Analogs of Insulin-like Peptide 3 (INSL3) B-chain Are LGR8 Antagonists in Vitro and in Vivo*

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In insulin-like peptide 3 (INSL3) is a member of the insulin superfamily that plays an important role in mediating testes descent during fetal development. More recently, it has also been demonstrated to initiate oocyte maturation and suppress male germ cell apoptosis. These actions are mediated via a specific G-protein-coupled receptor, LGR8. Little is known regarding the structure and function relationship of INSL3, although it is believed that the principal receptor binding site resides within its B-chain. We subsequently observed that the linear B-chain alone (INSL3B-(1–31)) bound to LGR8 and was able to antagonise INSL3 stimulated cAMP accumulation in HEK-293T cells expressing LGR8. Sequentially N- and C-terminally shortened linear analogs were prepared by solid phase synthesis and subsequent assay showed that the minimum length required for binding was residues 11–27. It was also observed that increased binding affinity correlated with a corresponding increase in α-helical content as measured by circular dichroism spectroscopy. Molecular modeling studies suggested that judicious placement of a conformational constraint within this peptide would increase its α-helix content and result in increased structural similarity to the B-chain within native INSL3. Consequently, intramolecularly disulfide-linked analogs of the B-chain showed a potentiation of INSL3 antagonistic activity, as well as exhibiting increased proteolytic stability, as assessed in rat serum in vitro. Administration of one of these peptides into the testes of rats resulted in a substantial decrease in testis weight probably due to the inhibition of germ cell survival, suggesting that INSL3 antagonists may have potential as novel contraceptive agents.

INSL3 is involved in female fertility but that there may be redundant signaling systems involved. It has recently been demonstrated that INSL3 gene expression is increased in patients with advanced thyroid carcinoma (13), although the significance of this is yet to be determined.

In rats, the action of gonadotropin on the maturation of oocytes in the ovary and the suppression of male germ cell apoptosis in the testis has recently been shown to be mediated by INSL3 (14). In mammals, it is now well accepted that INSL3 is a member of the relaxin peptide family (2). INSL3 binds with high affinity to LGR8 and has very low affinity for the paralogue receptor LGR7, which is the receptor for relaxin (3). It is now well accepted that INSL3 is a member of the relaxin peptide family (4), and LGR7 and LGR8 have been classified as relaxin family peptide receptors, RXFP1 and RXFP2, respectively (5). Importantly, relaxin can bind to and activate LGR8 in some species, although with lesser affinity and potency than INSL3 (6, 7).

Although the precise physiological functions of INSL3 have not been fully determined, it has been shown to play a pivotal role in testicular descent and ovarian function. In male mice, deletion of either the INSL3 (8, 9) or LGR8 (10) gene leads to cryptorchidism due to impaired development of the gubernaculum. The cryptorchid phenotype, with the testis being retained in the abdomen, results in disrupted spermatogenesis and infertility. Studies have indicated that INSL3 binds and activates LGR8 in the gubernaculum to elicit an increase in cAMP production (2) and growth of the structure (11). Overexpression of the INSL3 gene in female mice results in descent of the ovaries into the inguinal region due to an overdeveloped gubernaculum (12), consistent with the actions described above. Although female INSL3 null mice exhibit impaired fertility (9), female LGR8 null mice are fertile (10), suggesting that INSL3 is involved in female fertility but that there may be redundant signaling systems involved. It has recently been demonstrated that INSL3 gene expression is increased in patients with advanced thyroid carcinoma (13), although the significance of this is yet to be determined.

INSL3 is expressed primarily in the Leydig cells of fetal and adult testes and in the thecal cells of the ovary (1). The receptor for INSL3 has recently been identified and shown to belong to the family of leucine-rich repeat-containing G-protein-coupled receptors (LGRs) (2). INSL3 binds with high affinity to LGR8 and has very low affinity for the paralogue receptor LGR7, which is the receptor for relaxin (3). It is now well accepted that INSL3 is a member of the relaxin peptide family (4), and LGR7 and LGR8 have been classified as relaxin family peptide receptors, RXFP1 and RXFP2, respectively (5). Importantly, relaxin can bind to and activate LGR8 in some species, although with lesser affinity and potency than INSL3 (6, 7).

