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Design, synthesis and pharmacological evaluation of cyclic mimetics of the insulin-like peptide 3 (INSL3) B-chain

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Abstract: Insulin-like peptide 3 (INSL3) is a peptide hormone belonging to the relaxin-insulin superfamily of peptides that plays important roles in testes descent, oocyte maturation and the control of male germ cell apoptosis. These actions are mediated via a specific G-protein coupled receptor, LGR8. Previous structure–activity studies have shown that the key binding site of INSL3 is situated within its B-chain. Recent studies in our laboratory have led to the identification of a cyclic peptide mimetic 2 of the INSL3 B-chain, which we have shown to compete with the binding of [^{33}P]-relaxin to LGR8 expressed in HEK293T cells, and to inhibit cAMP-mediated signaling in these cells, i.e. it is an antagonist of INSL3. In order to further define the structure–activity relationships of cyclic analogues of the INSL3 B-chain, we used a structure-based approach to design a series of cyclic, disulfide-constrained INSL3 B-chain mimetics. To do this, we first created a model of the 3D structure of INSL3 using the crystal structure of human relaxin as a template. This model of INSL3 was then used as a template to design a series of disulfide-constrained mimetics of the INSL3 B-chain. The peptides were synthesized by solid-phase peptide synthesis using pseudoproline dipeptides to improve the synthesis outcome. Of the seven prepared INSL3 B-chain mimetics, three compounds were found to have partial displacement activity, while four were able to completely displace [^{33}P]-relaxin from LGR8, including compounds that were markedly shorter than compound 2. The best of these, mimetic 6, showed significantly greater affinity for LGR8 than compound 2, but still displayed around 1000-fold less affinity for LGR8 than native INSL3. Analysis of selected mimetics for their α-helical content using circular dichroism (CD) spectroscopy revealed that, generally, the mimetics showed less than expected helicity. The inability of the compounds to display true native INSL3 structure is likely contributing to their reduced receptor binding affinity. We are currently examining alternative INSL3 B-chain mimetics that might better present key receptor binding residues in the native INSL3-like conformation. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insulin-like peptide 3 (INSL3); LGR8; helix mimetic; cyclic peptide; disulfide; circular dichroism; radioligand binding; peptide synthesis

INTRODUCTION

Insulin-like peptide 3 (INSL3) is a member of relaxin-insulin superfamily of hormones, along with insulin, insulin-like growth factors I and II, relaxins 1, 2 and 3, and INSL4, 5 and 6. INSL3 binds with high affinity to and activates the leucine-rich repeat G-protein coupled receptor LGR8 [1,2]. It has only very low affinity for the paralagous receptor LGR7, which is the high affinity receptor for relaxin [3]. In contrast, relaxin can also effectively bind to and activate LGR8, albeit with slightly lower affinity than INSL3 [4,5]. LGR7 and LGR8 (recently reclassified as the relaxin family peptide receptors, RXFP1 and RXFP2 [6]) belong to the LGR family of receptors, which includes receptors for the follicle stimulating hormone and the luteinizing hormone.

INSL3, via its receptor LGR8, likely plays key roles in testicular descent and germ cell function. INSL3 is expressed by the Leydig cells of the testis in both the fetus and adult, as well as by the thecal cells of the ovaries [7–9]. Male mice deficient for the INSL3 gene [10,11] are phenotypically similar to male LGR8 gene deficient mice [12], in that both exhibit bilateral cryptorchidism resulting from impaired development of the gubernaculum, resulting in disruption of spermatogenesis and thus infertility. Consistent with these studies, overexpression of INSL3 in female mice causes the ovaries to descend into the inguinal region owing to an overdeveloped gubernaculum [13]. Furthermore, it has been shown that INSL3 acts as a paracrine mediator of the action of the luteinizing hormone on the maturation of oocytes in the ovaries and the suppression of male germ cell apoptosis in the testes [14]. These data indicate potential roles for INSL3-like agonists as regulators of fertility, and LGR8 antagonists as novel contraceptives.

