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Design and Development of Analogues of Dimers of Insulin-Like Peptide 3 B-Chain as High-Affinity Antagonists of the RXFP2 Receptor

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ABSTRACT:

Insulin-like peptide 3 (INSL3) is one of 10 members of the human relaxin–insulin superfamily of peptides. It is a peptide hormone that is expressed by fetal and postnatal testicular Leydig cells and postnatal ovarian thecal cells. It mediates testicular descent during fetal life and suppresses sperm apoptosis in adult males, whereas, in females, it causes oocyte maturation. INSL3 has also been shown to promote thyroid tumor growth and angiogenesis in human. These actions of INSL3 are mediated through its G protein-coupled receptor, RXFP2. INSL3, a two-chained peptide, binds to its receptor primarily via its B-chain, whereas elements of the A-chain are essential for receptor activation. In an attempt to design a high-affinity antagonist with potential clinical application as an anticancer agent as well as a contraceptive, we have previously prepared a synthetic parallel dimer of INSL3 B-chain and demonstrated that it binds to RXFP2 with high affinity. In this work, we undertook full pharmacological characterization of this peptide and show that it can antaogonize INSL3-mediated cAMP signaling through RXFP2. Further refinement by truncation of 18 residues yielded a minimized analogue that retained full binding affinity and INSL3 antagonism. It is an attractive lead peptide for in vivo evaluation as an inhibitor of male and female fertility and of INSL3-mediated carcinogenesis. © *2010 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 96: 81–87, 2011.*

Keywords: *B-chain dimer; disulfide constraint; helicity; INSL3; RXFP2*

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INTRODUCTION

Insulin-like peptide 3 (INSL3) belongs to the insulin–relaxin superfamily of polypeptide hormones, which comprise insulin, relaxins-1, 2, 3, INSL3, INSL4, INSL5, and INSL6 and insulinlike growth factor I and II.[1,2] It was discovered in early 1990s and named Leydig cell insulin-like peptide (Ley-IL), because it was found in the Leydig cells of testis.[3] It has also been referred to as RLF (relaxinlike factor) due to its relaxinlike activity in a mouse interpubic ligament bioassay.[4] It is expressed by testicular Leydig cells acting as a marker for fully differentiated adult-type Leydig cells,[5] and, in females, it is produced by ovarian follicles and the corpus luteum.[6,7]

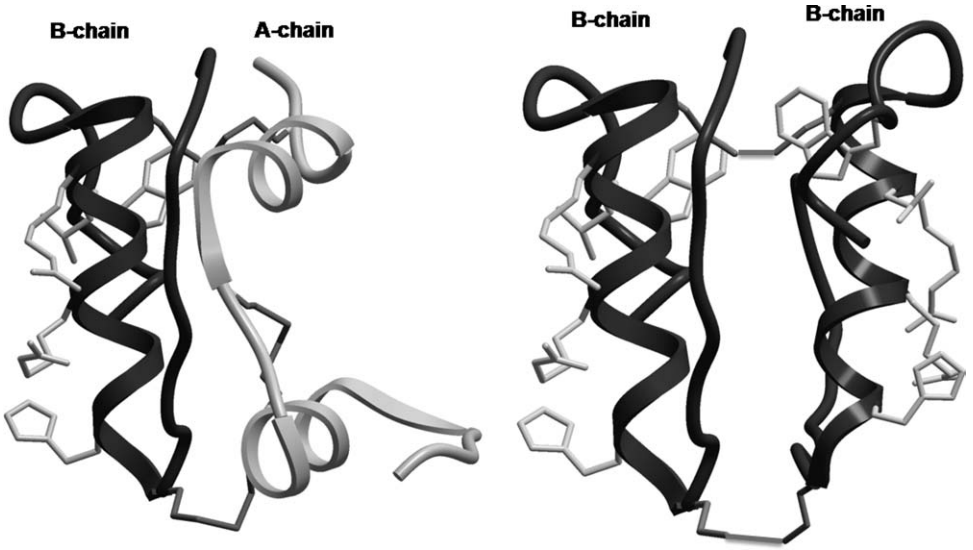


FIGURE 1 A: Solution NMR structure of human INSL3 (PDB:2H8B) and its sequence with the key receptor-binding residues highlighted. B: A model of parallel INSL3 B-chain dimer joined together via two cystine residues, which are involved in interchain disulfide bonds with A-chain in the native INSL3 structure.

