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Understanding insulin-like peptide 5 (INSL5) and relaxin family peptide receptor 4 (RXFP4): structure, signalling, and function

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Insulin-like peptide 5 (INSL5) is a member of the relaxin/insulin peptide family, predominantly produced by enteroendocrine L cells in the distal gut. Although initially characterised based on its structural similarity to insulin and relaxin peptides, INSL5 is now increasingly recognised as an important regulator of gastrointestinal physiology. Its endogenous receptor, relaxin family peptide receptor 4 (RXFP4), is primarily expressed in the gastrointestinal tract and signals mainly through G_i proteins. Recent studies have provided compelling evidence that the INSL5-RXFP4 signalling axis is an important regulator of colonic motility. In this review, we summarise current knowledge of the structure–function relationships of INSL5 and RXFP4, advances in the design of INSL5 analogues, and the signalling mechanisms underlying receptor activation. We also discuss the physiological and pharmacological evidence supporting a role for INSL5-RXFP4 signalling in colonic motility and highlight opportunities for the development of chemical probes and therapeutic leads targeting this system for the treatment of chronic constipation.

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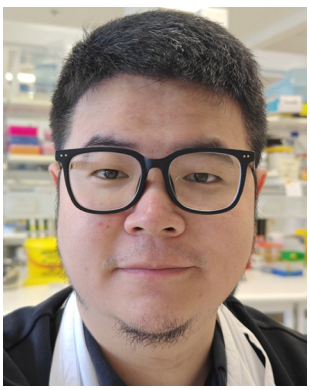

Huahao Wang

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1. Introduction

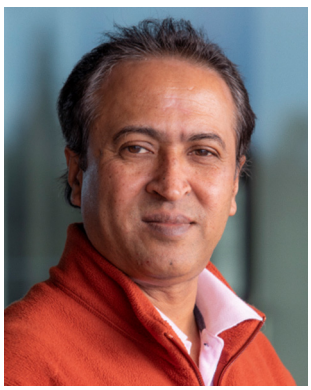
Insulin-like peptide 5 (INSL5) is a gut hormone belonging to the insulin/relaxin superfamily of peptides. This superfamily comprises structurally related peptide hormones that regulate metabolism, reproduction, growth, and neuroendocrine function.[1] Sequence and genomic analyses have identified ten human members: insulin, insulin-like growth factors I and II (IGF-I and IGF-II), and seven relaxin-related peptides, namely relaxin-1 (H1 relaxin), relaxin-2 (H2 relaxin), relaxin-3 (H3 relaxin), and insulin-like peptides 3 to 6 (INSL3–6).[2] These peptides signal through two main receptor classes. Insulin and the IGFs act on tyrosine kinase receptors, whereas relaxin peptides signal *via* G protein-coupled receptors (GPCRs) known as relaxin family peptide receptors (RXFPs).

All family members are initially translated as preprohormones containing a signal peptide, an A-chain, a B-chain, and a connecting C-peptide.[3–6] Except for IGF-I and IGF-II, which retain their C-peptide and remain single-chain proteins, these precursors undergo proteolytic cleavage and oxidative folding to generate the mature, biologically active two-chain hormones.[7] The conserved structural framework is defined by six cysteine residues that form three disulphide bridges, one intra-A-chain and two inter-chain.[8] The relaxin family peptides in particular are stabilised by a compact tertiary fold comprising three α-helices, with one central helix in the B-chain and two flanking helices in the A-chain (Fig. 1).[8] This disulphide-stabilised scaffold supports a broad spectrum of biological activities across the family *via* activating their corresponding receptors.[9] Established native ligand-receptor pairings (Fig. 1) include INSL3-RXFP2,[10] relaxin-3-RXFP3,[5] and INSL5-RXFP4.[11] Relaxin-1 and relaxin-2 both signal through RXFP1,[12,13] while the native receptors for INSL4 and INSL6 remain unidentified.[14] Within the relaxin subgroup, sequence variation is particularly evident in the B-chain motif R-X-X-X-R-X-X-I/V, which is a major determinant of RXFP1 activity.[2,15,16]

Several relaxin family peptides display cross-reactivity across RXFP subtypes, reflecting their shared structural motifs and complicating the assignment of distinct physiological roles. For example, relaxin-2 binds to and activates both RXFP1 and RXFP2,[10] and relaxin-3 can bind to and activate RXFP1, RXFP3, and RXFP4.[5,11,13] INSL5 can bind to both RXFP4 and RXFP3, but activates only RXFP4.

Against this backdrop of widespread cross-reactivity, INSL5 and its receptor RXFP4 stand out as one of the most recently characterised and comparatively selective ligand-receptor pairs within the superfamily, identified in 1999 and 2003, respectively.[11,20] INSL5 was first discovered by screening expressed sequence tag libraries for the conserved insulin B-chain cysteine motif in a human sigmoid colon cDNA library, yielding a clone that encoded a 135-amino-acid preprohormone with the canonical B-, C-, and A-chain organisation.[20] Comparative sequence analysis revealed 71% identity between the B- and A-chain regions of human and murine INSL5, consistent with a conserved two-chain, disulphide-linked architecture, characteristic of the insulin/relaxin superfamily.[20] Northern blot and RT-qPCR analyses revealed high INSL5 mRNA expression in human rectum, colon, and uterus, and in murine colon and thymus, indicating strong tissue-specific transcription of INSL5 and suggesting potential roles in gastrointestinal and possibly immune regulation.[20] Physiologically, INSL5 is predominantly secreted from enteroendocrine L-cells in the distal colon and activates RXFP4 in the colorectum.[11,21] The INSL5-RXFP4 axis has also been suggested to participate in appetite regulation,[22] insulin secretion and type 2 diabetes,[23] and colorectal cancer.[24] Early studies in *Rxfp4*-knockout (KO) mice demonstrated reduced refeeding after fasting, consistent with an orexigenic role for INSL5.[22] In contrast, later work reported minimal effects of exogenous INSL5 on food intake or body weight in lean and obese mice.[25] These divergent findings indicate that a role in appetite control remains incompletely defined. However, converging evidence from multiple research groups now points to a key role for the INSL5-RXFP4 system in regulating colonic motility.[21,26] This emerging view includes studies employing simplified INSL5 analogues, including both RXFP4 agonists and antagonists,[21,27] alongside small-molecule RXFP4 agonists,[28] which together have provided the necessary pharmacological tools that native INSL5 alone could not readily deliver. The structural complexity and poor synthetic tractability of native INSL5 initially constrained systematic structure–activity relationship (SAR) exploration and delayed the development of robust tool compounds.[29] The development of progressively simplified INSL5 analogues was therefore a critical milestone. These constructs overcame key synthetic bottlenecks while preserving RXFP4 activity, enabling detailed dissection of binding and activation motifs and supporting the parallel discovery of peptidomimetics and small molecules that target RXFP4 with varying degrees of selectivity.

The central aim of this review is to integrate current SAR and physiological knowledge on INSL5 and RXFP4. The sections



Mohammed Akhter Hossain

Professor M. Akhter Hossain leads the Insulin Peptides Laboratory at the Florey Institute of Neuroscience and Mental Health, University of Melbourne. He completed his PhD at the Tokyo Institute of Technology (2001) and undertook post-doctoral training in France and Australia. His research focuses on developing chemical methods for insulin and insulin-like peptides, as well as designing novel peptides and peptidomimetics for metabolic, gastrointestinal, and neurological disorders. He has published over 190 papers (2000–May 2026), holds five patents, and collaborates with major pharmaceutical companies, including Novo Nordisk, Takeda Pharmaceutical Company, and Novartis.