INSL3 is a small (6 kDa) protein that consists of two chains (A and B) linked by two intermolecular disulfide bonds, with a third intramolecular disulfide bond in the A-chain (Figure 1) [15]. This disulfide bonding pattern is common among the superfamily members,

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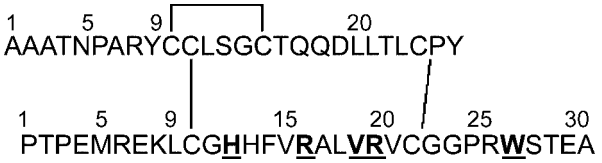

Figure 1 Primary structure and amino acid residue numbering of human INSL3. The residues in the B-chain implicated in the binding of INSL3 to its receptor LGR8 are underlined.

and is reflected in their common three-dimensional fold [16]. Recent structure–activity studies by others and us and using Ala-substituted analogs of INSL3 have demonstrated that the key residues responsible for interaction of INSL3 with the ectodomain of LGR8 reside in the B-chain helix of INSL3 (His^{12}, Arg^{16}, Val^{19} and Arg^{20}) [16,17] with an additional contribution from Trp^{27} located on the *C*-terminal tail of the B-chain [18] (Figure 1). Indeed, we have described a cyclic disulfide-constrained peptide based on the INSL3 B-chain that contains these key residues. The peptide competes with the binding of either radiolabeled relaxin or INSL3 to LGR8 and inhibits INSL3-mediated production of cAMP *in vitro*. Furthermore, *in vivo* administration of the peptide directly into the testes causes a reduction in testis weight, consistent with an inhibition of germ cell survival. While this latter observation renders the peptide a potential lead compound for subsequent development as a novel contraceptive agent, the peptide has relatively low affinity/potency (pK_i of approximately 10^{-6} M for displacement of ^{125}I-INSL3 from LGR8). This has led us to hypothesize that the cyclizing constraint used is not optimally positioned to present the B-chain residues in a native, INSL3-like conformation for binding. Furthermore, the peptide is relatively large (27 residues); a smaller cyclic INSL3 antagonist would have obvious advantages as a lead compound for further development.

To refine the structure–activity requirements for the binding of peptide mimetics of the INSL3 B-chain to LGR8, we describe in this paper a structure-based approach to design variously sized cyclic disulfide-constrained mimetics of the INSL3 B-chain, starting from a homology model of INSL3 based on the known three-dimensional structure of relaxin [19]. Following their synthesis and purification, selected cyclic B-chain mimetics were subjected to conformational analysis by circular dichroism (CD) and were used in radioligand binding studies to determine their ability to bind to the INSL3 receptor, LGR8.

MATERIALS AND METHODS

Materials

Molecular modeling was performed using the SYBYL molecular modeling software (Tripos, version 7.0, St Louis, MO, USA) on a Silicon Graphics O2 workstation. 9-Fluorenylmethoxycarbonyl (Fmoc) L-α-amino acid derivatives and Rink amide resin were purchased from Auspep Pty.Ltd, Australia. The pseudoproline derivatives Fmoc-Val-Ser ($\psi^{Me,Me}$Pro)-OH and Fmoc-Leu-Ser ($\psi^{Me,Me}$Pro)-OH were purchased from Novabiochem, USA. Fmoc-PAL PEG-PS and Fmoc-L-Ser(tBu)-PEG-PS resins were obtained from Perseptive Biosystem, USA. C5 semipreparative and C18 Rocket HPLC columns were obtained from Alltech Associates, Australia. [^{33}P]-labelled human gene 2 (H2) relaxin was prepared as described previously [20]. Solvents and other chemicals were all peptide synthesis and analytical grade.

Molecular Modeling

A homology model of INSL3 was created using the sequence and X-ray crystal structure [19] of H2 relaxin as a template. To do this, the relaxin sequence was first modified to that of INSL3. The resultant crude INSL3 model was refined by subjecting it to energy minimization using the Tripos forcefield and Gasteiger–Marsili charges. The minimization was terminated upon reaching a root mean square (RMS) energy gradient of <0.05 kcal/mol (after approximately 10 000 steps). The B-chain of this energy-minimized model was subsequently used as a template for the design of cyclic B-chain mimetics. In the first step, the distances between C_β atoms of residues on the strand and adjacent α-helix of the B-chain were measured. Pairs of residues found to have a $C_\beta - C_\beta$ distance less than 10 Å were chosen as potential sites for the incorporation of a disulfide constraint. To form the disulfide constraints, the pair of residues was replaced by Cys and the disulfide bond formed. The resultant cyclic peptides were shortened *N*-terminally until the position of the newly incorporated Cys, and acetylated; the *C*-terminus was shortened until Trp^{27} and amidated, as a similarly *C*-terminally modified analogue of INSL3 has been shown to exhibit near-native binding to LGR8 [17]. Where present, the two other B-chain Cys residues normally involved in interchain disulfide bonds with the A-chain (i.e. Cys^{10} and Cys^{22}) were replaced with Ser to prevent the formation of undesired disulfide bonds during later synthesis. The cyclic B-chain mimetics prepared in this way were minimized as described above and superimposed onto the INSL3 B-chain. The goodness of fit between the minimized mimetics and the INSL3 B-chain was assessed by measuring the RMS deviation of the distance between corresponding α- and β-carbon atoms.