INSL3 is a circulating hormone that mediates the transabdominal phase of testicular decent during fetal life.[8–10] In adults, INSL3 acts as a paracrine factor in mediating gonadotropin actions.[11] Luteinizing hormone, which is released by the anterior pituitary gland, stimulates INSL3 transcripts in ovarian theca and testicular Leydig cells. INSL3 then binds RXFP2 (previously known as LGR8) expressed in germ cells to activate the inhibitory G protein, thus leading to decreases in cAMP production. This, in turn, leads to the initiation of meiotic progression of arrested oocytes in preovulatory follicles in vitro and in vivo and suppresses male germ cell apoptosis in vivo.[11] INSL3 has recently been shown to be involved in bone metabolism, as *RXFP2*$^{-/-}$ knockout mice show a considerable reduction in their bone mass, mineralizing surface, and bone formation compared to wild-type mice.[12]

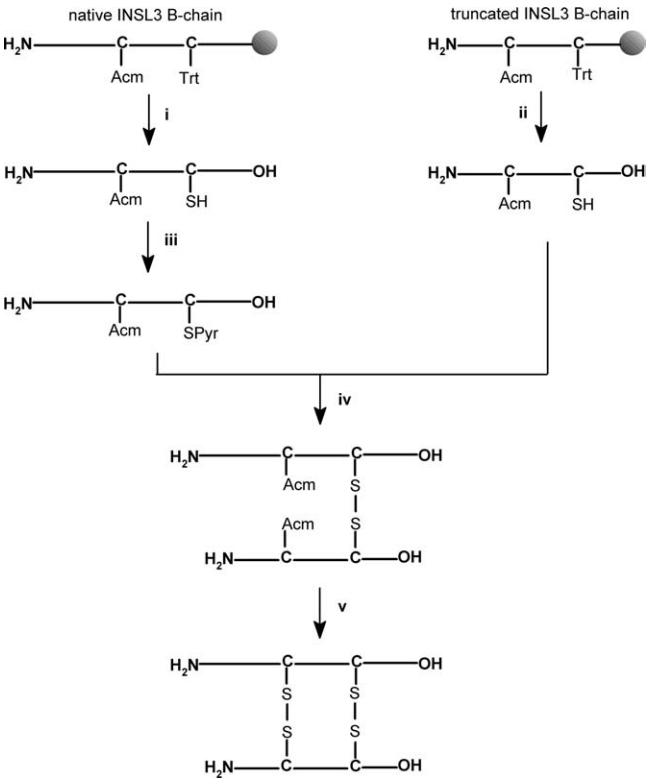
INSL3 may also play a role in the pathobiology of some forms of human cancers, such as thyroid carcinoma, as its expression is upregulated in hyperplastic and neoplastic human thyrocytes.[13] A recent study has confirmed that INSL3 does indeed promote thyroid tumor growth and angiogenesis.[14] The tumor-promoting effect of INSL3 has been linked to the ability of INSL3 to drive early tumor-cell invasiveness by enhancing the metabolic activity and elastin-degrading potential of thyroid carcinoma cells.[15]

Structure-activity studies using Ala scanning and truncations have shown that INSL3 binds to its receptor principally through residues (His^{B12}, Arg^{B16} Val^{B19}, Arg^{B20}, and Trp^{B27}) located within the B-chain.[16] Amongst these residues, Trp^{B27} appears to be the most crucial as its deletion or substitution leads to a significant loss of receptor-binding affinity of INSL3.[16–18]

Our previous studies have focused primarily on the development of analogues of INSL3 B-chain with antagonistic activity. These are valuable tools for the study of the physiological role of INSL3 in vivo. In addition, such antagonists may have significant clinical application in the area of fertility management as well as anticancer agents. These single B-chain analogues showed micromolar RXFP2 receptor-binding affinity, which is lower than the nanomolar affinity of the native INSL3,[18,19] probably due to their significantly reduced secondary structure, which appears to be required for high affinity interaction with the receptor. However, simple dimerization of the B-chain (see Figure 1) yielded an analogue, which was shown to retain the same degree of α-helicity as native INSL3 itself. This peptide also showed a binding affinity for RXFP2 that was comparable to that of native INSL3. In this study, we have further pharmacologically characterized this peptide and used it as a lead for further modification to develop a high-affinity domain-minimized antagonist of INSL3.