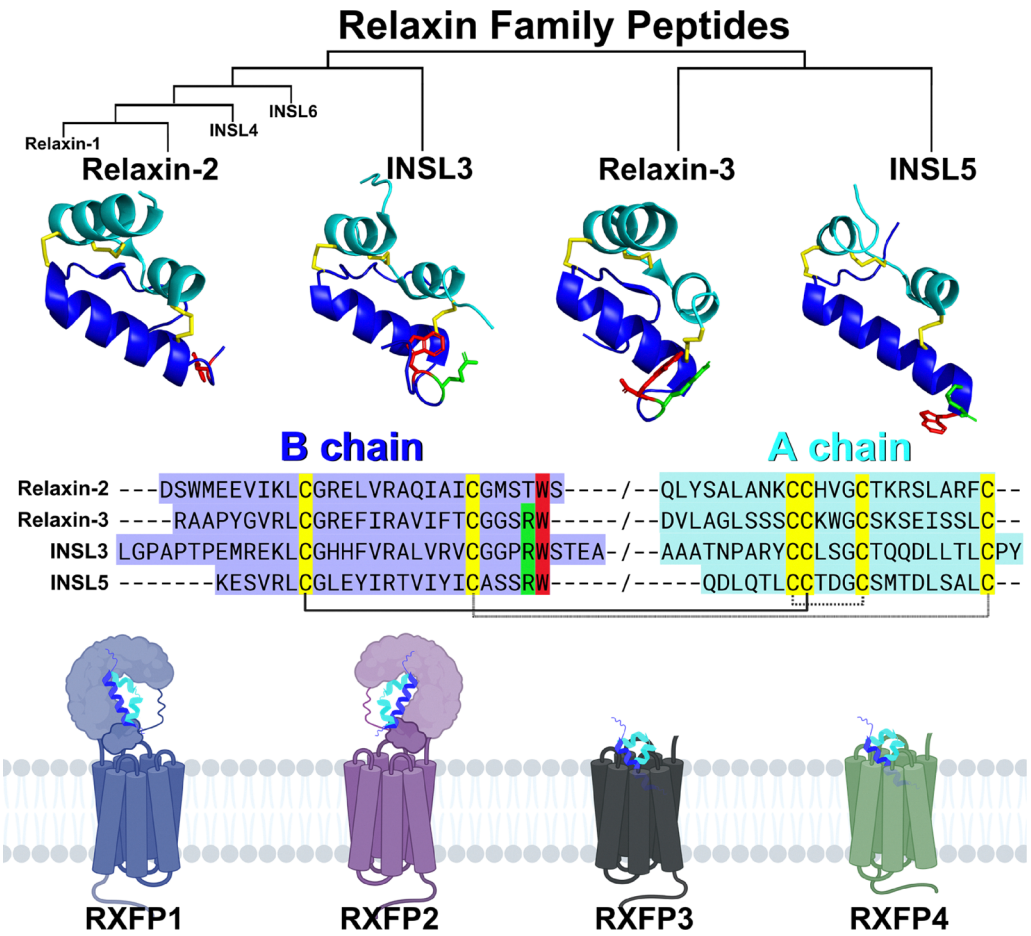


Fig. 1 The relaxin family peptides and their receptor targets. Comparison of the primary sequences of human relaxin-2, relaxin-3, INSL3, and INSL5, their 3D structural organisation, and GPCR targets. The A- and B-polypeptide chains are shown in cyan and blue, respectively; conserved Cys residues are shown in yellow, whereas C-terminal Arg and Trp residues within the B-chain are shown in green and red, respectively. The figure was generated using the following Protein Data Bank entries: 2MV1 (solution NMR structure of human relaxin-2),[17] 2FHW (solution NMR structure of human relaxin-3),[18] 2H8B (solution NMR structure of INSL3),[18] 2KBC (solution NMR structure of INSL5),[19] BioRender and PyMOL (the PyMOL Molecular GraphicsSystem, Version 3.0 Schrödinger, LLC).

that follow first characterise RXFP4 expression, signalling, and pharmacology, then detail the molecular and structural features of INSL5 and how these have been exploited in analogue design, including how stepwise analogue engineering and receptor pharmacology have defined key binding and activation motifs and expanded the set of INSL5-derived peptides and non-peptidic RXFP4 ligands. Finally, the review examines the physiological roles and therapeutic implications of the INSL5-RXFP4 axis in colon motility regulation.

2. RXFP4: expression, signalling, and pharmacology

RXFP4 belongs to a class A GPCR that serves as the principal signalling partner for INSL5 and defines the downstream biology of this ligand-receptor axis. A detailed understanding of RXFP4 expression, signalling, and ligand pharmacology is essential for interpreting the physiological roles of the INSL5-RXFP4 axis and for rational therapeutic targeting. RXFP4 is a GPCR within the relaxin family peptide receptor group (RXFP1–4), annotated initially as GPR100 or GPCR142. It was independently discovered in 2003 by two groups that identified sequences homologous to RXFP3 in human genomic databases.[5,30] Although initially proposed to function as a bradykinin receptor,[30,31] RXFP4 was subsequently confirmed as a relaxin-family receptor based on its activation by INSL5 as well as the related peptide relaxin-3.[5,11] Multiple studies have since validated INSL5 as the native selective high-affinity ligand for RXFP4.[29,32–35]

2.1. Tissue distribution

Available data point to a predominantly gastrointestinal expression of both RXFP4 and INSL5. Transcriptomic and next-generation sequencing analyses in human tissues have detected RXFP4 transcripts in the small intestine, colon, and rectum, with minimal expression in the pancreas.[36] Characterisation of cell expression and localisation studies in humans indicates that the major site of Rxfp4 expression is colonic enteroendocrine cells (EEC) that express tryptophan hydroxylase

(enterochromaffin, EC, cells) and, to a lesser extent, in I cells.[43] In mice, *Rxfp4* is enriched in the colon and lung, with negligible expression in the duodenum and small intestine.[36] Although *Rxfp4* transcripts are detectable in mouse pancreas, expression levels are very low, corresponding to approximately 4% of those observed in NCI-H716 enteroendocrine cells and less than 0.3% of *Glp1r* expression in the pancreas.[37] Localisation studies in the mouse revealed *Rxfp4* to be expressed in the majority of EC cells of the distal colon, with few other EEC expressing the gene *Rxfp4* was not found in enteric neurons, but positive nerve fibres, most immunoreactive for CGRP, were present. This is consistent with these being sensory axons from the dorsal root ganglion cells, in which the expression of *Rxfp4* has been reported.[38]

Early *in situ* hybridisation studies localised *Rxfp4* mRNA to the submucosal and myenteric plexuses of the mouse colon,[22] but this has not been confirmed in the later studies summarised above, and it appears to be an artefactual localisation.

At the sequence level, RXFP4 is highly conserved among humans and non-human primates (97% homology in monkeys), with lower but still substantial homology in cows (85%) and pigs (87%). In contrast, mouse RXFP4 shares around 74% homology with human RXFP4, and *Rxfp4* is a pseudogene in rats.[39]

The ligand for RXFP4, INSL5, is almost completely confined to L-type EEC of the distal colon,[22,40] although transcripts have also been reported in the uterus and thymus.[20] Colonic L-cells also express GLP-1 and PYY, which are commonly released at the same time as INSL5.[40] INSL5-containing L cells and RXFP4-expressing EC cells are often in close proximity to each other.[41]

2.2. Signalling pathways

INSL5 is co-secreted with glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from enteroendocrine L-cells in the distal colon and rectum.[40] These L-cells express multiple GPCRs involved in sensing luminal metabolites and related chemical signals, including free fatty acid receptor 1 (FFAR1), G protein-coupled receptor 119 (GPR119), and G-protein-coupled bile acid receptor (GPBAR1), which mediate the detection of luminal metabolites. Distal L-cells also express angiotensin II type 1 (AT1R) and vasopressin 1B (AVPR1B) receptors, through which angiotensin II and arginine vasopressin can stimulate GLP-1 and PYY release.[40,42] Pharmacological activation of FFAR1, GPBAR1, and the calcium-sensing receptor (CaSR) *via* Gq-mediated pathways elicits Ca^{2+} transients in INSL5-producing L-cells and promotes hormone secretion, with robust responses to forskolin, IBMX, and glucose.[40] These data position INSL5 within a broader network of nutrient and hormonal cues that modulate L-cell output.

RXFP4 itself is primarily an inhibitory $G\alpha_{i/o}$-coupled receptor. Its activation by INSL5 inhibits adenylate cyclase activity, reducing intracellular cyclic adenosine monophosphate (cAMP) levels (Fig. 2).[5,11,20] In addition to inhibition of cAMP signalling, RXFP4 activation can engage ERK1/2, consistent with downstream MAPK pathway activation (Fig. 2).[14,35,43] As a $G_{i/o}$-coupled receptor, RXFP4 is expected to signal *via* $G_{\beta\gamma}$ subunits, including activation of GIRK channels and suppression of cellular excitability and calcium entry. Consistent with this, INSL5 has been reported to inhibit glucose-stimulated insulin secretion and calcium mobilisation in MIN6 murine insulinoma cells.[32,40] Accordingly, calcium responses observed in studies involving INSL5-expressing cells should not be interpreted as direct evidence of canonical RXFP4-mediated Ca^{2+} mobilisation, but may instead reflect indirect effects, pathway cross-talk, or distinct downstream signalling in specific cell types.[44] Thus, although RXFP4 is fundamentally a $G_{i/o}$-coupled receptor, its physiological output may vary across tissues, including excitatory actions in colorectal enteroendocrine cells that ultimately promote 5-HT release.[45]

2.3. Ligand selectivity and cross-reactivity

Despite low circulating concentrations, INSL5 is a highly potent and efficacious agonist at RXFP4, supporting a physiologically relevant role for this ligand–receptor pair in the gastrointestinal tract.[5,46,47] However, circulating INSL5 may not necessarily reflect a primarily classical long-range endocrine role, and in some settings may instead represent ''overflow'' from a locally acting gut messenger, analogous to the clinical use of circulating noradrenaline or serotonin as markers of local release. Consistent with this, clinical studies have reported associations between circulating INSL5 and metabolic or sex hormone-related parameters, including insulin, androgens, and inflammatory markers, as well as with bile acid diarrhoea.[47–49] These correlations raise the possibility that INSL5 participates in systemic energy and reproductive homeostasis, but the causal mechanisms remain to be defined. As with other members of the relaxin family, the pharmacology of RXFP4 is complicated by off-target effects of ligands. Due to high structural similarity, relaxin-3 can bind to and activate RXFP4. However, with slightly lower potency and much lower selectivity than INSL5.[5,11] Unlike the loop or ''lay-flat'' α-helix conformations adopted by other reported class A GPCR-binding peptides, relaxin-3 anchors in the RXFP4 orthosteric binding pocket bordered by TMs 2, 3, 5-7, and ECLs 1-3.[50] As shown in Fig. 3, the B chain of relaxin-3 (magenta) exhibits a single α-helix conformation to penetrate the orthosteric pocket, while the A chain (transparent magenta) sits above the orthosteric pocket to interact with the extracellular half of the B chain as well as the extracellular surface of RXFP4. The cryo-EM structure of the RXFP4-INSL5 complex was revealed in 2023, with the Protein Data Bank (PDB) ID 7YJ4.[51] It shows that the positioning of INSL5 within the complex is generally similar to that of relaxin-3 in its binding mode (Fig. 3). The B chain of INSL5 (blue, Fig. 3) deeply inserts into the orthosteric pocket (bordered by TMs 2–7 and ECLs 1–3) of RXFP4, while the A chain (transparent blue, Fig. 3) is located above and interacts with the extracellular surrounding residues of the B chain and RXFP4. Unlike the flexible C-terminus of the B chain of relaxin-3 due to two glycines (positions 23 and 24 in relaxin-3, *i.e.*, G^{B23} and G^{B24}), the B chain of INSL5 adopts a single amphipathic α-helix conformation from E^{B10} to W^{B24} (Fig. 3 and 4),[51] which may explain why relaxin-3 activates RXFP4 but INSL5 does not activate RXFP3.

