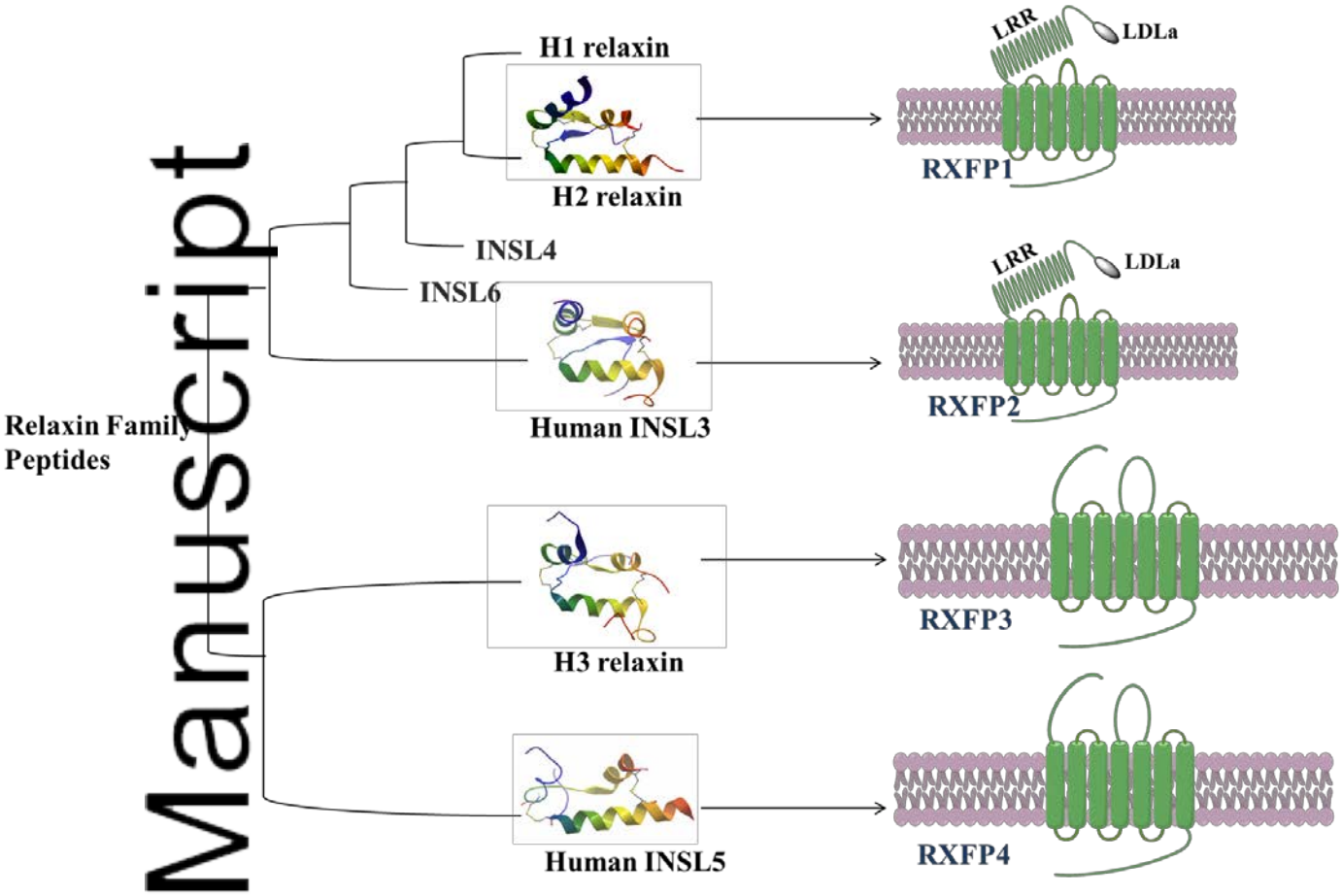


Figure 3

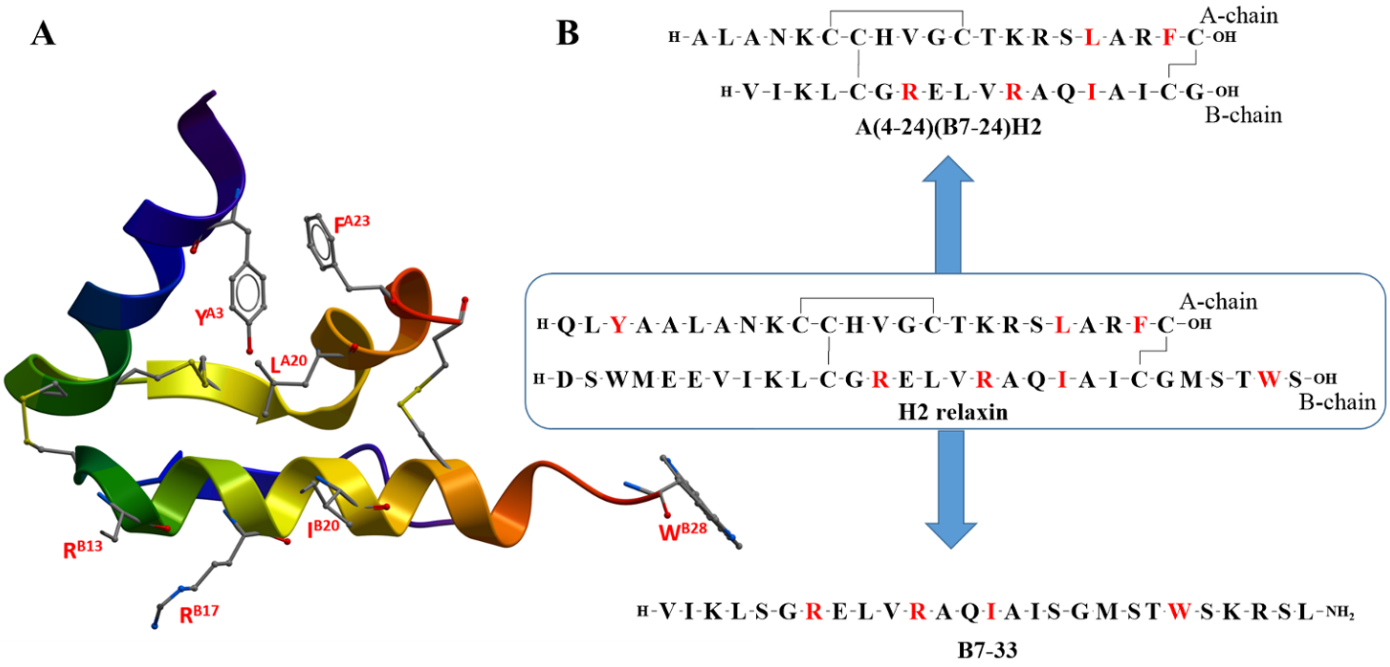


