



(86) Date de dépôt PCT/PCT Filing Date: 2004/06/18
(87) Date publication PCT/PCT Publication Date: 2004/12/29
(45) Date de délivrance/Issue Date: 2013/09/17
(85) Entrée phase nationale/National Entry: 2005/12/19
(86) N° demande PCT/PCT Application No.: AU 2004/000798
(87) N° publication PCT/PCT Publication No.: 2004/113381
(30) Priorité/Priority: 2003/06/20 (AU2003903124)

(51) Cl.Int./Int.Cl. *C07K 14/62* (2006.01),
A61K 38/22 (2006.01), *A61K 38/28* (2006.01),
A61K 38/30 (2006.01), *A61P 15/00* (2006.01),
A61P 15/08 (2006.01), *A61P 15/14* (2006.01),
A61P 19/00 (2006.01), *A61P 3/10* (2006.01),
A61P 35/00 (2006.01), *C07K 14/575* (2006.01),
C07K 14/64 (2006.01), *C07K 14/65* (2006.01)

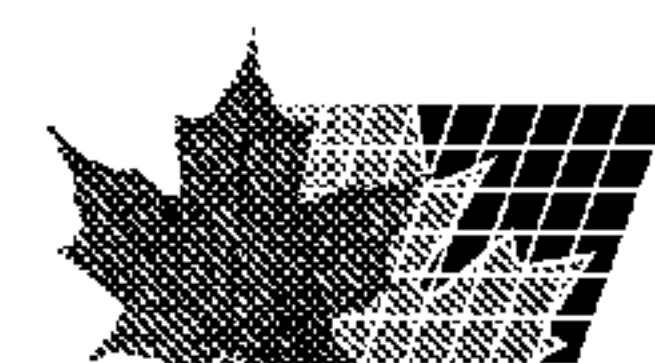
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(54) Titre : ANALOGUES PEPTIDIQUES DE LA SUPERFAMILLE DES RELAXINES
(54) Title: RELAXIN SUPERFAMILY PEPTIDE ANALOGUES

(57) Abrégé/Abstract:

Monomeric, cyclic peptide analogues of relaxin superfamily proteins, based on B-chains of relaxin superfamily proteins, and methods of making them, are described, as well as pharmaceutical compositions containing them and methods of utilising them.



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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
29 December 2004 (29.12.2004)

PCT

(10) International Publication Number
WO 2004/113381 A1

(51) International Patent Classification⁷: **C07K 14/62**,
14/64, 14/65, 14/575, A61K 38/22, 38/28, 38/30, A61P
3/10, 15/00, 15/08, 15/14, 19/00, 35/00

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(21) International Application Number:
PCT/AU2004/000798

(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(22) International Filing Date: 18 June 2004 (18.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2003903124 20 June 2003 (20.06.2003) AU

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

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Declaration under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii)) for all designations*

Published:

— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: RELAXIN SUPERFAMILY PEPTIDE ANALOGUES

(57) Abstract: Monomeric, cyclic peptide analogues of relaxin superfamily proteins, based on B-chains of relaxin superfamily proteins, and methods of making them, are described, as well as pharmaceutical compositions containing them and methods of utilising them.

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RELAXIN SUPERFAMILY PEPTIDE ANALOGUES

Field of the Invention

This invention relates to generally to conformationally constrained, monomeric peptide
5 analogues of the relaxin superfamily of heteromeric proteins and in particular, to
monomeric cyclic analogues of the B-chain of relaxin superfamily proteins, methods of
making these analogues, and uses thereof.

Background of the Invention

10 The relaxin superfamily currently comprises 10 members with a relatively high degree of
sequence homology. These family members include insulin, insulin-like growth factors I
and II, relaxin 1, 2 and 3, and the insulin-like hormones INSL3, 4, 5 and 6. The relaxin
superfamily members have a wide range of biological activities which are well described
in the art.

15

The critical action of insulin has been described for decades. Insulin has a key role in
cellular metabolism. Many subjects with diabetes require daily administration of insulin.