This promiscuity reflects shared sequence and structural motifs within the insulin/relaxin superfamily and suggests that

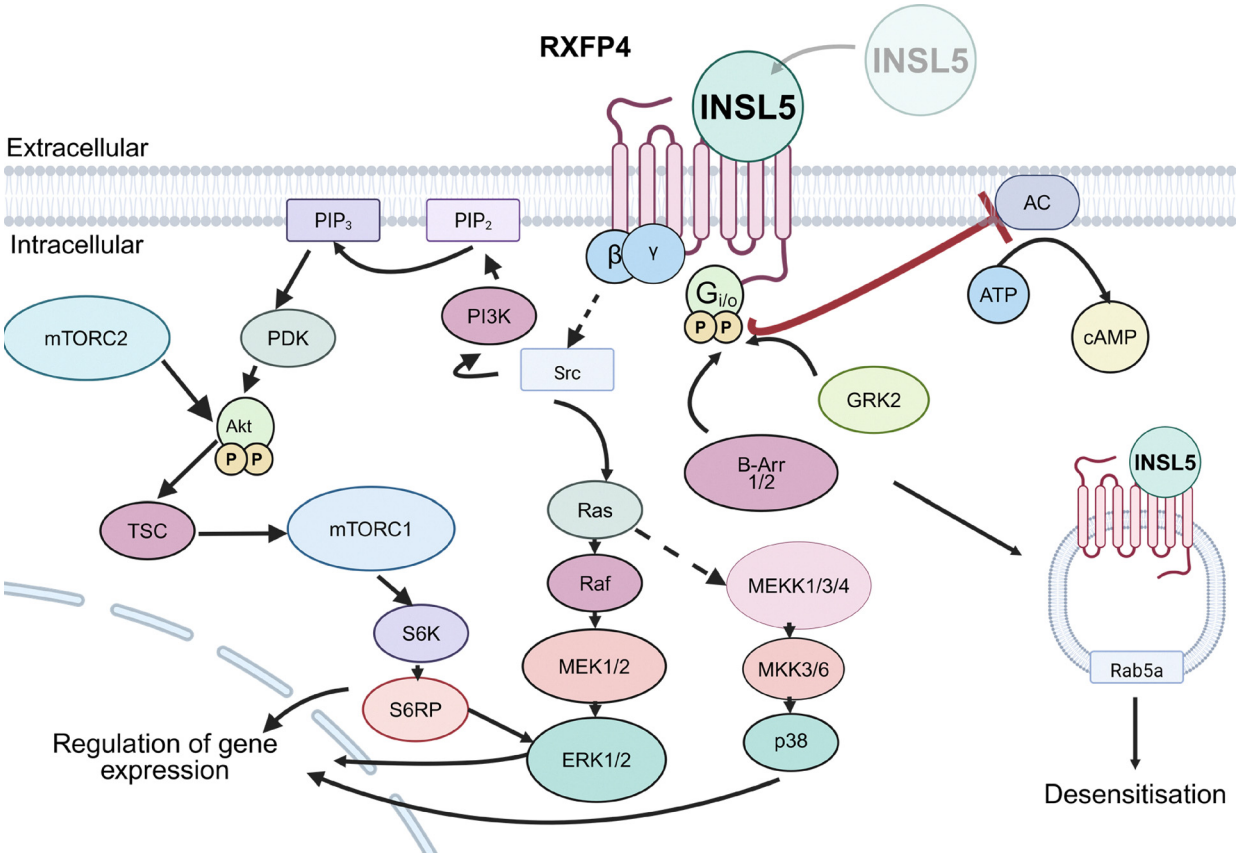


Fig. 2 Schematic representation of RXFP4 signalling. Binding of INSL5 to RXFP4 initiates $G_{i/o}$-protein coupling and downstream signalling cascades. Key pathways include inhibition of adenylate cyclase (AC) and reduced cAMP production, activation of ERK1/2, Ribosomal protein S6(S6RP), and p38. Partially activated pathways are indicated with dotted lines. Akt, protein kinase B; ATP, adenosine triphosphate; B-Arr, β-arrestin; cAMP, cyclic adenosine monophosphate; ERK1/2, extracellular signal-regulated kinase 1/2; $G_{i/o}$, inhibitory/stimulatory G protein subunits; GRK, G-protein-coupled receptor kinase; MEK, mitogen-activated protein kinase kinase; MEKK, mitogen-activated protein kinase kinase; MKK, mitogen-activated protein kinase kinase; mTORC, mechanistic target of rapamycin complex; PI3K, phosphoinositide 3-kinase; PIP, phosphatidylinositol phosphate; Rab5a, Ras-related protein Rab-5A; Ras, rat sarcoma (GTPase); Raf, rapidly accelerated fibrosarcoma (kinase); S6K, ribosomal protein S6 kinase; Src, proto-oncogene tyrosine-protein kinase; TSC, tuberous sclerosis complex. The figure was generated using BioRender.

partial functional overlap between RXFP3 and RXFP4 signalling could occur, particularly in species or tissues where both receptors are expressed. At the same time, comparative expression studies indicate that RXFP4 is generally more weakly and diffusely expressed than INSL5, especially in mice, which may contribute to species-specific differences in INSL5-RXFP4 signalling and complicate the interpretation of KO phenotypes.[52]

From an evolutionary standpoint, INSL5 and RXFP4 are conserved in humans, mice, monkeys, cows, and fish, whereas the *Insl5* and *Rxfp4* genes in rats and dogs do not encode intact open reading frames and are therefore regarded as non-functional pseudogenes in these species.[39,53,54] Comparative analyses further suggest that retention of this ligand–receptor pair varies across mammalian lineages, although the physiological significance of this pattern remains uncertain and should not be over-interpreted.[53] Defining the anatomical landscape of RXFP4 has also been technically challenging. Progress has been limited by the scarcity and variable specificity of antibodies and antisera suitable for colocalisation studies with enteroendocrine markers.[55–57] More recently, however, genetically engineered reporter systems have helped overcome some of these limitations and have provided clearer insight into RXFP4-expressing cell populations in the gut, particularly in studies by Lewis *et al.* and Koo *et al.*[41,58] Nevertheless, the available molecular, pharmacological, and evolutionary evidence supports a high-affinity, co-evolved interaction between INSL5 and RXFP4, providing a strong framework for the structural and analogue-design studies discussed in subsequent sections.

3. Molecular and structural features of INSL5

Understanding the molecular and structural features of INSL5 is central to explaining its interaction with RXFP4 and to guiding rational analogue design. INSL5 retains the canonical disulphide-stabilised scaffold of the insulin/relaxin superfamily but shows distinctive folding behaviour and receptor-recognition motifs that shape both its biosynthesis and biological activity. This section outlines the structural organisation

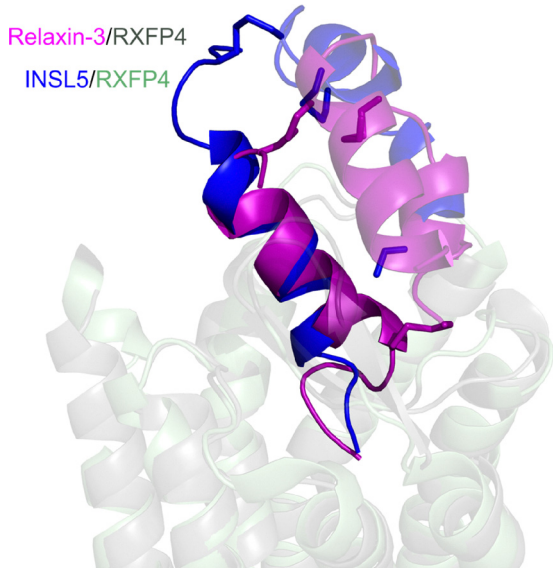


Fig. 3 Conformational comparison of relaxin-3/RXFP3 (magenta/grey) and INSL5/RXFP4 (blue/pale green). The visualisation of the 9KFK (cryo-EM structure of the relaxin-3/RXFP4 complex)[50] and 7YJ4 (cryo-EM structure of the INSL5/RXFP4 complex) PDB models was performed in PyMOL.[51]