The results demonstrate that INSL3 has a potential clinical application as a specific regulator of fertility and LGR8 antagonists have the potential to be highly specific contraceptives.

To date, there have been few structure-function studies for INSL3,
with a paucity of information regarding the residues or regions required for specific receptor binding. Analogos of INSL3 with modifications in the B-chain at positions 25 and 27 have shown that Trp27 is essential for proper positioning of the indole moiety. Further evidence of this is provided by the recent finding that an inactive mutant of human INSL3 identified in cryptorchid patients has a B-chain Pro25 → Ser substitution (18). Together, these limited data indicate that an LGR8 binding site probably resides within the C terminus of the B-chain of INSL3 and specifically within residues 22–27. Given that the B-chain of INSL3 therefore most likely contains the primary binding site, we undertook to determine whether it alone possesses biological activity, and if so, how much of the B-chain is required and whether conformationally constrained analogs could be designed and prepared which would mimic the action of the native peptide.

**EXPERIMENTAL PROCEDURES**

**Materials**—9-Fluorenylemethoxycarbonyl (Fmoc) L-amino acid derivatives for peptide synthesis were purchased from Auspep Pty. Ltd. (Melbourne, Australia). HPLC columns were obtained from Vydac, Hesperia, CA. Synthetic human INSL3 was prepared as described previously (19, 20) and labeled with 125I by PerkinElmer Life Sciences. Recombinant H2 relaxin was a gift from BAS Medical (San Mateo, CA).

**Primary structure and amino acid residue numbering of human INSL3.**

![Figure 1](Image)

**A-chain**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AAATNPARYCCLGLVQDNLTCIPY |

**B-chain**

| 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|
| PTPEMKRLCHHVFRLVRCGPRWSTEA |

**CD Spectroscopy**—CD spectra were measured on a Jasco J-710 (Tokyo, Japan) instrument at room temperature in a 1-mm path length cell. Peptides were made to a concentration of 0.01 μM (determined by amino acid analysis) in either phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer containing 120 mM NaCl, pH 7.4) or spectroscopy grade trifluoroethanol (TFE) (19). Curves were smoothed by the algorithm provided by Jasco. The CD spectra obtained were evaluated by comparing them to the spectra of known peptide conformations (23), and α-helicity was calculated according to the algorithm of Greenfield and Fasman (24).

**Molecular Design**—Using SYBYL molecular modeling software (Tripos Inc., St Louis, MO) on a Silicon Graphics O2 workstation, a model of INSL3 was prepared using the x-ray crystal structure of H2 relaxin as a template (25) as follows. The H2 relaxin sequence was modified to that of INSL3, and the resultant peptide subjected to energy minimization using the Tripos forcefield and Gasteiger-Marsili charges. By examining the B-chain of the energy-minimized model, 2 residues were identified (Glu4 and Arg59) with Cβ atoms within 4.5 Å of each other. These residues were then replaced with cysteine and a disulfide bond formed to give the cyclic analog 15. The second cyclic analog (analog 14), incorporating the entire B-chain, was cyclized in the same position as in analog 15.

**Ligand Binding Assays**—The method to study the binding of 33P-labeled H2 relaxin and 125I-INS3 to HEK-293T cells stably transfected with either human LGR7 or LGR8 has been described previously (3, 26). Data are expressed as mean ± S.E. of percent specific binding of triplicate determinations made from at least three independent experiments. Data were analyzed using Graphpad PRISM (Graphpad Inc., San Diego, CA) and a non-linear regression one-site binding model was used to plot curves and calculate pKₐ values. Final pooled pKₐ data were analyzed using one-way ANOVA coupled with Bonferroni’s multiple comparison test for multiple group comparison.

**Functional cAMP Assays**—cAMP stimulation assays using HEK-293T cells stably transfected with human LGR8 were performed as described previously (26). Briefly, cells were preincubated in the presence of 0.25 mM 3-isobutyl-1-methylxanthine (Sigma) before stimulation with INSL3 analogs in the presence or absence of 1 nM INSL3 for 30 min. Additionally, INSL3 concentration response curves were performed with various concentrations of human INSL3 in the absence or presence of 1 μM of peptides 14 or 15. At the end of the incubation, cells were lysed for measurement of cAMP in the cell lysate using a well characterized cAMP enzyme-linked immunosorbent assay (Biotrak EIA, Amersham Biosciences) (27). All experiments were repeated at least three times with triplicate determinations within each assay. Results are plotted as mean ± S.E. of percent normalized response compared with 1 nM INSL3. Data were analyzed using one-way ANOVA coupled with Bonferroni’s multiple comparison test for multiple group comparison.