Figure 4

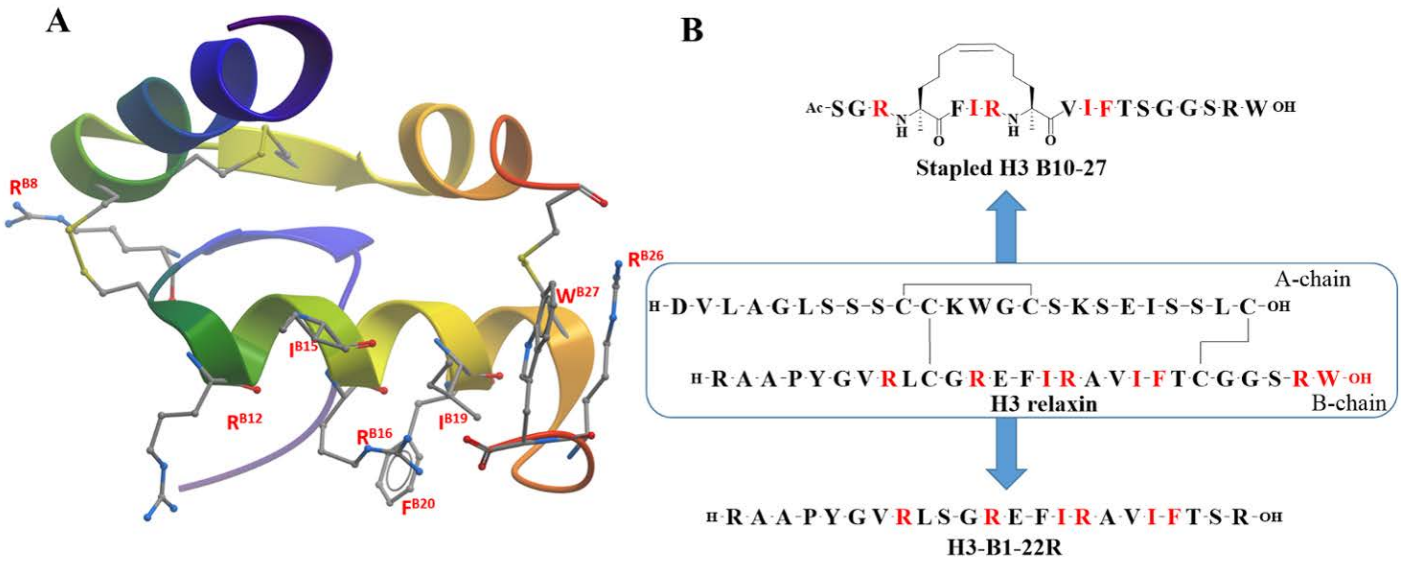


Figure 5