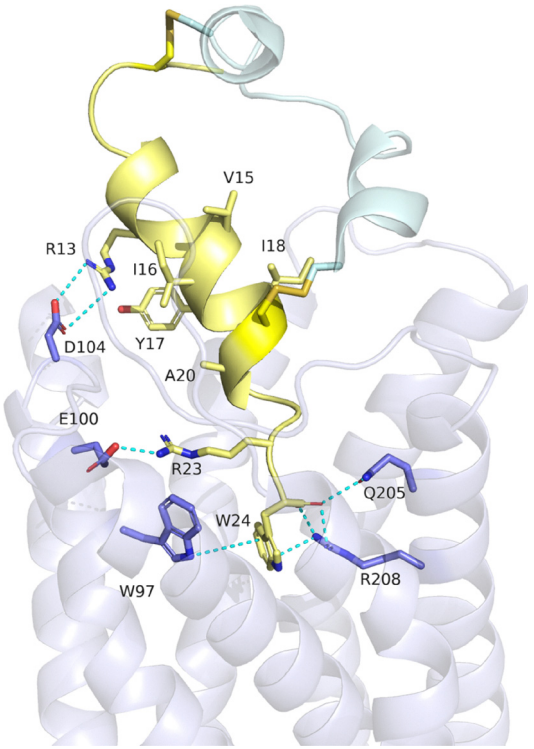


Fig. 4 Structure of INSL5 bound to RXFP4. The cartoon representation of INSL5, showing the A-chain in light blue and the B-chain in yellow, with three disulphide bonds depicted as stick connections in orange. Critical B-chain residues are shown as sticks; RXFP4 is shown in pale purple, featuring TM domains and ECLs (semi-transparent) and binding pocket residues as sticks. Critical interactions between the B-chain of INSL5 and the RXFP4 orthosteric binding pocket residues within a 4 Å distance are shown as dashed lines. The visualisation of 7YJ4 (cryo-EM structure of the INSL5/RXFP4 complex)[51] PDB models was performed in PyMOL.

of INSL5, the key residues responsible for RXFP4 engagement, and how advances in synthesis and peptide engineering have transformed a chemically intractable hormone into a tractable platform for the development of RXFP4 agonists and antagonists.

3.1. Receptor interaction motifs

Structure–activity and mutagenesis studies indicate that multiple residues along the INSL5 B-chain contribute to RXFP4 binding, whereas the C-terminal region of the B-chain is particularly important for receptor activation. A free C-terminal carboxylate is critical for receptor engagement; amidation or truncation of the B-chain terminus markedly reduces potency, whereas amidation of the A-chain has little effect, underscoring the asymmetric contribution of the two chains to receptor activation.[59] The A-chain functions mainly as a structural scaffold that presents the B-chain helix in the correct orientation and stabilises the overall fold rather than directly contributing extensive contact points.

Nuclear magnetic resonance (NMR) analyses first revealed that the C-terminal segment of the B-chain adopts an extended α-helical conformation not present in relaxin-3.[60] Alanine substitutions at A^{B20} and S^{B21}, which stabilise this helical region, abolish RXFP4 binding and activity.[61] Additional alanine-scanning and charge-reversal studies identified K^{B6}, R^{B13}, Y^{B17}, R^{B23} and W^{B24} as indispensable for receptor interaction.[61] In particular, positively charged R^{B13} and R^{B23} form electrostatic interactions with a cluster of negatively charged residues (E^{100} and D^{104}) in RXFP4 (Fig. 4); charge-exchange mutations at these positions in either the ligand or the receptor abolish signalling.[51] This was confirmed by the recently available cryo-electron microscopy (cryo-EM) structures of the INSL5-RXFP4-G_i complex, which have refined this interaction at atomic resolution.[51] The B-chain forms a continuous α-helix that penetrates deep into the transmembrane (TM) bundle, with its C-terminal residues R^{B23}-W^{B24} sitting at the core of the orthosteric pocket (Fig. 4). R^{B23} forms a salt bridge with $E100^{2.63}$ and hydrogen-bonding contacts with nearby residues, anchoring the ligand along TM2 and TM3 (Fig. 4).[51] W^{B24} occupies the geometric centre of the binding cavity, where its indole ring engages in a cation-π interaction with $R208^{5.42}$ and π-stacking with $W97^{2.60}$ and other aromatic residues in the upper TM bundle (Fig. 4).[51]

These critical C-terminal R^{B23}-W^{B24} residues stabilise an active-state conformation characterised by outward displacement of TM6 and an inward adjustment of TM5, typical of G_i-coupled class A GPCRs.[51] These structural insights are fully consistent with earlier functional data. R^{B23} and R^{B13} provide the positive charges necessary for long-range electrostatic attraction to the acidic EXXXD motif of RXFP4, whereas W^{B24} contributes aromatic contacts that deepen the ligand within the pocket (Fig. 4).[61] A^{B20} and S^{B21} help to maintain the rigidity of the C-terminal helix; their replacement with alanine disrupts the helix, while targeted glycine substitutions at these positions can fine-tune local flexibility and improve receptor complementarity

(see Section 3.3).[61] In contrast, the N-termini of the A- and B-chains are structurally more flexible and contribute little to receptor engagement, a conclusion supported by NMR, truncation studies, and the cryo-EM structures.

3.2. Synthetic challenges

The native structure of insulin-like peptide 5 (INSL5) presents formidable challenges for chemical synthesis and has been a major barrier to both structural and medicinal chemistry studies. Human INSL5 is a 45-residue, two-chain peptide comprising a 21-residue A-chain and a 24-residue B-chain, linked by two interchain disulphide bonds and one intrachain A-chain disulphide bond. Early studies established that both chains are difficult to access by standard Fmoc-based solid-phase peptide synthesis (SPPS), owing to their poor physicochemical properties, sequence-dependent side reactions, and the need for precise oxidative folding to generate the native hormone.[29]

The A-chain and B-chain syntheses fail for different reasons. In the A-chain, the Asp-Gly motif is particularly problematic because it is highly susceptible to base-induced aspartimide formation during Fmoc deprotection, which can lead to backbone cyclisation, by-products, and sequence heterogeneity.[29] This liability required the use of specialised building blocks such as Fmoc-(Dmb)Gly-OH, together with a pseudoproline dipeptide, to suppress side reactions and reduce aggregation during synthesis.[29] By contrast, the B-chain becomes increasingly difficult as elongation proceeds because of its pronounced hydrophobicity and tendency to aggregate on the resin, which promotes incomplete deprotection, poor coupling efficiency, and amino acid deletions.[29] Notably, standard optimisation strategies such as microwave-assisted synthesis, longer coupling times, and alternative coupling reagents were insufficient to overcome these problems. Instead, a key breakthrough came when piperidine was replaced with DBU for Fmoc deprotection, indicating that incomplete deprotection, rather than inefficient coupling alone, was a major contributor to the poor outcome of B-chain synthesis.[29]

Beyond chain assembly, formation of the mature hormone requires precise installation of three native disulphide bonds. This is intrinsically challenging because the two chains must not only be synthesised successfully, but also combined and oxidatively folded with the correct regioselective connectivity. Chemical synthesis of INSL5, therefore, depends on orthogonal cysteine protection strategies and stepwise oxidation protocols to minimise the formation of mispaired disulphide isomers.[29,33,43] In the first successful total synthesis of native human INSL5, Hossain *et al.* employed a classical stepwise regioselective disulphide-forming strategy using orthogonally protected cysteines, culminating in iodine-mediated deprotection and oxidation of the final Acm-protected pair. Although this route produced a correctly folded, biologically active peptide, the isolated yield was extremely low, reported as 0.8% relative to the starting A-chain, highlighting the substantial synthetic burden associated with native INSL5.[29] Consistent with this, subsequent studies confirmed that both human and murine INSL5 remained difficult synthetic targets, with similarly challenging chain synthesis and disulphide assembly behaviour.[29,33,43]

These early studies made it clear that the synthetic difficulty of INSL5 is not due to a single bottleneck, but rather to the cumulative effects of chain aggregation, side reactions, poor solubility of intermediates, and complex oxidative folding. As a result, large-scale production of native INSL5 was expensive, technically demanding, and poorly suited to routine medicinal chemistry optimisation or broader pharmacological use. The low yields and operational complexity also limited access to sufficient quantities of peptide for high-resolution structural studies and analogue development.