MATERIALS AND METHODS

9-Fluroenylmethoxycarbonyl (Fmoc) protected L-α-amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from GL Biochem (Shanghai, China). *N*,*N*-dimethylformamide, piperidine, methanol, diethylether, dichloromethane, and acetonitrile were from Merck (Melbourne, Australia), and trifluoroacetic acid (TFA) was obtained from Auspep (West Melbourne, Australia). Fmoc-L-Ala-PEG-PS, Fmoc-L-Cys(Trt)-PEG-PS, Fmoc-L-Phe-PEG-PS, Fmoc-L-Pro-PEG-PS, Fmoc-L-Trp-PEG-PS, and Fmoc-L-Tyr(tBu)-PEG-PS resins were purchased from Applied Biosystems (Melbourne, Australia). 3,6-



SCHEME 1 Synthesis of INSL3 B-chain dimers. Both B-chains were assembled on solid support with two different side-chain protecting groups for cysteines. (i, ii) TFA cleavage (94:2.5:2.5:1, TFA: H_2O:DoDt:TIPS), (iii) conversion of SH to SPyr. Peptide dissolved in TFA and DPDS was added (4-fold excess over peptide). (iv) Formation of first inter-chain disulfide bond. B-chain with free thiol was dissolved in H_2O and added to the B-chain with SPyr which was dissolved in 100 mM NH_4HCO_3 (pH 8.5). (v) Formation of second interchain disulfide bond. Peptide in glacial acetic acid (2 mg/ml) followed by the addition of 60 m*M* HCl (0.1 ml/mg) and 20 mM I_2 (42 eq/Acm).

Dioxa-1,8-octanedithiol (DODT), triisopropylsilane, and diisopropylethylamine were from Sigma-Aldrich (Sydney, Australia). 2,2′-Dipyridyl disulfide (DPDS) (Fluka-Switzerland), NH_4HCO_3 (BDH Laboratory Supplies, Poole, UK), and trifluoromethanesulfonic acid were from MP Biomedicals (Sydney, Australia). Dulbecco's modified Eagle's medium, RPMI 1640 medium, 2 m*M* L-glutamine, fetal calf serum, and penicillin/streptomycin were obtained from Trace Biosciences (Castle Hill, NSW). Bovine serum albumin and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid were obtained from Sigma-Aldrich (Seven Hills, NSW). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), disodium hydrogen phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), and sodium azide were obtained from AJAX Chemicals (Sydney, NSW). All other reagents were obtained from Sigma-Aldrich (Sydney, Australia).

Solid-Phase Peptide Synthesis

The linear precursor peptides were synthesized as C-terminal acids on PEG-PS preloaded resins with 0.19–0.22 mmol/g loadings using Fmoc solid-phase chemistry.[19] The side chain-protecting groups of trifunctional amino acids used were TFA-labile, except for acetamidomethyl (Acm)-protected cysteine. All peptides were synthesized on a 0.1-mmol scale on a CEM Liberty™ microwave peptide synthesizer (AI Scientific, Scarborough, QLD) using a fivefold excess of amino acid and HBTU and a 10-fold excess of DIEA.[19] Each coupling and deprotection step was carried out at 75°C using 25 W microwave power for 5 min and 60 W microwave power for 3 min, respectively. To prevent racemization, His and Cys residues were coupled for 1 h without microwave power.

The cleavage of peptides from the solid support was carried out using a TFA:H_2O:DODT:TIS (94:2.5:2.5:1, 20 ml) mixture for 90 min. Cleaved peptides were precipitated in ice-cold diethyl ether and collected by centrifugation (3000 rpm, 3 min); the pellet was washed by resuspending it in ice-cold diethyl ether and centrifuging it again three times.

In the native B-chain sequence, Cys^{B10} was trityl-protected and Cys^{B22} was Acm-protected. To form the first interchain disulfide bond, Cys^{SH} was converted to Cys^{SPyr} by dissolving the B-chain (1 μmol) and DPDS (4 μmol) in TFA and stirring at room temperature for 15 min (Scheme 1).

The truncated INSL3 B-chains and INSL6 B-chain sequences were synthesized with the first cysteine in the sequence protected with trityl-protecting group and the second cysteine protected with Acm. The trityl group was removed during cleavage of peptide from the solid-support to generate Cys^{SH}, which could then be used to react with Cys^{SPyr} on the native B-chain to form the first interchain disulfide bond. This was done by dissolving the B-chain with a free thiol in H_2O and adding it to the B-chain Cys^{SPyr} in 0.1 M NH_4HCO_3. The formation of the disulfide bond was monitored by analytical RP-HPLC.