**Serum Stability Studies**—Proteolytic stability of peptides was assessed by incubating the peptide (0.7 mg/ml) at 37 °C in a 10% solution in PBS of freshly prepared rat serum (pooled from four rats). Samples (20 μl) were removed at various time points, and acetonitrile (40 μl) was added to precipitate plasma proteins, which were removed by centrifugation (9000 rpm, 3 min). The amount of intact peptide remaining in the supernatant of each sample was determined using RP-HPLC, by measuring the area under the curve of the peak at the appropriate retention time compared with a standard curve of concentration for each peptide. The half-life (t₁/₂) for each peptide is expressed as the mean of the individual half-lives determined from the single exponential curve fit for three independent experiments.

**Intratubular Injections**—It has been demonstrated previously that INSL3, acting through LGR8, suppresses testicular germ cell apoptosis.
induced by a GnRH antagonist (14). To examine the effect of the INSL3 B-chain analogs on testis function in vivo, male Sprague-Dawley rats at 28 days of age were used, based on the increased expression of LGR8 at that developmental stage (14). The cyclic INSL3 B-chain analog 15 or the linear analog 16 (20 μg/50 μl in 71 mM acetic acid) were injected into the left testis, while the right testis remained uninjected (n = 6 and n = 3 animals, respectively). For controls (n = 6 animals), vehicle alone was injected into the left testis. The animals were sacrificed 4.5 days after the injection and the testes dissected and weighed. Results are presented as mean ± S.E. of the percentage weight of the left testis in comparison with the right testis. Data were analyzed using one-way ANOVA coupled with Bonferroni’s multiple comparison test for multiple group comparison.

RESULTS

Design and Synthesis of Linear INSL3 B-chain Analogs—To allow a study of the structure-activity requirements of the INSL3 B-chain, we prepared a series of linear B-chain analogs of varying lengths (analogs 1–13, Table 1). The full-length analog 9 consists of the sequence of the entire INSL3 B-chain (31 amino acids), except that the Cys residues at positions 10 and 22 (normally involved in formation of two disulfide bonds to the INSL3 A-chain) are replaced by Ser, to prevent unwanted disulfide formation. Analogs 1–8 are a series of N-terminally truncated analogs of the full-length peptide 9; analogs 10–13 are the corresponding C-terminally truncated peptides. Each of the B-chain analogs 1–13 were synthesized as the C-terminal amide by routine solid phase peptide synthesis methods, and purified to homogeneity by RP-HPLC, in yields of 10–20% compared with the crude peptide.

Competing Binding Studies with N- and C-terminally Truncated Linear B-chain Analogs—The ability of the linear B-chain analogs 1–13 to bind to the INSL3 receptor LGR8 was assessed in competition binding studies with 33P-relaxin and 125I-INSL3, using HEK-293T modified to stably express LGR8 (Table 1, Fig. 2). As reported previously (3), INSL3 potently displaced 33P-relaxin from these cells, with a pKᵢ of 9.6 ± 0.17. Surprisingly, INSL3 B-chain analogs were able to displace 33P-relaxin binding from LGR8 expressing cells although their affinity was 104-fold less than native INSL3. Hence the full-length linear B-chain analog 9 demonstrated a pKᵢ of 5.64 ± 0.12 (Table 1, Fig. 2, A–D). Removal of 2 (analog 8, Fig. 2A), 5 (analog 7), or 8 (analog 6, Fig. 2A) residues from the N terminus had limited effect on the ability of the compounds to compete with 33P-relaxin at LGR8 compared with the full-length analog 9. However, analog 5, shortened N-terminally by a further 3 residues, showed significantly reduced affinity (p < 0.01), while removal of 3 more residues gave analog 4, which displayed only partial displacement (Fig. 2B). Further N-terminus shortening (i.e. analogs 1–3) effectively abolished the ability of the compounds to displace 33P-relaxin (Fig. 2B). C-terminal truncation by 1–4 residues (analogs 10–13) appeared to have no effect on the ability of linear B-chain analogs to displace 33P-relaxin (Fig. 2C). Similar pKᵢ values were obtained for selected compounds when the alternative LGR8 radioligand 125I-INSL3 was used (i.e. INSL3 pKᵢ = 9.58 ± 0.07; see analogs 8 and 3, Table 1 and Fig. 2F). None of the linear B-chain analogs 1–13 were found to bind to LGR7 (data not shown).