A major advance was later reported by Zaykov *et al.* in 2018, who developed a high-yield, nonconventional synthetic strategy that significantly improved practical access to native human INSL5.[25] Rather than focusing solely on disulphide connectivity, this work recognised that poor solubility and cumulative physical loss of intermediates during purification were also major determinants of the low yield of earlier routes. To address these issues, the authors introduced a temporary isoacyl dipeptide into the B-chain to improve solubility and handling, while the A-chain intramolecular disulphide was formed through thiol displacement of an StBu-protected cysteine. The A- and B-chain fragments were then combined under conditions that avoided intermediate purification, and the final disulphide was formed after hydrofluoric acid deprotection followed by DTNP-mediated oxidation and rearrangement of the isoacyl linkage to the native amide. This route delivered native human INSL5 in a markedly improved yield of 23% from purified A- and B-chains, demonstrating that advances in INSL5 synthesis depend not only on disulphide chemistry itself but also on careful management of intermediate solubility, concentration, and purification losses.[62]

A further refinement was described by Zaykov *et al.* in 2019 during preparation of native INSL5 for *in vivo* pharmacology studies.[25] In this work, the authors developed a more convergent [1+2] strategy in which the A-chain intramolecular disulphide was formed first, after which the remaining A-chain cysteines were activated and directly combined with the B-chain to form the two interchain disulphide bonds in a more operationally straightforward manner. The route proceeding through the activated A-chain afforded native INSL5 in 15% yield from purified A-chain, whereas the alternative route proceeding through the activated B-chain gave 8% yield.[25] Although these yields were lower than those obtained in the 2018 high-yield route, the method offered practical advantages in terms of step economy and simplicity. Importantly, this study also isolated an inverse disulphide isomer that retained full agonist activity, albeit with substantially lower potency, further underscoring both the complexity of INSL5 oxidative folding and the partial structural tolerance of the RXFP4 ligand recognition system.[25]

Taken together, these studies show that the synthetic challenge of INSL5 is multifactorial. Major obstacles include aspartimide formation in the A-chain, aggregation and incomplete deprotection in the B-chain, and the difficulty of achieving

native disulphide pairing without excessive material loss. At the same time, the evolution of INSL5 synthesis illustrates a clear conceptual shift in the field. Early work focused on establishing chemically correct access to the native hormone, whereas later studies increasingly prioritised process efficiency, intermediate solubility, reduced purification burden, and operational simplicity. Although the reported yields are not strictly directly comparable because they were calculated from different reference points, native human INSL5 synthesis improved from 0.8% in the original stepwise route to 23% in the nonconventional high-yield approach, while the later convergent strategy provided 15% or 8%, depending on the direction of chain activation.[25,29,62]

These persistent synthetic constraints provided a strong rationale for the next phase of the field: simplification of the INSL5 scaffold. More recent efforts have therefore focused on developing shorter and more synthetically accessible analogues that preserve RXFP4 activity while reducing disulphide complexity and improving manufacturability. Supported by structure–activity studies and, more recently, structural insights from NMR and cryo-EM, this shift has enabled increasingly rational analogue design, in which minimal, targeted modifications can be introduced to generate tool compounds and therapeutic candidates with improved synthetic tractability and optimised pharmacological profiles.

3.3. INSL5-based analogues

3.3.1. Truncation and simplification guided by structure. NMR and mutagenesis had already indicated that the N-termini of both chains contribute little to RXFP4 binding, whereas key residues cluster around the C-terminal B-chain helix.[33] Building on these observations, the Hossain laboratory designed A13, a two-chain, two-disulphide analogue that became a central platform for further analogue development (Fig. 5).[35] A13 incorporates three major modifications relative to native INSL5: truncation of the A-chain N-terminus, removal of the intra-A-chain disulphide bond, and replacement of T^{A15} with Lys ($T^{A15}K$) in the A-chain.[32] These changes simplify the folding landscape and improve solubility without perturbing the critical B-chain C-terminus. A13 can be synthesised with a 17.4-fold higher yield than native INSL5 while retaining full agonist activity at RXFP4 *in vitro*.[35] *In vivo*, A13 accelerates colorectal propulsion in wild-type (WT) mice but not in *Rxfp4*-KO animals, confirming that its physiological effects are mediated by RXFP4.[21] Further truncation of six residues from the B-chain N-terminus yielded A13:B7-24 (Fig. 5), which maintains binding affinity and potency similar to A13.[35,63] This finding aligns with structural data indicating that the N-terminus of the B-chain is flexible and dispensable for receptor engagement.[33] Together, A13 and A13:B7-24 demonstrate that a minimal two-chain, two-disulphide scaffold can fully recapitulate RXFP4 activation, provided the C-terminal helix and key contact residues are preserved. Both SAR work and the INSL5-RXFP4 cryo-EM structure have guided refinement of this minimal scaffold. Cryo-EM analyses subsequently revealed that the B-chain helix penetrates deep into the transmembrane domain core, allowing R^{B23} and W^{B24} to fully occupy the orthosteric pocket and establish a dense network of salt bridges, hydrogen

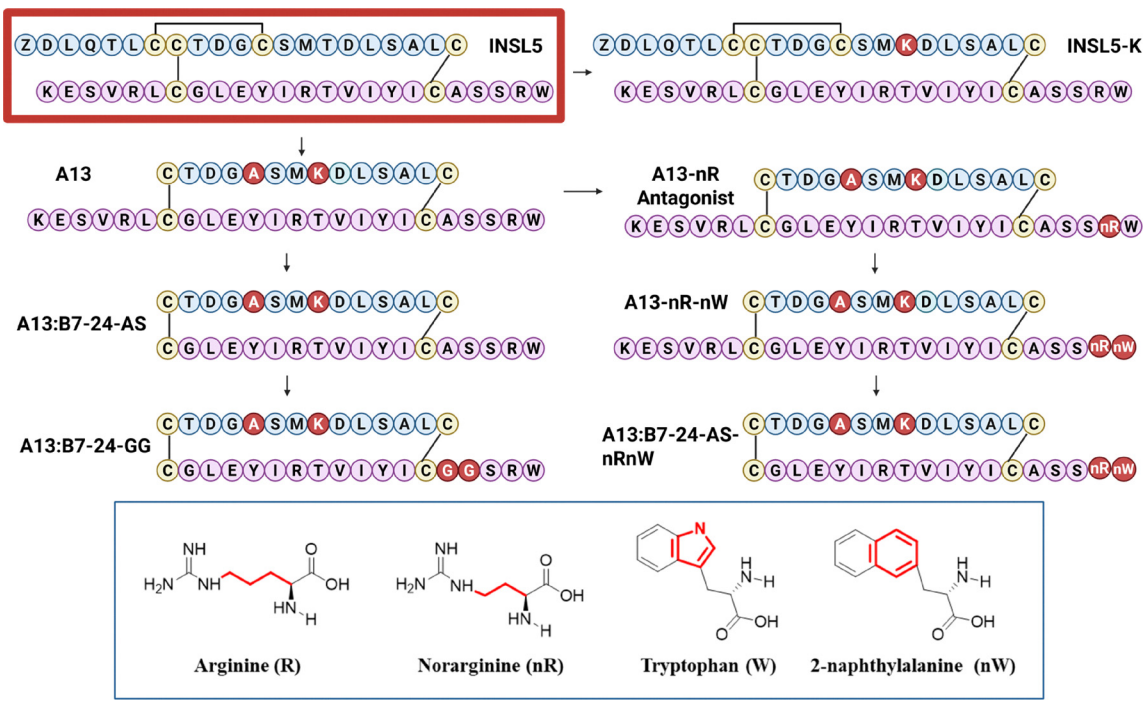


Fig. 5 Structure of RXFP4 peptide agonists and antagonists. The native two-chain, three-disulphide-bond INSL5 structure is compared to related peptides, which have a trend of structural minimisation. A and B polypeptide chains are shown in blue and pink, respectively; cystine residues are highlighted in yellow; mutations are highlighted in red; Figure generated using Biorender.

bonds, and π interactions with E100$^{2.63}$, W97$^{2.60}$, R208$^{5.42}$, F291$^{7.35}$, and H299$^{7.43}$.[51]

Based on these structural insights, the Hossain laboratory introduced glycine substitutions at A^{B20} and S^{B21} in the A13:B7-24 scaffold to generate A13:B7-24-GG (Fig. 5).[64] These substitutions increase local backbone flexibility at the C-terminal helix and allow the RW motif to adopt an optimised conformation within the RXFP4 pocket, reminiscent of the flexible Gly-Gly motif in relaxin-3 but embedded in an RXFP4-selective scaffold. A13:B7-24-GG exhibits a 19.5-fold higher synthesis yield than native INSL5, approximately 4-fold higher potency at RXFP4 (EC_{50} = 1.17 nM *vs.* 4.57 nM), and more than 7-fold enhanced selectivity at RXFP4 over RXFP3.[64] In the same study, a single-mutation analogue, INSL5-K (T^{A15}K modification on the native scaffold), similarly exhibits around a 10-fold increase in potency (EC_{50} = 0.45 nM) with slightly reduced selectivity.[64] These data confirmed that T^{A15}K in A13 is a beneficial modification for RXFP4 engagement, and highlight how strategic truncation, removal of non-essential disulphide bonds, and helix-tuning substitutions can markedly improve manufacturability and pharmacological performance while preserving native-like efficacy.