The formation of second interchain disulfide bonds was carried out by iodine oxidation. The appropriate single disulfide-linked peptide was dissolved in acetic acid (2 mg/ml) followed by the addition of 60 m*M* HCL (0.1 ml/mg) and 20 m*M* I_2 (42 eq/Acm). The reaction mixture was stirred at room temperature for 1 h, and the progression of the reaction was monitored by RP-HPLC. After the completion of reaction, the mixture was poured onto chilled diethyl ether to precipitate the peptide, which was collected by centrifugation.[20] The peptide was dissolved in 20 m*M* aqueous ascorbic acid to quench the remaining iodine (Scheme 1).

The analysis and purification of peptides were carried out by RP-HPLC using Waters XBridge™ columns (4.6 × 250 mm, C18, 5 μm) and (19 × 150 mm, C18, 5 μm), respectively. HPLC solvents contained 0.1% TFA with H_2O as solvent A and acetonitrile as solvent B. A gradient of 1% change in buffer B per min over 30 min was used.

The characterization of peptides was achieved using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany). The matrices used were either sinapinic acid or α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Germany), made up in 75% acetonitrile containing 0.1% TFA. The peptide content was determined using vapor-phase acid hydrolysis followed by amino acid analysis as previously described.[18]

Circular Dichroism Spectroscopy

Some of the synthetic B-chain dimer analogues were selected for further analysis of their secondary structure. The peptides (0.1 μ*M*) were dissolved in phosphate-buffered saline (PBS: 10 m*M* potas-

sium phosphate buffer containing 137 m*M* NaCl pH 7.4). The CD measurements were carried out using a JASCO model J815 spectropolarimeter between the wavelengths of 195 and 250 nm at room temperature with a resolution and bandwidth of 0.1 nm and a cell of 0.1-cm path length (P). The recorded spectra in millidegrees of ellipticity (θ) were converted to mean residue ellipticity in deg cm^2 $dmol^{-1}$ as previously described.[18]

Ligand-Binding Assay

Whole-cell receptor-binding experiments were conducted in a competitive manner using human embryonic kidney (HEK)-293T cells stably transfected with RXFP2 and europium-labeled INSL3 as described previously.[21] Briefly, the media were aspirated, and cells were washed with 200 μl of PBS once. The cells were incubated with 300 p*M* of Eu-DTPA-INSL3 (K_d: 0.892 n*M*) per well in the presence of increasing concentrations of peptide analogues. Nonspecific binding was determining with an excess (500 n*M*) of unlabeled INSL3. The binding data were analyzed using a one site-binding model in GraphPad PRISM 4 (GraphPad, San Diego). The data were expressed as the mean ± SEM (standard error of mean) of the percentage of total specific binding of triplicates from at least three independent experiments. The inhibition constants (K_i) were determined from IC_{50} values using the Cheng–Prusoff equation, and statistical comparison of pK_i values were determined using one-way ANOVA followed by Bonferroni's multiple comparison test for multiple group comparisons.

Functional cAMP Assay

The level of peptide-induced cAMP accumulation was measured using a reporter gene assay in HEK-293T cell line cotransfected with RXFP2 and a pCRE-β-galactosidase reporter plasmid as previously described.[22] The cotransfected cells were incubated with increasing concentrations of INSL3 and the B-chain dimers for 6 h, after which the medium was removed and cells frozen at −80°C overnight. The amount of cAMP-driven β-galactosidase expression was measured in cell lysates as described.[22] Each concentration point was performed in triplicate, and the data expressed as the mean ± SEM of three independent experiments.

RESULTS AND DISCUSSION

INSL3 binds to its receptor RXFP2 with high-affinity primarily by interactions of specific B-chain residues of INSL3[16,17,23–25] with residues in the leucine-rich repeats of the receptor ectodomain.[26] The A-chain is essential for receptor activation. However, the mechanism by which this occurs has yet to be fully elucidated although it is thought to involve interaction of INSL3 with the transmembrane helices of the receptor.[27] In our previous studies, we focused on the development of an antagonist using only the B-chain of INSL3. However, the B-chain alone does not adopt significant α-helical structure, which, itself, is thought to be important for presentation of the key receptor-binding residues in a correct orientation to the binding surface on the receptor. As a consequence, analogues based on the B-chain alone have only low affinity for RXFP2. We have previously used various strategies to increase the α-helicity in the B-chain of INSL3, such as the inclusion of intrachain disulfide and lactam constraints as well as the incorporation of helicogenic residues and N-caps. Unfortunately, none of these approaches induced significantly increased α-helicity, and therefore all the resulting analogues possessed low affinity for the RXFP2 receptor.[18,19] On the other hand, replacing the A-chain (Figure 1A), which is involved in *activating* the receptor, with another B-chain resulted in a parallel B-chain dimer (analogue **1**, Figure 1B) that not only had an improved level of α-helicity but also significantly increased receptor-binding affinity (Table I).[18] In this study, we undertook to further pharmacologically characterize this peptide dimer to determine if it was an antagonist of the INSL3 receptor, RXFP2. It was also then further modified by either truncating it or replacing one of the B-chains with the B-chain of INSL6, another member of the insulin–relaxin superfamily, with the goal of both increasing its receptor-binding potency and reducing is molecular size.