Effects of N- and C-terminally Truncated Linear B-chain Analogs on INSL3-mediated cAMP Production—Stimulation of LGR8 stably transfected HEK-293T cells with INSL3 or relaxin is known to produce a concentration-dependent increase in cAMP (3, 26). In contrast, none of the linear INSL3 B-chain analogs 1–13 were able to promote cAMP production in LGR8 stably transfected HEK-293T alone (data not shown). However, when assayed in competition with INSL3, many of the linear B-chain analogs 1–13 were found to inhibit INSL3-mediated cAMP accumulation (Fig. 3, A and B). In general, the data obtained reflected the binding of the compounds to LGR8. When added to cells at a concentration of 10 μM in competition with INSL3 (1 nM), the full-length B-chain analog 9 gave a 25% inhibition of cAMP accumulation. Progressive N-terminal truncation steadily reduced the extent of inhibition, such that the shortest analogs 1–4 were unable to inhibit INSL3-mediated cAMP formation (Fig. 3A). In contrast, the C-terminally truncated analogs 10–13 showed similar inhibition to the full-length analog 9 (Fig. 3B). Similar inhibitory actions were observed when H2 relaxin was used as the stimulus for cAMP production (data not shown).

Conformational Analysis of N- and C-terminally Truncated Linear B-chain Analogs—Native INSL3 is known to adopt a predominately α-helical structure as assessed by CD spectroscopy (20), consistent with the hypothesis that INSL3 assumes a similar three-dimensional fold to relaxin. To determine whether the isolated linear INSL3 B-chain and analogs can also adopt an INSL3-like helical conformation, we determined the α-helix content of the linear B-chain analogs 1–13 (Table 1). In PBS, none of the linear INSL3 B-chain analogs showed appreciable α-helix content (maximum 9% shown by analogs 9 and 10, i.e. approximately 3 residues). The α-helical content increased somewhat when measured in the helix-inducing solvent TFE: the full-length analog 9 gave 16% helicity (5 residues), C-terminally truncated analogs 10–13 showed slightly reduced helicity, while N-terminal truncation (see analogs 1–6) rapidly abolished α-helicity. In general terms, the α-helical content measured by CD spectroscopy, while less than expected if the B-chain analogs were adopting a native relaxin-like conformation, correlated with the ability of the compounds to displace 33P-relaxin from LGR8.

Design and Synthesis of Cyclic Disulfide-constrained B-chain Analogs—Given the ability of linear INSL3 B-chain analogs to act as competitive LGR8 antagonists, we explored the possibility that conformationally con-
strained cyclic B-chain analogs might show increased potency by increasing the likelihood that the analogs would adopt a native, INSL3-like conformation. To do this, we first built a model of the three-dimensional structure of INSL3 based on the structure of relaxin, as the structure of INSL3 is yet to be determined by experimental methods. We then examined this model for potential sites to insert a disulfide constraint between residues of the...
B-chain β-strand (residues 1–9) and α-helix (residues 13–26). Using this structure-based approach, Glu3 and Arg26 were chosen as the sites for the insertion of a Cys3-to-Cys disulfide constraint. Two cyclic analogs incorporating this Cys3-to-Cys26 disulfide were designed: the full-length analog 14 and the N- and C-terminally shortened analog 15. An additional linear analog 16 was also designed, in which the 2 Cys residues of the cyclic analog 15 were replaced by Ser. The synthesis of these analogs proceeded routinely.