3.3.2. Design and optimisation of INSL5-based antagonists. The availability of A13 as a robust agonist scaffold enabled systematic engineering of RXFP4 antagonists. Using A13 as a template, the Hossain laboratory first generated A13-nR (Fig. 5) by substituting R^{B23} with the arginine mimetic norarginine (nR), which possesses a shorter side chain while retaining a terminal guanidinium group.[27] A13-nR antagonised agonist-induced increases in colon motility in RXFP4-expressing mice and inhibited colorectal propulsion triggered by intracolonic short-chain fatty acids, indicating that RXFP4 signalling contributes to bacterial product-induced diarrhoea and that INSL5-based antagonists may have therapeutic potential in this setting.[27] However, A13-nR displayed partial agonism *in vitro* and did not fully suppress cAMP response-element reporter activity, with modest antagonist potency and incomplete blockade of RXFP4 signalling.[27]

Subsequent work refined this antagonist scaffold by adding additional modifications at W^{B24} and by further truncating the B-chain N-terminus. Replacing W^{B24} with its mimetic 2-naphthylalanine (abbreviated as nW) in the A13-nR scaffold generated A13-nRnW (Fig. 5), which displayed improved RXFP4 affinity, greater antagonist potency, and enhanced inhibition of agonist responses compared to A13-nR.[65] An N-terminally truncated variant, A13:B7-24-AS-nRnW, preserved similar binding and antagonistic profiles while benefiting from a shorter and synthetically simpler scaffold, *e.g.*, achieving high RXFP4 affinity in the low-nanomolar range (approximately 3 nM), markedly reduced partial agonist activity, antagonist potency around 10 nM, and maximal inhibition of agonist responses of roughly 80%.[65] Mechanistically, molecular dynamics (MD) simulations suggest that these antagonists maintain the salt bridge between nR^{B23} and E100$^{2.63}$ but induce a distinct orientation of the B24 aromatic side chain compared to what is observed with agonists.[65] Instead of rotating towards TM5 to form a stable cation–π interaction with R208$^{5.42}$, the modified aromatic ring undergoes restricted "in-place" flips that fail to stabilise the active conformer of the receptor. This loss of the W^{B24}-R208$^{5.42}$ cation-π contact correlates with reduced TM5 inward movement and impaired receptor activation, providing a structural explanation for the agonist-to-antagonist transition.

Collectively, these studies demonstrate that the B-chain C-terminus, particularly residues A^{B20}, S^{B21}, Y^{B18}, R^{B23}, and W^{B24}, acts as a finely tuneable control module for RXFP4 signalling. Small changes in side-chain length, aromaticity, or backbone flexibility at these positions can flip the receptor response from full agonism to potent antagonism without substantially compromising affinity due to affinity coming from other B-chain residues.

3.4. Small-molecule agonists

The discovery of RXFP4-active INSL5 analogues has been accompanied by separate efforts to identify small-molecule RXFP4 agonists through high-throughput screening. One such campaign identified a guanidine-containing small molecule, **Compound 1**, which acted as an agonist at both RXFP3 and RXFP4.[66] Subsequent medicinal chemistry optimisation improved potency, leading to later analogues such as **Compound 4** (Fig. 6), which displayed enhanced RXFP4 activity. Although **Compound 4** still retains some activity at RXFP3, it remains the most potent and selective small-molecule RXFP4 agonist reported to date.[51]

Cryo-EM structures of RXFP4 bound to **Compound 4** revealed that its central guanidinium group forms a salt bridge with E100$^{2.63}$, analogous to R^{B23} in INSL5, while an aromatic moiety occupies the same central hydrophobic cavity as W^{B24} and engages W97$^{2.60}$, F291$^{7.35}$, and H299$^{7.43}$. This structural similarity confirms that small molecules can faithfully reproduce the critical electrostatic and aromatic interactions that drive RXFP4 activation. However, because the underlying binding pocket around E100$^{2.63}$ is highly conserved between RXFP3 and RXFP4, early guanidinium-based agonists retained dual activity at both receptors.

A subsequent medicinal chemistry study systematically optimised this active moiety and identified several highly potent dual RXFP3/4 agonists, including **Compound 10d**.[67] Structure–activity analysis showed that the rigid amidinohydrazone core was essential for activity, while para-substitution of the distal aromatic ring strongly influenced potency. **Compound 10d**, incorporating a 5-cyano,7-methyl indole and a para-methoxycarbonyl phenethyl substituent, acted as a full agonist at RXFP4 with an EC_{50} of 2.7 nM and Emax of 97%, but showed almost identical potency at RXFP3 (EC_{50} 3.1 nM, Table 1), indicating that it is a potent dual RXFP3/4 agonist rather than an RXFP4-selective ligand. The same study also highlighted a key limitation of this class, namely that improved potency did not translate into greater RXFP4 selectivity, and broader drug-like properties remained suboptimal. Another high-throughput screening campaign discovered **JK0621-D008**, with its S-enantiomer JK-1 showing activity on RXFP4 and some off-target activity on RXFP3 (Fig. 6 and Table 1).[68]

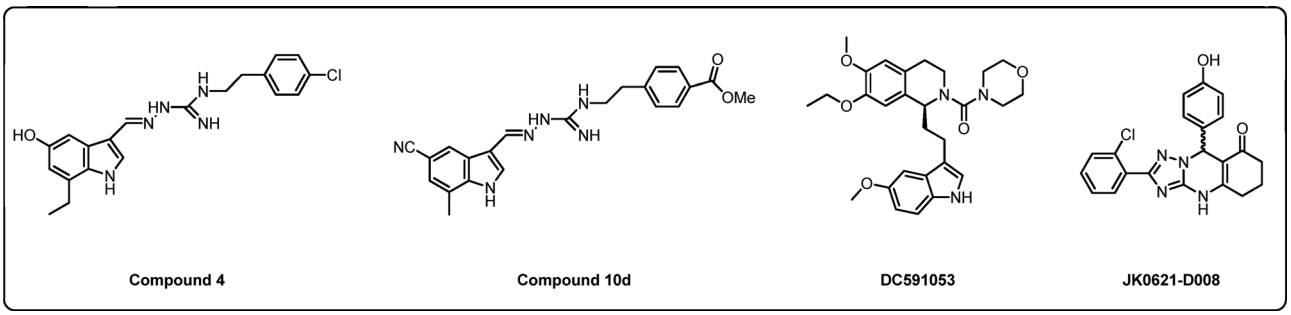


Fig. 6 Chemical structures of selected small molecules targeting RXFP4.

Table 1 RXFP3 and RXFP4 receptor binding affinity (K_i) and cAMP potency (EC_{50}) of selective major RXFP4 ligands

	RXFP3		RXFP4		
Ligand Name	SmBiT-R3/I5 K_i	cAMP EC_{50}	SmBiT-R3/I5 K_i	cAMP EC_{50}	Ref.
H3 relaxin	1.32 nM	0.83 nM	1.41 nM	1.15 nM	70
INSL5	81.28 nM	No activity	3.29 nM	4.57 nM	71
A13	195.12 nM	No activity	7.76 nM	7.94 nM	71
A13:B7-24-AS	426.12 nM	No activity	8.71 nM	8.32 nM	72
A13:B7-24-GG	602.56 nM	4786.30 nM	2.29 nM	1.17 nM	64
A13-nR	—	—	14.13 nM	>10 000 nM	27
A13-nRnW	—	—	3.47 nM	>10 000 nM	65
A13:B7-24-AS-nRnW	—	—	3.09 nM	No activity	65
INSL5-K	12.30 nM	No activity	1.26 nM	0.45 nM	64
Compound 4	—	12.8 nM	—	1.6 nM	66
Compound 10d	—	3.1 nM	—	2.7 nM	67
JK-1	—	—	17400 nM[a]	31600 nM	68
DC591053	No activity	No activity	112 nM[a]	57.5 nM	51

[a] Measured using competitive europium (Eu)-labelled ligand.

Although these studies establish that RXFP4 is amenable to nonpeptide ligand discovery, the small-molecule field remains in its early stages. To date, only a limited number of RXFP4-active small molecules have been reported, and most have shown substantial activity at the related receptor, RXFP3, highlighting the continuing challenge of achieving strong receptor selectivity within this closely related receptor pair. Even the most potent examples, including **Compound 4** and **Compound 10d** referred to above, are best regarded as useful lead structures rather than fully optimised RXFP4-selective chemical probes. Current small-molecule agonists remain incompletely characterised with respect to pharmacokinetics, metabolic stability, safety, and *in vivo* target engagement. Importantly, whereas peptide-based antagonist development has advanced substantially, small-molecule RXFP4 antagonists have not yet been reported. Thus, major challenges in medicinal chemistry remain before small molecules can match the selectivity and mechanistic reliability currently offered by peptide ligands.

More recently, **DC591053** was reported as another small-molecule RXFP4 agonist, a tetrahydroisoquinoline-based RXFP4-selective agonist (Fig. 6).[51] **DC591053** retains the overall spatial arrangement of the INSL5 C-terminal pharmacophore: its indole-like aromatic group sits in the same central pocket as W^{B24} and engages $W97^{2.60}$, $F291^{7.35}$, and $H299^{7.43}$, while additional polar substituents form hydrogen bonds with $Q205^{5.39}$ and $T295^{7.39}$.[51] Unlike the guanidinium-containing compounds, **DC591053** features a urea-based polar group that interacts more weakly with $E100^{2.63}$ and instead relies on a network of RXFP4-specific contacts in TM3, TM5, TM7, and the extracellular loops (ECLs) to achieve subtype selectivity. Mutagenesis of these non-conserved residues disproportionately reduces the potency of DC591053, confirming that it exploits RXFP4-specific microenvironments rather than the fully conserved acidic cluster.[51]

Functionally, **DC591053** is a full agonist at RXFP4 but is approximately an order of magnitude less potent than INSL5.[51] Its pharmacokinetic and toxicity profiles remain to be fully characterised. Interestingly, **DC591053** closely resembles **compound 16** reported by Zhang *et al.* in a medicinal chemistry study of RXFP3/RXFP4-active small molecules, raising the possibility that it originated from, or was inspired by, a related screening library.[69]

Taken together, structural and medicinal-chemistry studies illustrate that the active moiety for RXFP4 activation can be encoded in either a compact peptide scaffold or a small-molecule framework that replicates the geometry and electrostatics of the R^{B23}-W^{B24} motif. Progress in truncation, helix tuning, and residue-specific mimetics has produced INSL5-based agonists with improved manufacturability and, crucially, a new class of high-affinity antagonists with minimal partial agonism. In parallel, cryo-EM-guided design has delivered small-molecule peptidomimetics that validate these principles

in a drug-like format. However, these advances have so far been confined to agonist discovery, and small-molecule RXFP4 antagonists have not yet emerged. Together, these advances provide a coherent structural and mechanistic foundation for targeting the INSL5-RXFP4 axis in different disease models.