Peptide Synthesis

Linear precursor peptides were synthesized either in batch mode using Rink amide resin, or in a continuous flow manner using Fmoc-PAL PEG-PS on a 0.1 mmol scale, except peptide **3**, which was prepared as the free acid on Fmoc-L-Ser(tBu)-PEG-PS. Fmoc-protected L-α-amino acids (0.3 mmol) were coupled using HBTU [21,22] (0.3 mmol) and DIPEA (0.45 mmol) in DMF (5 ml) for 30 min. Pseudoproline dipeptides (0.2 mmol) [23] were coupled manually as for individual amino acids. Fmoc-protecting groups were removed by treating the resin-attached peptide with piperidine (20% v/v) in DMF for 20 min. The 2,4,6-trinitrobenzenesulphonic acid (TNBSA) test [24] was carried out to qualitatively confirm the success of coupling and deprotection. The resin-bound peptides were acetylated at *N*-terminus by treating them with a

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solution of acetic anhydride (0.3 mmol) and DIPEA (0.45 mmol) in DMF. Linear peptides were obtained by treating the resin with TFA/TIPS/H_2O (18 : 1:1 (v/v), 10 ml) for 90 min. The cleaved peptides were precipitated in ice-cold diethyl ether and collected by filtration or centrifugation. The crude linear peptides were purified by reversed-phase (RP) HPLC on a C5 semipreparative column using 1%/min gradient of acetonitrile in 1% aqueous TFA over 30 min. Linear peptides were cyclized by treating them with 1% DMSO in 0.1 M aqueous NH_4HCO_3, with the reaction monitored by RP-HPLC using a C18 Rocket column and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The desired cyclic B-chain mimetics were purified from the reaction mixture by semipreparative RP-HPLC.

Peptide Characterization

The successful synthesis of peptides was confirmed by mass spectrometry: mimetics **4**, **5** and **6** were characterized by MALDI-QTOF (Applied Biosystems, USA); mimetic **8** was characterized by surface-enhanced laser desorption time-of-flight (SELDI-TOF) (Ciphergen, USA); mimetics **3**, **7** and **9** were characterized by MALDI TOF-TOF (Bruker Bioflex, Germany) mass spectrometry in the linear mode at 19.5 kV. Peptides were quantitated by amino acid analysis of a 24-h acid hydrolyzate using a GBC instrument (Melbourne, Australia).

Circular Dichroism (CD) Spectroscopy

CD spectra of INSL3 B-chain mimetics **5**, **6**, **8** and **9** were recorded on an Aviv 62DS CD spectrophotometer (Aviv Associates Inc., NJ) between the wavelengths of 195–250 nm at room temperature in a 1 mm path-length quartz cuvette. Peptides were made to a concentration of 0.1 μM (determined by amino acid analysis) in either phosphate buffered saline (PBS; 10 mM potassium phosphate buffer containing 120 mM NaCl, pH 7.4) or spectroscopy grade trifluoroethanol (TFE) [25]. The recorded spectra in millidegrees of ellipticity (θ) were converted to mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$.

Competition-binding Assays

The ability of the cyclic INSL3 B-chain mimetics to compete with the binding of [^{33}P]-labelled H2 relaxin to HEK-293T cells stably transfected with human LGR8 was carried out using a method described by us previously [3,26]. Data from these competition-binding assays were expressed as mean ± SEM of percentage specific binding of [^{33}P]-labelled H2 relaxin from triplicate determinations made from at least three independent experiments. Data were analyzed using Graphpad PRISM (Graphpad Ins., San Diego, USA), and a nonlinear one-site model was used to plot curves and calculate pK_i values. Final pooled pK_i data were analyzed using one-way ANOVA coupled with Bonferroni's multiple comparison test for multiple group comparison.