**Competition Binding Studies of Cyclic Disulfide-constrained B-chain Analogs**—The cyclic B-chain analogs 14 and 15 were both able to displace 33P-relaxin from LGR8 with almost identical pKi values (5.98 ± 0.06 versus 5.99 ± 0.02) (Table 1). Neither cyclic analog demonstrated a statistically significant difference in affinity to its linear counterpart (analogs 9 and 16, respectively, Fig. 2, D and E). Analog 15 also displaced 125I-INSL3 from LGR8 with a similar pKi to that which it displaced 33P-relaxin (Table 1, Fig. 2F).

**Effects of Cyclic Disulfide-constrained B-chain Analogs on INSL3-mediated cAMP Production**—The cyclic B-chain analogs 14 and 15 were effective inhibitors of the INSL3-mediated production of cAMP, causing a 54 and 64% inhibition, respectively, at 10 µM (Fig. 3B). Both cyclic analogs caused a rightward parallel shift in the INSL3 concentration response curve for cAMP production (Fig. 3C), and their inhibitory effect is concentration-dependent (Fig. 3D), but they did not affect cAMP accumulation in the absence of INSL3 (data not shown). Taken together, these findings indicate that cyclic analogs 14 and 15 were acting as competitive antagonists at LGR8. Interestingly, none of the linear analogs tested were as effective in reducing cAMP production as the cyclic analogs (e.g. linear analog 5, Fig. 3, A and D), despite the fact that there was generally not a marked difference in the binding of linear and cyclic analogs to LGR8.

**Conformational Analysis of Cyclic Disulfide-constrained B-chain Analogs**—The cyclic analogs 14 and 15 showed comparable α-helical content in either PBS or TFE as assessed by CD spectroscopy, and considerably more than their linear counterparts 9 and 16, or indeed any of the linear peptides tested (Table 1). Nevertheless, the degree of α-helicity exhibited by 14 and 15 was still considerably less than would be expected if the cyclic analogs were to adopt a truly native conformation (~40–50% helicity based on homology with relaxin).

**Proteolytic Stability of INSL3 B-chain Analogs**—To help determine the optimal INSL3 B-chain analog to use in *in vivo* studies, we compared the proteolytic stability *in vitro* of the cyclic analogs 14 and 15 and the linear analog 16 (Fig. 4). When incubated in 50% rat plasma in PBS at 37 °C, the cyclic analog 15 (t1/2: 29 ± 5 min) was significantly more stable than its linear counterpart 16 (t1/2: 6.7 ± 0.5 min; p = 0.018, unpaired Student’s *t* test), a finding that has been observed widely in the field of peptide design (e.g. Ref. 28). It is interesting to note that cyclic analog 14 is not as stable (t1/2: 5.2 ± 0.1 min) as cyclic analog 15. However, the degradation curves for the two peptides reaches a similar plateau (~30% of the area of the parent compound), which differs to that of the linear peptide 16 (11%), which may be the result of a similar pattern of terminal proteolytic “clipping” of the cyclic peptides.

**Effects of a Cyclic Disulfide-constrained B-chain Analog on LGR8 Function in Vivo**—Analog 15 was tested in parallel to the linear analog 16 for its ability to antagonize INSL3 activity *in vivo*. Injection of analog 15 into one testis of male Sprague-Dawley male rats at 28 days of age resulted in a significant decrease (*p* < 0.001) in the weight of this testis in comparison to the uninjected testis (80.9 ± 2.3%; *n* = 6; Fig. 5). In contrast, injection of vehicle (96.6 ± 1.7%; *n* = 6) or analog 16 (97.8 ± 2.0%; *n* = 3) did not result in a significant decrease in testis weight (Fig. 5). Importantly, the decrease in testis weight in response to treatment with analog 15 (80.9 ± 2.3%) is very similar to that seen previously in response to GnRH antagonist treatment (79.3 ± 3.4%; Fig. 5) (14).