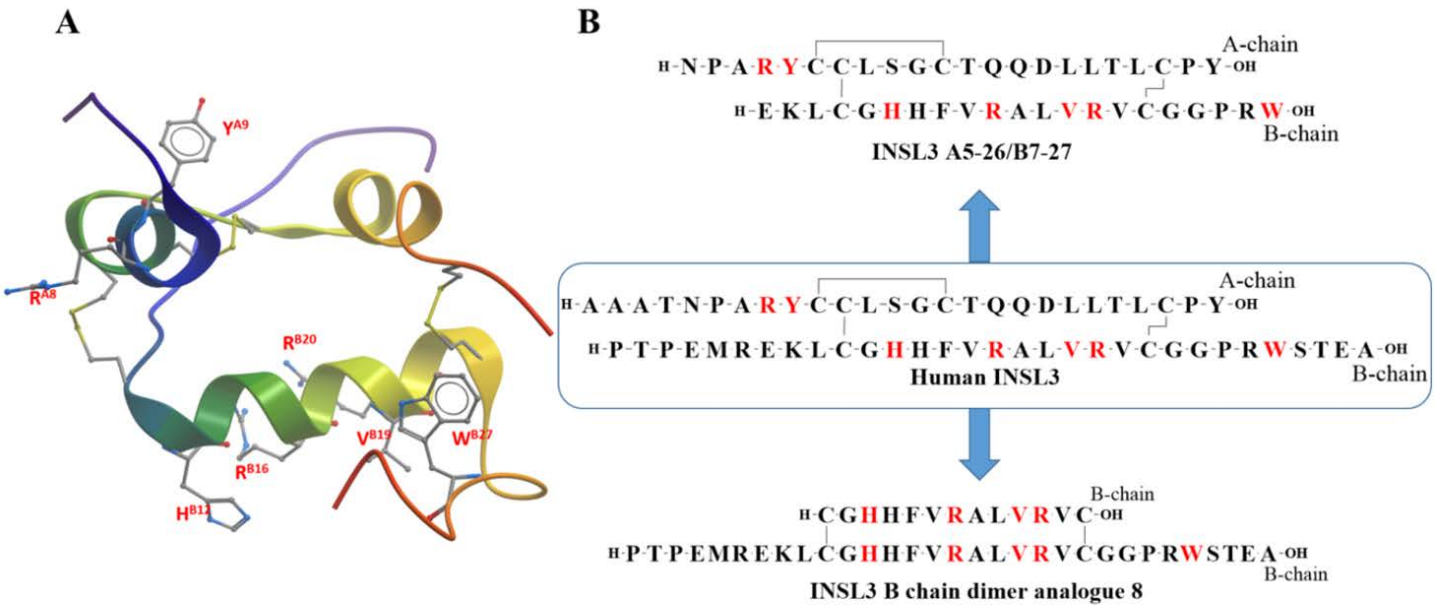
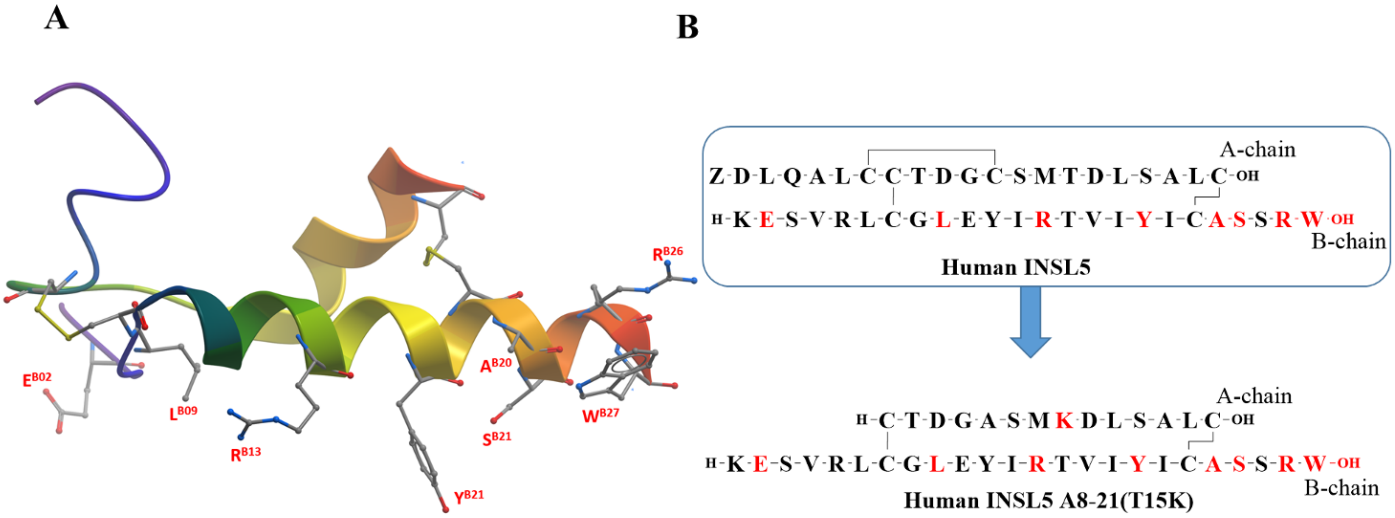