RESULTS

Molecular Design

A model of the 3D structure of INSL3 (Figure 2) was prepared using homology modeling techniques, starting from the X-ray crystal structure of human relaxin-2. As anticipated, the INSL3 model obtained in this way showed a high degree of similarity to the relaxin structure, with an RMS deviation of backbone atoms of 1.02 Å. The B-chain from this INSL3 model (compound **1**, Table 1) was subsequently used as a template to assist in the design of a series of *N*- and *C*-terminally truncated, cyclic disulfide-constrained INSL3 B-chain mimetics (compounds **3–9**, Table 1). The likely similarity of the putative INSL3 B-chain mimetics **3–9** to the 'native' INSL3 B-chain **1** was assessed by measuring the RMS deviation of the distances between corresponding $C_\alpha - C_\beta$ atoms. Compound **2**, a cyclic B-chain mimetic previously described by us [27], showed relatively low similarity to the INSL3 B-chain, as evidenced by its high RMS value. In contrast, placement of the disulfide bond at a more favorable position yielded mimetic **3** (an analogue of **2**), with an improved RMS value. Mimetic **4** is *C*-terminally truncated to, and amidated at, Trp^{27}. In mimetic **5**, this Trp residue has been removed, to determine whether it also plays a role in LGR8 binding as it has been shown to do in intact INSL3. Mimetic **7** is an analogue of mimetic **6** in which the hydrophobic Ala^{17} residue – whose side chain is normally buried by the A-chain in intact INSL3 – has been replaced by a more polar and thus potentially more favorable Ser. Mimetics **8** and **9** are characterized by subsequently smaller cycles accompanied by truncations to the *N*-terminal Cys, thus yielding cyclic B-chain mimetics of further reduced size.

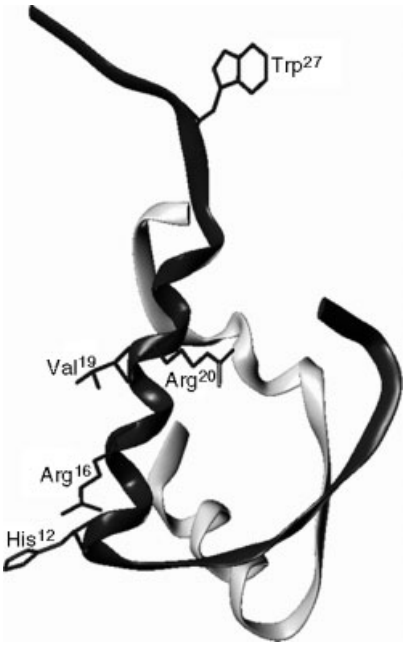

Figure 2 Model of the 3D structure of human INSL3 derived by homology modeling techniques from the crystal structure of relaxin. The A-chain is shown in gray and the B-chain in black. Shown are the side chains of the residues in the B-chain implicated in binding to LGR8.

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Table 1 Primary sequence, root mean square deviation (RMSD) in Å and MS data of cyclic INSL3 B-chain analogues described in this study

Analogues	Sequence	RMSD (Å)	m/z $[M+H]^+$ expected	
1	H-PTPEMREKL**C**GHHFVRALVRV**C**GGPRWSTEA-OH	—	—	—
2[a]	Ac-TP**C**MREKLSGHHFVRALVRVSGGP**C**WS-OH	3.163	—	—
3	Ac-**C**PEMREKLSGHHFVRALVRVSG**C**PRWS-OH	1.413	3178	3176
4	Ac-**C**PEMREKLSGHHFVRALVRVSG**C**PRW-NH_2	0.754	3090	3088
5	Ac-**C**PEMREKLSGHHFVRALVRVSG**C**PR-NH_2	0.754	2906	2905
6	Ac-**C**PEMREKLSGHHFVRALVR**C**SGGPRW-NH_2	1.49	3048	3046
7	Ac-**C**PEMREKLSGHHFVRSLVR**C**SGGPRW-NH_2	1.49	3064	3062
8	Ac-**C**KLSGHHFVR**C**LVRVSGGPRW-NH_2	0.662	2390	2392
9	Ac-**C**SGHHFVR**C**LVRVSGGPRW-NH_2	0.593	2191	2193

[a] Previously published compound [27].