**DISCUSSION**

In this study, we synthesized the linear INSL3 B-chain and examined its ability to bind to the INSL3 receptor LGR8 and its effects on cAMP accumulation in LGR8 expressing cells. We found that the peptide, while devoid of INSL3-like activity, is able to bind to LGR8 and act as an antagonist of INSL3 actions. The ability of the individual INSL3 B-chain analogs to bind to LGR8 was somewhat surprising given that we had recently reported that the linear B-chain of H2 relaxin was unable to bind to its receptor LGR7 (29). Comprehensive studies with relaxin have delineated a set of primary binding residues in the B-chain, which form the so-called binding cassette (RXXRXX/T/V) (30, 31). Although a similar displaced motif is present in INSL3, it has only a very low affinity for LGR7 (6). Furthermore, the placement of a typical relaxin binding cassette into sheep INSL3 did not result in a peptide with significantly enhanced relaxin activity (27). Structure function studies with human INSL3 have defined additional residues at the C-terminal end of the B-chain important for binding to LGR8. Hence, the indole ring of Trp27 in the human INSL3 cassette (RXXRXX) is a necessary binding residue in the B-chain, which form the so-called binding cassette (RXXRXX/T/V) (30, 31). Although a similar displaced motif is present in INSL3, it has only a very low affinity for LGR7 (6). Furthermore, the placement of a typical relaxin binding cassette into sheep INSL3 did not result in a peptide with significantly enhanced relaxin activity (27). Structure function studies with human INSL3 have defined additional residues at the C-terminal end of the B-chain important for binding to LGR8. Hence, the indole ring of Trp27 in the human INSL3 peptide is essential for high affinity binding and Pro7 appears to be essential for the conformation of the C-terminal region and hence for the binding to LGR8. Hence, the indole ring of Trp27 in the human INSL3 peptide is essential for high affinity binding and Pro7 appears to be essential for the conformation of the C-terminal region and hence for the “presentation” of Trp27 (17, 32). Hence it is likely that the presence of the Trp residue in the C-terminal region of all the INSL3 B-chain analogs in this study is sufficient to confer measurable binding affinity on...
the analogs to LGR8. However, as the absence of Trp²⁷ in human INSL3 does not result in complete loss of binding it is likely that other B-chain residues participate in binding to LGR8 (17).

The truncation of at least 8 residues from the N terminus of the INSL3 B-chain analogs could be tolerated without appreciable loss of binding affinity. This region corresponds to the strand of the strand-helix structure of the relaxin B-chain. Although the three-dimensional structure of INSL3 is yet to be determined experimentally, the complete conservation of cystine residues and reasonable degree of homology with relaxin, along with the recently demonstrated similarity of the three-dimensional structures of H2 relaxin and relaxin-3 (33), suggests that it is highly likely that INSL3 shares an overall common fold with the other members of the family. Thus the data with the N-terminally truncated INSL3 B-chain analogs support the view that this N-terminal putative strand is not required for binding to LGR8. Further N-terminally truncated analogs, which would correspond to removal of residues of the α-helix in relaxin, showed reduced affinity (analogs 4 and 5) or no affinity or INSL3 antagonist action (analogs 1-3). The relatively abrupt loss of activity with these shortened analogs could be the result of the direct removal of residues required for binding, or the loss of secondary structure (i.e. α-helix), resulting in inappropriate presentation of key residues. Indeed, in the x-ray structure of H2 relaxin (25), the N-terminal strand of the relaxin B-chain makes a series of hydrophobic contacts with residues of the B-chain helix, thus acting to stabilize the helix. In support of the latter, we observed a loss of α-helicity as measured by CD spectroscopy with N-terminal shortening of the B-chain analogs, even though we would not be expected to be removing helical residues per se. In either case, it appears as though residues present in the putative α-helical region of INSL3 are required for the B-chain analogs to exhibit binding to LGR8 and INSL3 antagonism.

Given the ability of the linear INSL3 B-chain analogs to bind to LGR8 and act as INSL3 antagonists, we reasoned that cyclic analogs, prepared by joining the N and C termini of the B-chain, might show improved potency. By tethering the strand to the helix, such a modification would be expected to further enhance the helix stabilizing properties of the strand. From a structural point of view, this strategy appears to have been vindicated: the two disulfide-constrained cyclic peptides 14 and 15 showed considerably greater α-helical structure than their linear counterparts, whether in water or TFE. The effects of this increased helicity on biological activity were less definitive. The pKᵦ values of the linear and cyclic analog counterparts did not differ. However, the cyclic analogs did seem to exhibit greater antagonistic activity than their linear counterparts, as assessed by their ability to inhibit INSL3-mediated cAMP production via LGR8. The reason for the enhanced antagonist action of the cyclic peptides is not clear but might arise from slight differences in the mode of interaction of the linear and cyclic B-chain analogs with LGR8.