Table 1 outlines the RXFP3 and RXFP4 receptor-binding affinity (K_i) and cAMP potency (EC_{50}) of all the RXFP4 ligands discussed in this section.

4. Physiological functions of the INSL5-RXFP4 axis in colon motility and therapeutic implications

Relaxin family peptides have diverse physiological roles, including reproductive regulation, vascular and connective tissue remodelling, and modulation of the central nervous system (CNS).[14,73,74] The specific physiological functions of the INSL5-RXFP4 system, except in the distal colon, remain unclear. To date, this signalling axis has been implicated in several physiological processes, including appetite regulation,[22] insulin secretion and type 2 diabetes,[23] and colorectal cancer.[24] However, evidence in these areas is often context-dependent and, in some cases, conflicting. Early studies suggested that INSL5 may act as a peripheral signal of energy deficit, with fasting-responsive expression and possible orexigenic effects, but these findings have not been consistently reproduced and appear to be strongly influenced by nutritional state, microbiota, and co-secreted L-cell hormones such as GLP-1 and PYY.[22,25,40,58] Likewise, proposed roles in glucose regulation and reproductive function, including fasting adaptation, β-cell support, sperm function, and endocrine-metabolic disorders such as polycystic ovary syndrome (PCOS), remain incompletely defined, and observations vary across models and genetic backgrounds.[47,55,75,76] By contrast, accumulating evidence now points to a more clearly defined and physiologically important role for the INSL5-RXFP4 system in the distal gut, where its actions can be linked directly to a specific enteric function.

INSL5 and its receptor play a physiological role in regulating fecal propulsion in the distal large intestine and, thus, defecation. Expression of INSL5 (gene and protein) is restricted to L-type enteroendocrine cells (EEC) of the large intestine and is absent from other regions of the gastrointestinal tract.[22,40] Within the large intestine, INSL5 is present in L cells of the distal colon, but is absent or at very low levels in the proximal colon.[77] The proximal colon has a major role in mixing the content of the gut, which is in a liquid or semi-liquid form in these regions, allowing bacterial involvement in digestive processes, absorption of bacterially produced nutrients, including short-chain fatty acids (SCFA) and B vitamins, and absorption of water and electrolytes. There is a transition to the distal colon and rectum, which have a fecal storage function and from where feces are expelled in humans and other species. Thus, the location of INSL5-containing EEC in the distal colon and rectum suggests a role in fecal expulsion. This has indeed been found to be the case: both activation of RXFP4 with the INSL5 analogue A13 and stimulation of hormone release from colonic INSL5-containing EEC caused defecation.[21,58]

Colonic L cells express receptors for microbial products, including free fatty acid receptors, as well as expressing bile acid receptors.[77–80] Instillation of a SCFA mixture into the colon accelerates colonic emptying.[81,82] Acceleration of colonic propulsion by SCFAs in mice is inhibited by the RXFP4 receptor antagonist, INSL5-A13NR, confirming that INSL5 plays a physiological role in stimulating propulsion.[27] There is also an involvement of 5-HT, acting through 5-HT_3 receptors, because enhanced propulsion caused by SCFAs or by stimulation of colorectal L cells is inhibited by 5-HT_3 receptor antagonists, and stimulation of colorectal propulsion by the RXFP4 agonist A13 is antagonised by the 5-HT3 receptor antagonist, alosetron,[41,58,82] supporting the involvement of serotonin activation of enteric reflex pathways. Related chemogenetic/DREADD studies have also shown that selective activation of distal colonic L-cells engages a neural pathway that alters colonic output.[58] Importantly, emerging human evidence further supports the clinical relevance of this pathway, because INSL5 release has been associated with bile acid diarrhea.[26] In human volunteers, bile acid introduced into the distal colon caused the release of INSL5 and accelerated defecation.[26] Moreover, patients suffering from bile acid diarrhea had elevated levels of circulating INSL5, and in patients with plasma INSL5 greater than 100 pg mL^{-1}, the 5-HT_3 receptor antagonist, ondansetron, improved the stool score (reduced its water content). Together, these findings strengthen the view that INSL5-RXFP4 signalling contributes to colonic propulsion through a 5-HT3 antagonist-sensitive mechanism. It is notable that some earlier studies had reported circulating levels of INSL5 of >1000 pg mL^{-1}, suggesting that there was immunoassay cross-reactivity in the earlier studies.[26]

In mouse, the colorectal L cells are in close proximity to the 5-HT-containing EEC, which express RXFP4.[41] Thus, localisation and physiological evidence indicate that INSL5 released from the distal colon EEC acts on RXFP4 receptors on adjacent 5-HT expressing EEC to cause 5-HT release. Surprisingly, RXFP4 receptors are expressed by 5-HT EEC of the proximal small intestine, where INSL5 does not occur, and activation of the receptors in the proximal small intestine EC cells inhibits 5-HT release.[83] It is pertinent that the EC cells of the GI tract form transcriptionally distinct subgroups, those of the upper small intestine being different from those of the distal colon, both molecularly and structurally.[84,85] It will be important to investigate signalling downstream of RXFP4 in the distal colon and rectum 5-HT-containing EEC.

Defecation is dependent on the activation of enteric nervous system circuits responsible for colorectal propulsion,[86] so it is pertinent that 5-HT released from the mucosa acts on sensory nerve endings of enteric neurons to stimulate the enteric neural circuits.[85,87] The observation that ondansetron reduces fecal water content in patients with bile acid diarrhea is consistent with 5-HT also enhancing fluid secretion.[26,88]

We deduce that the INSL5/RXFP4 physiological pathway involves chemicals in the gut lumen, including SCFA and bile

acids, stimulating L-type EEC of the distal colon and rectum to release INSL5, which acts on RXFP4 of neighbouring 5-HT-containing EC cells (Fig. 7). 5-HT is released and stimulates the mucosal endings of enteric sensory neurons through which propulsive reflexes are initiated, and defecation ensues.[86]

The therapeutic implications of these findings are substantial. Constipation remains a major unmet clinical problem, particularly in older individuals, in patients receiving opioid therapy, and in neurological disorders associated with impaired defecation.[89] Current therapies are often nonspecific, only partially effective, or limited by adverse effects.[90–92] The INSL5-RXFP4 axis offers a new therapeutic entry point because it appears to stimulate propulsion through a native colonic signalling pathway rather than through generalised secretory or stimulant-laxative mechanisms. In this regard, the 2025 study by Yan *et al.* is highly informative. Using a loperamide-induced constipation model, the authors showed that both native INSL5 and the small-molecule RXFP4 agonist **DC591053** improved multiple constipation-related endpoints, including faecal water content, faecal weight, pellet number, and colonic transit time.[28] These effects were accompanied by restoration of colon morphology and modulation of several mechanistically relevant biomarkers, including increased circulating 5-HT, reduced vasoactive intestinal peptide (VIP) and nitric oxide, reduced transient receptor potential vanilloid 1 (TRPV1) and CGRP expression, and reduced aquaporin 3 (AQP3) expression in the colon.

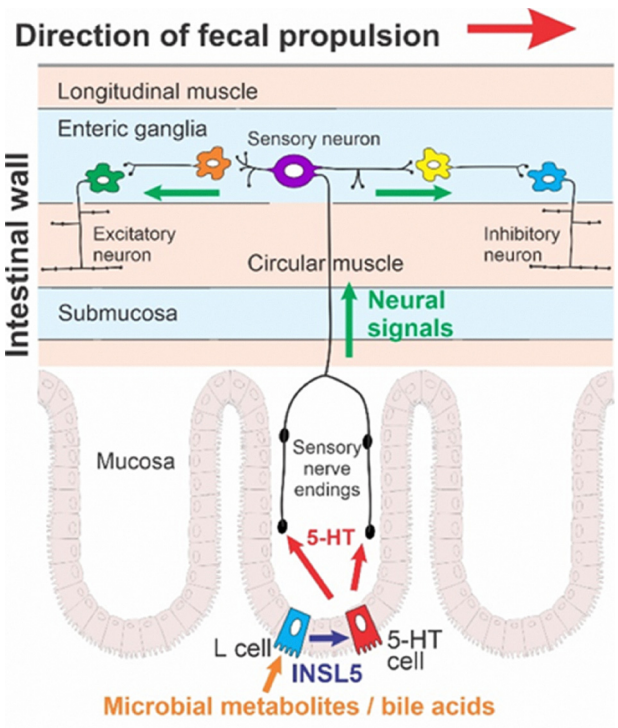


Fig. 7 Proposed mechanism by which luminal bacterial products stimulate propulsive reflexes in the colon. Bacterial metabolites in the colonic lumen, such as free fatty acids, activate receptors on enteroendocrine cells (EECs), particularly L-cells, triggering the release of hormones including INSL5. INSL5 then acts on RXFP4-expressing 5-HT-containing enterochromaffin cells, promoting 5-HT release. The released 5-HT activates enteric neural circuitry that drives propulsive motility.