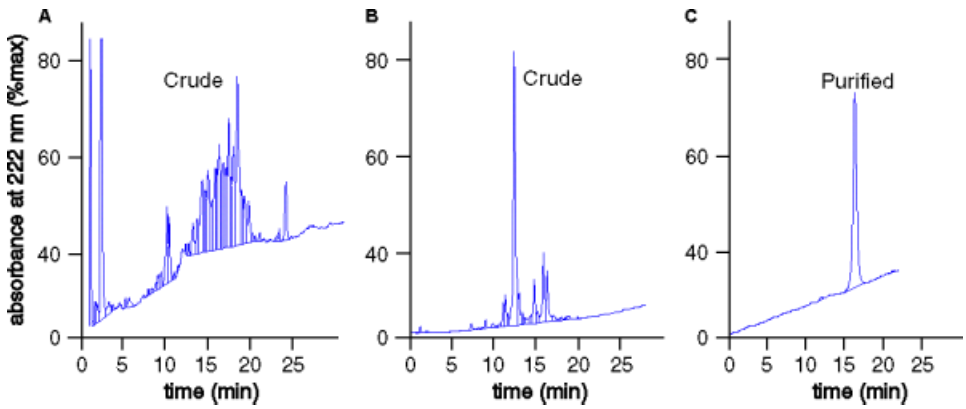

Figure 3 RP-HPLC traces of the linear peptide **5**. Panel A: Chromatogram of crude peptide synthesized without pseudoproline dipeptides, indicating multiple reaction products. Panel B: Chromatogram of crude peptide resynthesized using pseudoproline dipeptides, indicating improved synthesis outcome. Panel C: Chromatogram of the purified peptide.

Peptide Synthesis

The designed INSL3 B-chain mimetics **3–9** were synthesized as the *C*-terminal amide or acid and acetylated at the *N*-terminus. An initial attempt to synthesize the cyclic mimetic **5** gave a complex HPLC trace, indicating the presence of multiple products (Figure 3(A)). Resynthesis using a double coupling protocol as well as increasing the time of coupling and deprotection for each amino acid did not significantly improve the outcome (data not shown). However, when mimetic **5** was resynthesized using the pseudoproline dipeptides Val-Ser ($\psi^{Me,Me}$Pro)-OH and Fmoc-Leu-Ser ($\psi^{Me,Me}$Pro)-OH [28] in place of the Val-Ser and Leu-Ser sequences, a dramatically improved chromatogram was obtained (Figure 3(B)). Mass spectrometry (Table 1) confirmed the principal peak in the chromatogram to be the target peptide, which was easily purified (Figure 3(C)). Because the use of pseudoproline dipepeptides in the synthesis of mimetic **5** led to such a dramatic improvement in the quality of the crude peptides, subsequent mimetics were synthesized routinely using this approach. After cyclization, using 1% DMSO in 0.1 M NH_4HCO_3, all peptides were purified to homogeneity by RP-HPLC, in yields of 10–20% compared to the crude peptides.

Competition-binding Studies

The cyclic INSL3 B-chain mimetics **3–9** were assessed for their ability to bind to the INSL3 receptor LGR8 in competition-binding studies with [^{33}P]-relaxin, using HEK-293T cells modified to stably express LGR8 (Table 2 and Figure 4). As described previously [27], INSL3 potently displaced [^{33}P]-relaxin from these cells, with a pK_i of 9.6 ± 0.17. In this assay, we had previously reported that the INSL3 B-chain mimetic **2** is almost 10^4-fold less potent than native INSL3 [27]. The INSL3 B-chain mimetics **3–5** showed only partial displacement of [^{33}P]-relaxin (Figure 4(A)). On the other hand, mimetics **6–9** were full displacers of [^{33}P]-relaxin from LGR8 (Figure 4(B)). Mimetic **6** showed higher affinity for LGR8 binding compared to the previously described mimetic **2** ($p < 0.001$), whereas mimetics **7**

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Table 2 pK_i against [^{33}P]-relaxin and % α-helix in PBS and in 20% TFE of cyclic INSL3 B-chain analogues