The peptides synthesized in this study represent a new class of antagonists for the members of the relaxin-insulin superfamily. Furthermore, they represent some of the first antagonists characterized for the relaxin family peptide receptors LGR7 and LGR8. The previously characterized soluble ectodomain of LGR7 (7BP), which has been demonstrated to block relaxin action in vitro and in vivo (34), presumably acts by competing with membrane-bound receptor for ligand binding. However, the related LGR8 soluble ectodomain protein (8BP) does not demonstrate INSL3 binding or antagonist activity (35). Recently potent INSL3 antagonists have been described, which consist of the human INSL3 peptide without the N-terminal 7–9 residues of the A-chain (36). Given the pure antagonist actions of these A-chain truncated INSL3 analogs and the B-chain analogs described in this study, it appears as though initial LGR8 binding takes place through residues in the B-chain, while the A-chain, perhaps residues from toward its N terminus, activate intracellular signaling pathways. These data fit well with the hypothesis derived from chimeric receptor studies, in which the primary ligand binding site is in the ectodomain of the receptors, whereas there is a secondary binding site in the transmembrane domains (37). Hence it is likely that the primary binding interaction with the ectodomain is driven by the B-chain, and activation of the receptor through the secondary binding site in the transmembrane domains is driven by the A-chain. Further evidence for this hypothesis is provided by recent studies, which have clearly demonstrated that residues in the B-chain relaxin binding cassette interact with residues in the leucine-rich repeats of the LGR7 ectodomain (38).

It is interesting to note that the cyclic B-chain analogs described in this study bear some gross structural resemblance (i.e. disulfide-constrained strand-helix) to inhibitors of binding of IGF-1 to IGF binding proteins and receptors (39, 40). The IGF binding inhibitors were derived from naive phage display libraries and therefore show no similarity to IGF-1. However, it was possible to replace most of the helix stabilizing strand in these inhibitors with an i + 7 or i + 8 covalent diaminopentane-derived bridge between Glu residues (41). The incorporation of this or similar constraints into the INSL3 B-chain analogs might offer a means of further reducing their size and increasing their potency.

Recent studies have demonstrated that intratesticular administration of INSL3 can reverse germ cell apoptosis in the rat testis resulting from treatment with the GnRH antagonist ganirelix (14). The anti-apoptotic actions of LH on male germ cells are well documented; however, as LH receptors are not expressed on germ cells, but are expressed on Leydig cells, it has been hypothesized that LH stimulates a Leydig cell factor to induce its effects (42). These authors further demonstrated that treatment with LH was able to increase INSL3 production by Leydig cells and that LGR8 was expressed on germ cells (14). It was subsequently shown that INSL3 mediates the action of LH on germ cells (14). It could therefore be postulated that administration of an LGR8 antagonist should result in the inhibition of LH actions on germ cells and hence increased germ cell apoptosis. We therefore tested the effects of selected B-chain analogs on INSL3 activity in rat testis. Local administration of the cyclic B-chain analog 15 directly into the rat testis was found to cause a reduction in testicular weight to a similar extent to that seen with rats treated with the GnRH antagonist, consistent with an action of analog 15 in inhibiting LH-induced endogenous INSL3 signaling in this tissue. The linear B-chain analog 16 proved to be a valuable control for this activity, especially in relation to potential peptide toxicity effects, as it had no effect on testis weight, despite exhibiting a similar ability to bind to LGR8 in vitro. Importantly, 16 was not as effective at inhibiting INSL3-mediated cAMP production in vitro, and furthermore, it was considerably less stable toward proteolytic degradation than the cyclic analog 15, as determined from plasma stability studies in vitro. Hence it is likely that the cyclic analog 15 is binding to LGR8 expressed on germ cells and inhibiting INSL3 induced anti-apoptotic effects of LH resulting in decreased testis weight. Further studies are in process to define the precise mechanisms by which the cyclic analogs reduce testis weight.

These analogs are among the first known selective antagonists of this unique class of G-protein coupled receptor, and although significant hurdles would need to be overcome regarding bioavailability, these preliminary studies highlight the potential for such INSL3 antagonists to be developed as novel male contraceptive agents that act directly on germ cells in contrast to the current strategies which target central GnRH secretion (43).
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