Relaxin is an insulin-like peptide having two separate chains (A and B) joined by two
20 interchain and one intrachain disulfide bond. The actions of relaxin include an ability to
inhibit myometrial contractions, stimulation of remodelling of connective tissue and
induction of softening of the tissues of the birth canal. Additionally, relaxin increases
growth and differentiation of the mammary gland and nipple and induces the breakdown of
collagen, one of the main components of connective tissue. Relaxin decreases collagen
25 synthesis and increases the release of collagenases (Unemori *et al* (1990) *J. Biol. Chem.*
265, 10682-10685). These findings were recently confirmed by the establishment of the
relaxin gene-knockout mouse (Zhao *et al* (1999) *Endocrinology* 140, 445-453), which
exhibited a number of phenotypic properties associated with pregnancy. Female mice
lacking a functionally active relaxin gene failed to relax and elongate the interpubic
30 ligament of the pubic symphysis and could not suckle their pups, which in turn, died within
24 hours unless cross-fostered to relaxin wild type or relaxin heterozygous foster mothers.

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Evidence has accumulated to suggest that relaxin is more than a hormone of pregnancy and acts on cells and tissues other than those of the female reproductive system. Relaxin causes a widening of blood vessels (vasodilatation) in the kidney, mesocaecum, lung and peripheral vasculature, which leads to increased blood flow or perfusion rates in these tissues (Bani *et al* (1997) *Gen. Pharmacol.* 28, 13-22). It also stimulates an increase in heart rate and coronary blood flow, and increases both glomerular filtration rate and renal plasma flow (Bani *et al* (1997) *Gen. Pharmacol.* 28, 13-22). Relaxin has also been found to inhibit histamine release and the accumulation of calcium, as well as promote nitric oxide synthesis, during cardiac anaphylaxis (Masini *et al.*, *Br J Pharmacol.* 2002 Oct;137(3):337-44). The brain is another target tissue for relaxin where the peptide has been shown to bind to receptors (Osheroff *et al* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 6413-6417; Tan *et al* (1999) *Br. J. Pharmacol* 127, 91-98) in the circumventricular organs to affect blood pressure and drinking (Parry *et al* (1990) *J Neuroendocrinol.* 2, 53-58; Summerlee *et al* (1998) *Endocrinology* 139, 2322-2328; Sinnahay *et al* (1999) *Endocrinology* 140, 5082-5086).

Important clinical uses arise for relaxin in various diseases responding to fibrotic breakdown, connective tissue remodelling, scleroderma, vasodilation, such as coronary artery disease, peripheral vascular disease, kidney disease associated with arteriosclerosis or other narrowing of kidney capillaries, or other capillaries narrowing in the body, such as in the eyes or in the peripheral digits, the mesocaecum, lung and peripheral vasculature, and neurological modification. Relaxin has also been implicated in depression of platelet aggregation and their release by megakaryocytes, and may thus be associated with clotting disorders.

The INSL peptides, such as INSL3, may be involved in various physiological actions, including descent of the gonads. In particular, INSL3 has been shown to be involved in maturation and descent of the testes (Ivell & Hartnung, *Mol Hum Reprod* 2003, 9(4): 175-181), as well as the survival of sperm cells, development of ovarian follicles and maturation of the oocyte (Kawamura *et al.*, 2004, *PNAS* 101, 7323-7328). Therefore,

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potential clinical applications of INSL3 agonists and antagonists include the treatment of fertility disorders, or the control of fertility levels.

INSL3 has been implicated in regulation of relaxin activity in the heart (Tan *et al.*, *Eur J Pharmacol.* 2002 Dec 20;457(2-3):153-60), and may thus play an important regulatory role in cardiovascular disease.

Furthermore, INSL3 polymorphisms have been hyperplastic and neoplastic disorders of the thyroid gland (Hombach-Klonisch *et al.*, *Int J Oncol* 2003 May; 22(5): 993-1001), suggesting a role for this relaxin superfamily member in the etiology of these pathologies.