Compounds	pK_i (Mean ± SEM)	% α-Helicity		
		PBS	TFE	Expected
2	5.99 ± 0.02	13	20	50
3	<5	—	—	—
4	<5	—	—	—
5	<5	6	19	52
6	6.65 ± 0.09	10	34	50
7	6.2 ± 0.1	—	—	—
8	6.34 ± 0.07	8	26	62
9	5.73 ± 0.09	4	9	68

and **8** had indistinguishable receptor binding affinity ($p > 0.05$) compared to mimetic **2**. Mimetic **9**, the shortest of all analogues prepared in this study, showed a slightly lower receptor binding affinity compared to the other full displacers.

CD Spectroscopy

The CD spectra of the INSL3 B-chain mimetics **5**, **6** (Figure 5(A)), **8** (Figure 5(B)) and **9** were measured in PBS (pH 7.4) in the absence and presence of the α-helix-inducing agent TFE (20%), allowing the calculation of α-helical content (Table 2). The mimetics showed a small degree of helical content in PBS, ranging from 4–10%. As anticipated, the addition of TFE increased the amount of helical content considerably, such that mimetics **6** and **8** showed 34% and 26% α-helix. We had previously reported that the B-chain mimetic **2** showed 20% α-helicity in the presence of 20% TFE [28]. The expected helicity of the peptides, based on the INSL3 homology model, ranged from 50 to 60%.

DISCUSSION

In this study, we continued our work to develop novel mimetics of the human INSL3 B-chain, with a view to obtaining compounds with improved affinity for the INSL3 receptor, LGR8. To do this, a structure-based approach was employed using a model of the 3D structure of INSL3, which was prepared from the X-ray crystal structure of the homologous protein relaxin. The B-chain of this model of the INSL3 structure was then used as a template to aid in the design of a series of seven novel, cyclic disulfide-constrained B-chain mimetics. Four of these mimetics were found

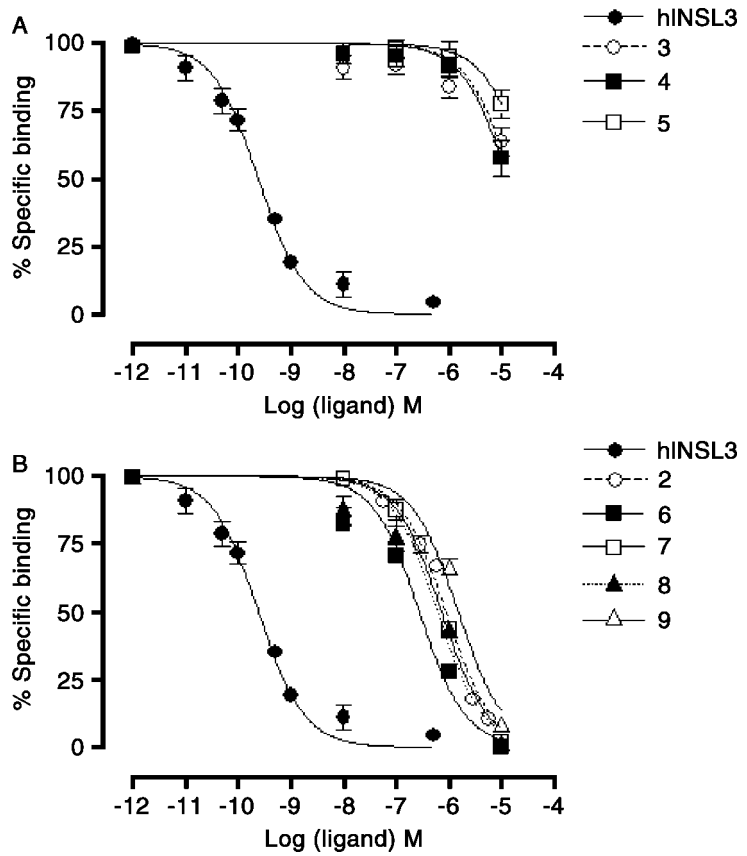

Figure 4 Competition-binding studies of INSL3 and selected INSL3 B-chain analogues. Binding studies were carried out in HEK-293T cells stably transfected with LGR8, using [^{33}P]-relaxin as the radioligand. Data were obtained from triplicate determinations from $n = 3–4$ independent experiments. Panel A: Partial displacers of radioligand. B: Full displacers of radioligand.