To investigate the effect of the orientation of the two B-chains on the receptor-binding affinity of the dimer, one of the B-chains was reversed to make an antiparallel dimer (analogue **2**). This analogue, despite having slightly higher α-helical content compared to the parallel dimer (analogue **1**) (Table I), had about 10-fold lower RXFP2 receptor-binding affinity. The drop in the receptor-binding affinity may be due to the perturbation of the binding residues, which are located in the helical region of the B-chain as a result of a change in orientation of the chains. The flexible C-terminal region of one of the B-chains may also be bending toward the helical region of the second B-chain and masking some of the key receptor binding residues, which are located in that region and required for high-affinity binding.

After establishing that the parallel B-chain dimer (analogue **1**) had the better receptor-binding affinity, it was then tested for its ability to antagonize INSL3-mediated cAMP activity at RXFP2. In the presence of 1 μmol of analogue **1**, the concentration response curve for INSL3 was shifted 100-fold ($pEC_{50} = 8.1 \pm 0.06$, $n = 3$ compared to $pEC_{50} = 10.14 \pm 0.12$, $n = 3$, in the absence of analogue **1**) (see Figure 2). Thus, the parallel B-chain dimer was the preferred lead antagonist for further modification.

We then investigated whether we could reduce the size of the parallel B-chain dimer (analogue **1**). Analogue **3** is similar to analogue **1**, except that 10 residues from the N-terminus of one of the two B-chains were deleted. However, this did not have any impact on the receptor-binding affinity of this analogue (Table I). The N-terminus of both of the B-chains was

Table I Primary Amino Acid Sequence of INSL3 and the B-Chain Dimer Analogues

Peptide	Sequence	Receptor Binding (pK_i, $n = 3$)	Receptor Activation (pEC_{50}, $n = 3$)	% α-Helicity
INSL3	**AAATNPARY**C**C**LSG**C**TQQDLLTL**C**PY	9.22 ± 0.07 ($n = 6$)	10.14 ± 0.12	33
1	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA	8.48 ± 0.08	No activity	32
2	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA	7.44 ± 0.05*	No activity	40
3	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA **C**GHHFVRALVRV**C**GGPRWSTEA	8.74 ± 0.02	No activity	–
4	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA **AAATNPARY**CGHHFVRALVRV**C**GGPRWSTEA	8.93 ± 0.21	No activity	–
5	**C**GHHFVRALVRV**C**GGPRWSTEA **C**GHHFVRALVRV**C**GGPRWSTEA	7.45 ± 0.07**	No activity	–
6	**C**GHHFVRALVRV**C**GGPRWSTEA **C**GHHFVRALVRV**C**	7.53 ± 0.15**	No activity	–
7	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA **C**GHHFVRALVRV**C**GGPRW	8.33 ± 0.46	No activity	–
8	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA **C**GHHFVRALVRV**C**	8.50 ± 0.47	No activity	32
9	PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA ISSARKL**C**GRYLVEIEKL**C**GHANWSQF	8.70 ± 0.40	No activity	34

Receptor binding affinity (pK_i, $n = 3$), receptor activity (pEC_{50}, $n = 3$), and α-helicity of the peptides in PBS.
*$P < 0.05$;
**$P < 0.001$ significantly different from INSL3.

then truncated by 10 residues in analogues **5** and in combination with truncation of the C-terminus of one of the chains in analogues **6**. Unfortunately, these truncations led to about a 10-fold decrease in the receptor-binding affinity of both analogues (Table I). However as analogue **6** demonstrated equivalent affinity to analogue **5**, it is likely the truncations at the C-terminus of the single B-chain truncated at the N-terminus would be tolerated. Hence, one of the two B-chain stands was left intact, and the second B-chain was truncated from both termini to give analogues **7** and **8** (Table I). Despite being much smaller, the truncated analogues **7** and **8** showed similar receptor-binding affinity to intact analogue **1**. Analogue **8**, which is shorter by 18 residues, is the most domain-minimized analogue of the INSL3 B-chain dimer.