Figure 6



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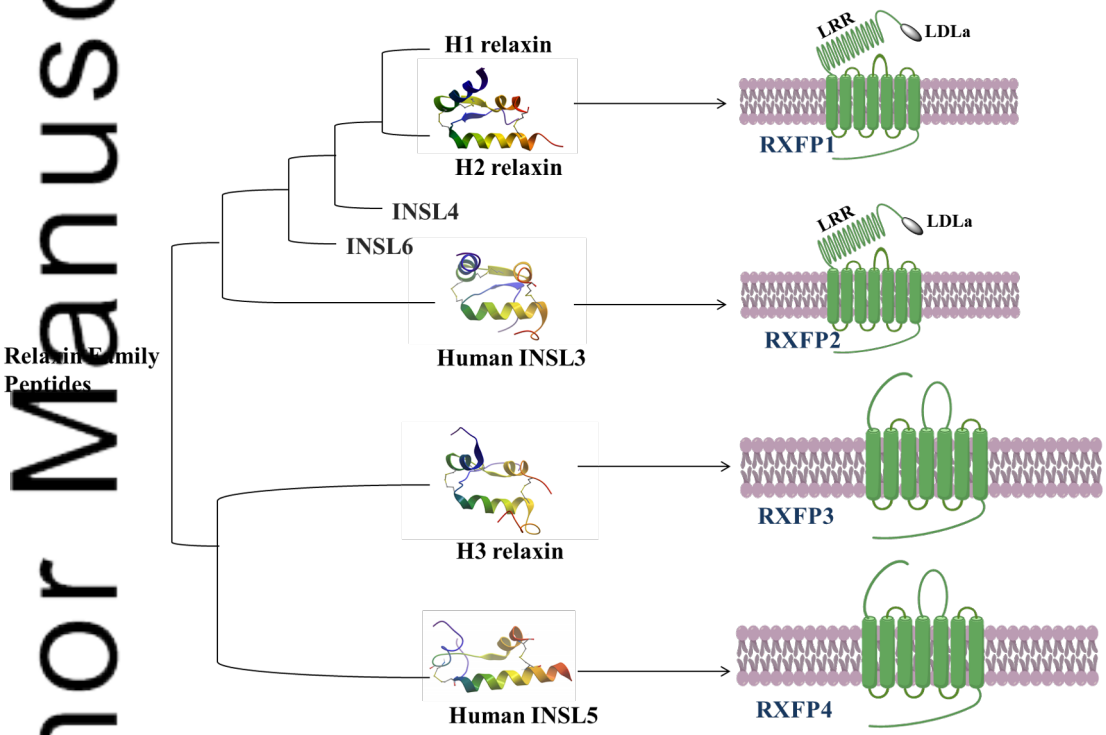

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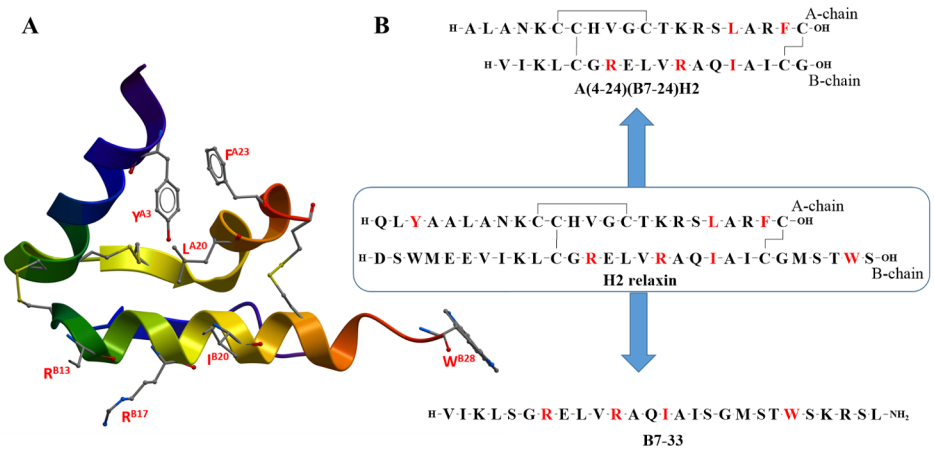