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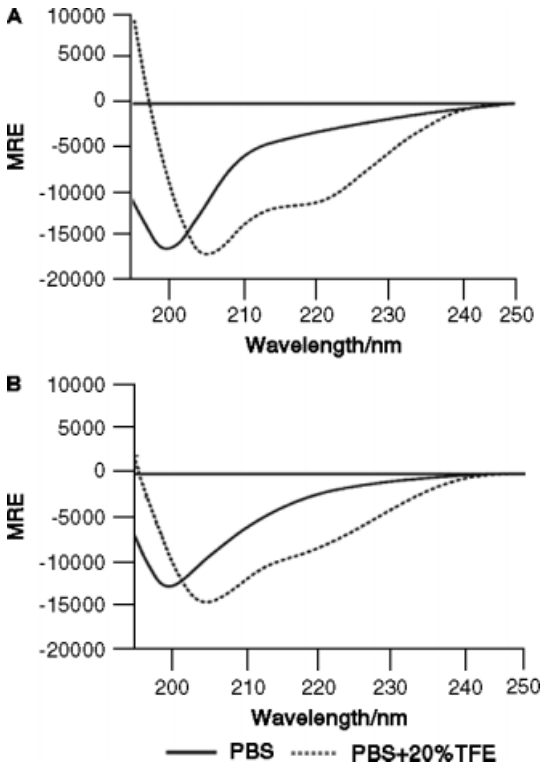

Figure 5 CD spectra of compound **6** (Panel A) and compound **8** (Panel B) in phosphate buffered saline (PBS; solid line) and PBS + 20% TFE (dotted line).

to completely displace the LGR8 ligand [^{33}P]-relaxin in cell-based receptor binding studies. One compound (mimetic **6**) was found to have significantly greater affinity for LGR8 than the cyclic B-chain mimetic **2** previously described by us [27]. Mimetic **6** also displayed the greatest helical content as assessed by CD spectroscopy, suggesting that, of the compounds examined, it has the most conformational similarity to native INSL3. Although mimetic **6** still has 10^3-fold less affinity than INSL3 for LGR8, the results from this study indicate future directions that could be taken to obtain INSL3 mimetics – agonists and antagonists – as lead compounds with therapeutic potential for the control of fertility.

As a template for the design of INSL3 B-chain mimetics, we used a homology model of the 3D structure of INSL3 based on the coordinates of the crystal structure of the related hormone relaxin. Shortly after completing this study, we solved and published the NMR-derived solution structure of INSL3 [16]. As expected, owing to the reasonably high degree of homology between relaxin and INSL3 – in particular, the absolute conservation of cystines – our homology model and the experimentally derived structures are similar. The major differences between the structures relate to mobility of the B-chain strand, and the B-chain *C*-terminus, both of which show considerable flexibility in the NMR structure. The dynamic nature of the *C*-terminus means that the position of one of the key residues for LGR8 binding, Trp^{27}, is not uniquely defined. In contrast, the positions of the other residues involved in LGR8 binding (His^{12}, Arg^{16}, Val^{19} and Arg^{20}) are well defined in the solution structure, and map very closely to their positions in the homology model. Furthermore, an analysis of the INSL3 NMR structure by us revealed that we would have likely proposed an identical set of cyclic, disulfide-constrained B-chain mimetics if we were to have used the NMR structure rather than the homology model as a template.

Initial attempts to synthesize peptide **5** using a standard Fmoc synthesis protocol resulted in crude material containing multiple peptide products that was not amenable to easy purification by RP-HPLC. This situation was not effectively resolved by standard approaches, e.g. double coupling of each amino acid, and modifying coupling and deprotection times. However, the use of pseudoproline dipeptides during linear peptide chain assembly yielded a crude compound following cleavage from the resin, which was of significantly greater purity and could be purified readily by HPLC. Pseudoproline dipetides, which can be incorporated into a peptide chain during synthesis in place of a Ser or Thr residue (and the residue *N*-terminal to it), act to create a kink in the growing peptide chain, which has been shown to disrupt the formation of β-sheets and other secondary structures which hinder coupling and deprotection steps during solid-phase peptide synthesis [28]. Upon treatment with TFA during resin cleavage and side chain deprotection, the pseudoproline ring opens, yielding a final linear product with the correct sequence. Owing to the dramatic improvement obtained with the synthesis of mimetic **5** (the first compound synthesized), all subsequent compounds were synthesized with pseudoproline dipeptides in place of Leu^9-Ser^{10} and Val^{21}-Ser^{22} where possible.