Importantly, like analogue **1**, none of the new compounds were able to activate RXFP2 (see Figure 3). This result was expected, as the analogues lack the receptor-activating A-chain. It has previously been shown that residues at the N-terminus of the A-chain (A1–A9) are essential for the activity of INSL3.[28] Therefore, it was postulated that addition of the relevant A-chain residues to the N-terminus of analogue **3** may yield a high-affinity RXFP2 agonist. Therefore, analogue **4** was synthesized and tested for its binding affinity and activity. Although it retained high-RXFP2-binding affinity, it was unable to activate the receptor. It is probable that the structure of analogue **4** does not present the critical residues of the A-chain in the correct orientation for receptor activation. Alternatively, it is possible that other A-chain residues

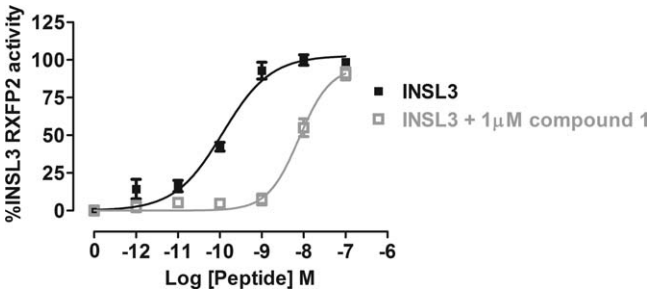


FIGURE 2 cAMP functional assay. INSL3 B-chain dimer (pEC_{50} = 8.1 ± 0.06, n = 3) at 1 μmol concentration causes a twofold rightward shift in the native INSL3 curve (pEC_{50} = 10.14 ± 0.12, n = 3).

are responsible for receptor activation as has been demonstrated recently.[29]

In the native INSL3 (Figure 1A), the helical region of the B-chain lies between the two cysteines, which are involved in forming interchain disulfide bonds with the A-chain. These two contact points seem to be important in aiding the helical structure of the B-chain and thus allow the binding residues to assume an appropriate orientation. We postulated that the helical region of the B-chain could also be stabilized with the B-chain sequence of other peptides in insulin–relaxin superfamily. To show that the high-affinity binding of the B-chain dimer is not due to the doubling effect of the key receptor-binding residues, analogue **9**, which is a chimeric dimer of INSL3 B-chain, and the INSL6 B-chain was synthesized. This analogue not only has similar receptor-binding affinity as the INSL3 B-chain homodimer but also has similar level of α-helicity (Table I). Therefore, as we have discussed earlier, although the disulphide links to the A-chain appear to be essential for providing the helical structure of the B-chain, this structure can be stabilized by substitution with another peptide sequence.

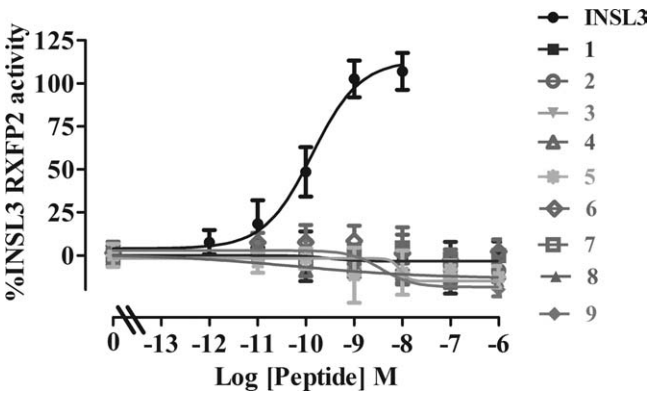


FIGURE 3 Receptor activation assay of native INSL3 and the B-chain dimers. INSL3 induces cAMP accumulation in HEK-293T cell expressing RXFP2, whereas the B-chain dimers are unable to activate the receptor.

In summary, it has been demonstrated that a synthetic parallel dimer of the B-chain of human INSL3 is a potent inhibitor of the native peptide's binding to its receptor, RXFP2. Removal of 18 residues from one strand of the dimer yielded analogue **8**, which is currently the smallest B-chain dimer analogue that retains high-affinity binding. It possesses significant promise for further refinement to improve its pharmacokinetic properties as a precursor to detailed in vivo evaluation as a regulator of male and female fertility and as an inhibitor of INSL3-mediated carcinogenesis.

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