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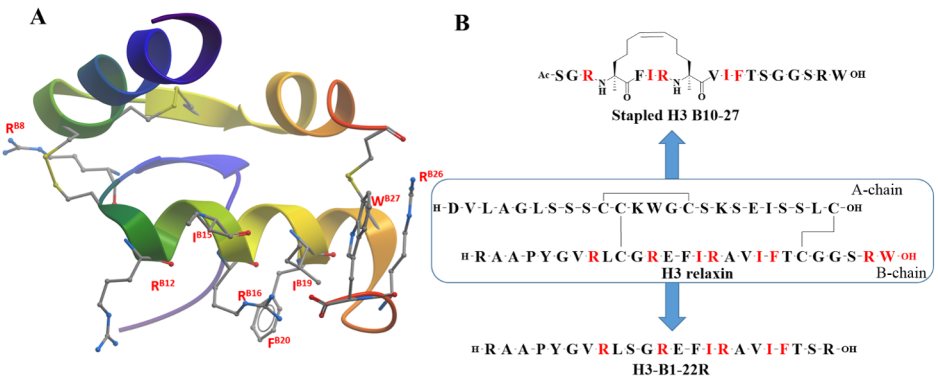

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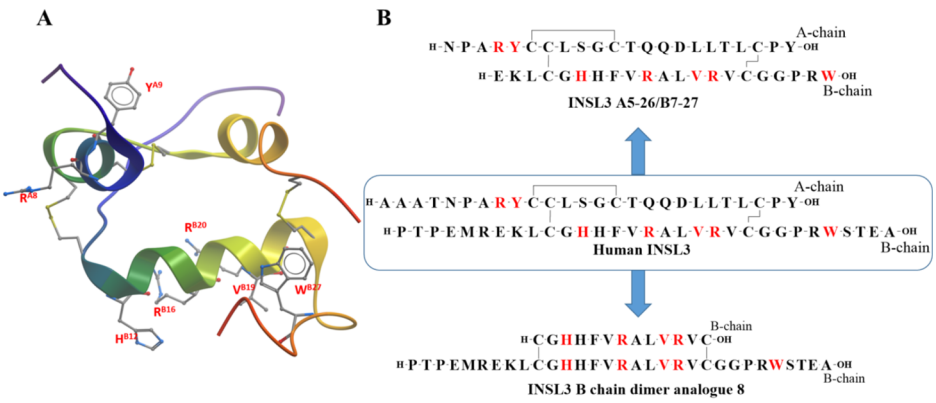

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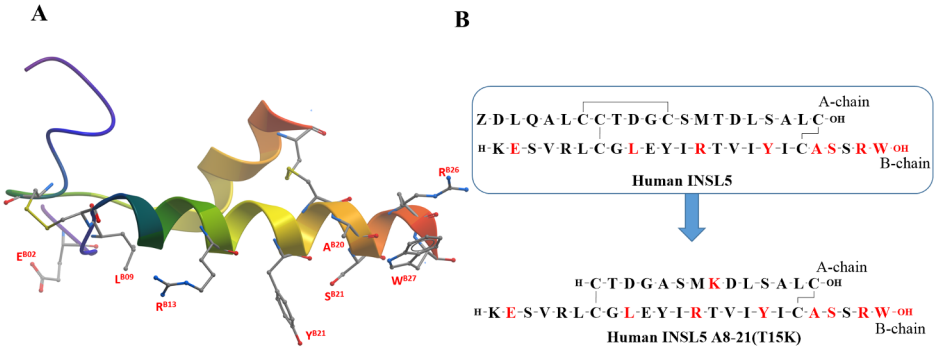

BPH_13684_f6.tif

Table of Links

TARGETS	
GPCRs	**Enzymes**[b]
RXFP1	ERK1
RXFP2	ERK2
RXFP3	
RXFP4	

LIGANDS	
relaxin-1	A(4-24)(B7-24)H2
relaxin-2	B-R13/17K 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 of Links 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 ([a,b]Alexander et al., 2015a,b).*