Three of the B-chain mimetics (compounds **3–5**) showed only partial displacement of [^{33}P]-relaxin from LGR8. A common feature of this group of compounds was the position of the disulfide constraint (replacing Thr^2 and Gly^{24} of the native INSL3 structure). From our modeling studies, we thought that this was likely to be a better position to place a disulfide constraint to maintain native INSL3 structure than the constraint used in the previously described mimetic **2** (Pro^3 and Arg^{26}), as evidenced by the lower RMSD values obtained. It is possible that the reduced activity of mimetics **3** and **4** is due to a disruption of the positioning, and/or reduction in the mobility, of Trp^{27}. Several studies have now identified Trp^{27} as one of the residues involved in the binding of the INSL3 B-chain to LGR8 [18,27], and that the positioning of this residue is important [17]. From the recently published solution structure of INSL3 [16], it is now clear that Trp^{27} and the residues preceding it are

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located in a highly mobile region of INSL3, likely facilitated by the two flexible residues Gly^{23} and Gly^{24}. It could be that the replacement of the Gly^{24} with Cys in mimetics **3** and **4** either forces Trp^{27} into an unfavorable position for binding to LGR8, or that the dynamic behavior of this part of the B-chain and its mimetics is required for LGR8 binding, and that the constraint prevents this. The fact that mimetics **3** and **4** are not significantly more potent antagonists of LGR8 binding than mimetic **5**, in which Trp^{27} has been completely omitted, supports this view.

Mimetics **6–9** all demonstrated complete displacement of [^{33}P]-relaxin from LGR8 comparable to that of our previously published cyclic B-chain mimetic **2** [27]. These results showed that residues could be removed from the *N*-terminus of the cyclic mimetics without adversely affecting receptor binding, consistent with our previous observations with linear *N*-terminally truncated peptides [27]. Indeed, mimetic **9** retains similar affinity for LGR8 compared to the previously reported compound **2** despite being eight residues smaller than compound **2** (seven residues absent from the *N*-terminus, one from the *C*-terminus). It is interesting to note that mimetic **6**, which has significantly greater affinity for LGR8 than compound **2**, also displays the greatest degree of α-helical structure in solution as determined by CD spectroscopy, at least when the CD measurements are made in the presence of the helix-inducing agent TFE. Nevertheless, the experimental helical content of all the compounds is less than the theoretical helical expectation (i.e. the percentage of α-helix that would be adopted if the mimetics were to adopt a native INSL3-like conformation – greater than 50%), suggesting that the favored conformation of the mimetics is only partly native. This observation is supported by the fact that the mimetics have at best 1000-fold less affinity for LGR8 than native INSL3. Furthermore, mimetic **7** – designed as an analogue of mimetic **6** in which Ala^{17}, normally buried by INSL3 A-chain, and thus potentially exposed to solvent in the absence of the A-chain, was replaced by a polar Ser residue – did not lead to improved affinity for LGR8. If the B-chain mimetics generally adopt only a partially native structure, then it is unlikely that this relatively subtle structural change would have a profound effect on binding affinity.

In summary, we have used a structure-based molecular design approach to produce a series of putative mimetics of the B-chain of INSL3. Despite the fact that these mimetics incorporate a disulfide conformational constraint that should encourage them to mimic a native INSL3-like conformation, the mimetics display generally lower helical content than would be expected, and show considerably reduced affinity for LGR8 compared to intact INSL3. It is generally well accepted that the A-chains of relaxin and INSL3 offer structural support to their respective B-chain to facilitate the correct presentation of B-chain residues, rather than contributing residues for binding *per se*. It is apparent from this systematic study that even a judiciously placed disulfide constraint in the B-chain of the mimetics is insufficient replacement for the missing A-chain support. We are currently examining INSL3 B-chain mimetics that offer alternative means of presenting the key residues for LGR8 binding in a native conformation, with a view to obtaining compounds with improved receptor affinity.

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