These observations suggest that RXFP4 agonism may influence not only motor reflexes but also epithelial water handling and the broader neurochemical environment associated with constipation. Increased 5-HT is consistent with the epithelial-neural relay model proposed by Koo *et al.*, while reduced nitric oxide and VIP would favour propulsion by reducing inhibitory influences on motility. Reduced AQP3 expression may contribute to increased faecal water content, which would improve stool hydration and facilitate transit. At the same time, reduced TRPV1 and CGRP may reflect decreased sensory stress or neural irritation within the constipated colon. Although some of these downstream changes are likely indirect, together they indicate that RXFP4 agonism has a multidimensional impact on the constipated bowel.

From a drug development perspective, the comparison between native INSL5 and **DC591053** is also important. Native INSL5 is pharmacologically effective but synthetically complex and poorly suited to routine therapeutic development. In contrast, a small-molecule agonist offers practical advantages in synthesis, stability, and oral administration. Yan *et al.* showed that oral **DC591053** can ameliorate constipation, although higher doses were required than for the injected peptide, suggesting a trade-off between convenience and potency. This provides proof of concept that RXFP4 is druggable beyond peptide ligands.

Nevertheless, several challenges remain before the INSL5-RXFP4 axis can be translated into therapy. Most mechanistic work has been conducted in mice, and the extent to which the same cellular localisation and mucosal circuitry operate in human colon remains to be fully established. The dominant preclinical readouts, particularly bead expulsion and loperamide-induced constipation, capture important aspects of propulsion but do not encompass the full complexity of chronic human constipation. In addition, because RXFP4 signalling may intersect with serotonergic pathways, there is a need to define the therapeutic window carefully to avoid excessive stimulation, cramping, or diarrhoea. Peptide-based RXFP4 agonists may be especially suitable for this indication. Although peptide drugs often have short circulating half-lives, this property may be advantageous for colonic motility disorders, where prolonged systemic exposure may be unnecessary or even undesirable. A short-acting peptide could provide sufficient local receptor activation to stimulate propulsion while limiting off-target effects.

Compared with the emerging colonic motility literature, evidence for roles of INSL5-RXFP4 in appetite and systemic metabolism is considerably less consistent. Several factors likely contribute to this variability. First, different studies have used distinct experimental frameworks, including acute pharmacological administration,[22,25] chronic dosing,[25] whole-body knockout models,[55,93] and indirect stimulation of distal L-cells, each of which interrogates different aspects of physiology. Acute peptide administration may reveal effects that are masked in constitutive knockout models by developmental compensation, whereas L-cell activation studies are complicated by the co-release

of GLP-1 and PYY, which can obscure or override any independent orexigenic action of INSL5. Second, species and strain differences are likely to be important, as phenotypes have varied between mouse models and RXFP4 is not even functional in rats. Third, nutritional state, microbiota composition, and background diet appear to strongly influence colonic INSL5 expression and distal L-cell output, suggesting that the INSL5-RXFP4 axis may function primarily as a context-dependent signal of energy deficit rather than as a dominant regulator of feeding under basal conditions. Taken together, these considerations suggest that the conflicting appetite and metabolic data do not necessarily rule out a physiological role for INSL5, but instead indicate that this role is conditional, experimentally sensitive, and likely subordinate to stronger co-secreted gut hormone signals in many settings. In contrast, the evidence linking INSL5-RXFP4 to colorectal motility is more coherent across pharmacological, genetic, anatomical, and physiological studies, making this currently the most compelling and experimentally supported function of the axis.

Although RXFP4 is emerging as a promising target for colorectal dysmotility, the translational path remains at an early stage. Much of the current evidence is still based on acute pharmacological studies and rodent models, and no RXFP4-directed therapy has yet entered clinical development. Peptide-based agonists currently offer the clearest pharmacological validation of the pathway because they retain high potency and receptor selectivity, but they also face familiar limitations associated with peptide therapeutics, including synthetic complexity, limited oral bioavailability, susceptibility to proteolytic degradation, and the need to optimise tissue exposure and dosing duration. By contrast, small-molecule agonists may offer advantages in oral delivery and manufacturability, but currently available compounds remain less mature in terms of receptor selectivity, pharmacokinetic characterisation, and safety assessment. Thus, while the INSL5-RXFP4 axis is an attractive therapeutic target, substantial work is still required to define the optimal ligand format, route of delivery, and safety profile for clinical translation.

Overall, the current literature supports a model in which INSL5 acts as an endogenous prokinetic signal in the distal large intestine. By activating RXFP4, predominantly on enterochromaffin cells, INSL5 promotes 5-HT-dependent activation of enteric propulsive reflexes and thereby enhances colorectal transit. This local endocrine-neural circuit provides a compelling explanation for how luminal stimuli can be translated into motor activity and identifies RXFP4 as a promising therapeutic target for constipation and related disorders of colorectal dysmotility.

5. Knowledge gaps and future directions

Despite considerable progress in ligand design and physiological characterisation, several important gaps still limit full understanding of the INSL5-RXFP4 system and its translational potential. At present, the clearest and most convincing evidence supports a role in colorectal motility, whereas evidence for proposed functions in appetite regulation, glucose homeostasis, and reproductive biology remains less consistent and is often highly context-dependent. Future work should therefore focus on integrating structural, pharmacological, and physiological evidence more closely, so that conclusions about RXFP4 biology are supported by reliable ligand tools, well-validated cellular targets, and disease-relevant models.

A major unresolved question concerns the precise cellular mechanism by which INSL5-RXFP4 signalling promotes colorectal propulsion.[21,27,28,41] Recent studies support a local distal colonic circuit involving L-cells, enterochromaffin cells, serotonergic signalling, and enteric reflex pathways (Fig. 7). However, the relative contributions of epithelial cells, enteric neurons, and extrinsic sensory inputs remain incompletely defined, particularly in humans. Clarifying the mechanisms will be essential because the optimal therapeutic strategy may differ depending on whether RXFP4 is best targeted at the mucosal surface, within enteroendocrine signalling networks, or more directly within enteric circuitry. High-resolution receptor mapping, spatial transcriptomics, and further functional studies in native human and animal preparations will therefore be important next steps. On the other hand, a major unresolved issue is why proposed roles of INSL5-RXFP4 in feeding and metabolism have proven difficult to reproduce across models. Future studies should directly compare pharmacological and genetic perturbations under controlled dietary and microbiota conditions, and distinguish effects of INSL5 itself from those of co-secreted L-cell hormones.

A second key gap is the limited pharmacological toolbox currently available for RXFP4. Native INSL5 has been crucial for defining receptor biology, but its synthetic complexity restricts widespread use. Simplified peptide agonists and antagonists have greatly advanced the field, yet additional ligands with improved selectivity and more clearly defined signalling profiles are still needed.[27,35,63,64,94] In particular, the development of full antagonists, labelled probes, biased ligands, and compounds suitable for *in vivo* target engagement studies would substantially strengthen future mechanistic work. Small-molecule agonists further demonstrate that RXFP4 activation is chemically tractable,[51,66–68] but currently available small molecules still require improvement in receptor selectivity, pharmacokinetics, and depth of functional characterisation. Thus, future medicinal chemistry efforts should focus not only on potency but also on selectivity, pathway profiles, and translational suitability.

Another important priority is the need to validate current findings in human systems. Much of the strongest functional evidence for RXFP4 in colon motility comes from murine studies, particularly bead expulsion assays and pharmacological constipation models. While these models have been highly informative, they do not fully capture the complexity of human colorectal dysmotility. It therefore remains important to establish whether RXFP4 expression patterns, downstream signalling mechanisms, and functional outcomes are conserved in the human colon. Studies using human tissues, organoids, and

ex vivo motility preparations will be especially valuable in addressing this translational gap.

From a therapeutic perspective, RXFP4 is emerging as a promising target for colorectal dysmotility, particularly constipation. However, translation will require a clearer understanding of long-term efficacy, receptor desensitisation, tissue selectivity, and potential off-target physiological consequences. It will also be important to determine whether gut-restricted RXFP4 modulation is preferable to systemic exposure, and whether chronic activation can improve propulsion without causing cramping, diarrhea, or unwanted metabolic effects. In this regard, comparative studies of peptide and small-molecule RXFP4 ligands in longer-term disease models will be especially informative.

Overall, the field is now entering a stage where mechanism-based therapeutic exploration is realistic, but several foundational questions still need to be addressed. The most important future directions are to define the endogenous cellular mechanism of RXFP4 action in the colon, expand the ligand toolbox, validate findings in human systems, and establish the therapeutic window for RXFP4-directed agents. Addressing these gaps will determine whether the INSL5-RXFP4 axis can be translated from an intriguing gut endocrine pathway into a clinically useful target for disorders of colorectal motility.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Supplementary data has been deposited with Protein Data Bank.[95]

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