The biological actions of the relaxin superfamily members are mediated through hormone receptors. In particular, the insulin receptor, the IGF-I and IGF-II receptors, GPCR135, GPCR142, as well as LGR7 and LGR8. LGR7 is the relaxin receptor, which binds all human relaxin peptides with high affinity, but has very low affinity for INSL3 (Bathgate *et al.*, *Trends in Endocrinology and Metabolism* 2003, 14: 207-213). LGR8 is the INSL3 receptor, which binds INSL3 with high affinity. Knockout mice which lack a LGR8 gene have identical phenotype to the INSL3 knockout mice. However in the human, LGR8 will also bind relaxin, although it has a higher affinity for INSL3 (Bathgate *et al.*, *Trends in Endocrinology and Metabolism* 2003, 14: 207-213).

The LGR8 receptor is expressed in the gubernaculum, ovary and testis, where in the latter organ it mediates INSL3's actions on testes descent (Bathgate *et al.*, *Trends in Endocrinology and Metabolism* 2003, 14: 207-213). In the ovary, LGR8 mediates follicular development and in both the testes and ovaries, it is implicated in male and female germ-cell maturation (Kawamura *et al.*, 2004, *PNAS* 101, 7323-7328). LGR8 is also expressed in the kidney, bone, peripheral-blood leucocytes, muscle, brain, thyroid, and uterus (Hsu *et al.*, *Science*, 295: 671-674). Hence, INSL3 has potential actions on kidney and thyroid function, central actions on the brain, modulation of muscle function, effects on bone and blood cell function and reproductive actions on the uterus.

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There is a need for analogues that are relaxin-superfamily analogues which bind at the biological receptors for the relaxin superfamily proteins. Ideally, such analogues would include ligands, such as agonists, reverse agonists, partial agonists, mixed agonists/antagonists and full antagonists, which bind at the relaxin superfamily member
5 receptors and initiate, inhibit, activate, or otherwise control, the biological activities of members of this protein superfamily. Analogues of the relaxin superfamily proteins could provide the opportunity for a wide range of therapeutic interventions.

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Summary of the Invention

In its broadest form, the present invention relates to peptide analogues of members of the relaxin superfamily of proteins, the analogues having a binding activity at a relaxin protein superfamily receptor, methods of producing the analogues, and pharmaceutical compositions, methods of treatment, and uses of the peptide analogues.

In a first aspect, the analogue is a conformationally constrained, monomeric cyclic peptide analogue of a B-chain of a relaxin superfamily member protein which binds to a biological target of the relaxin superfamily protein, the protein being selected from insulin, IGF-I, IGF-II, relaxin 1, relaxin 2, relaxin 3, INSL3, INSL4, INSL5 or INSL6.

- 10 The monomeric peptide analogues of the invention are produced by modification of a turn or loop moiety of the B-chain of the relaxin superfamily protein, the modification involving selection of at least a first and a second amino acid residue with an alpha-helix or beta-strand carbon separation distance of less than six Angstroms and cross-linking the first and second amino acids, wherein the cross-link conformationally constrains the analogue.
- 15 A specific aspect of the invention includes a monomeric, cyclic peptide analogue of a B-chain of a relaxin superfamily member protein which binds to a biological target of the relaxin superfamily protein, and modulates an activity of the biological target, wherein the analogue is produced by modification of a turn or loop moiety of the B-chain of the relaxin superfamily protein, the modification involving selection of at least a first and a second amino acid residue
- 20 with an alpha-helix or beta-strand carbon separation distance of less than six angstroms and cross-linking the first and second amino acids, wherein the cross-link conformationally constrains the analogue wherein the relaxin superfamily protein is selected from IGF-I, IGF-II, relaxin 1, relaxin 2, relaxin 3, INSL3, INSL4, INSL5 and INSL6.

In one aspect, the monomeric analogue is an INSL3 B-chain analogue modified from the sequence set forth in SEQ ID NO:7.

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In a further aspect, the INSL3 analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequences set forth in SEQ ID NO:7.

In a particularly preferred form, the first and/or second amino acids are substituted with amino
5 acids suitable for cross-linking.

According to another aspect, the monomeric analogue is the INSL3 analogue designated cINSLa in Figure 3.

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In a further aspect, the monomeric analogue is the INSL3 analogue designated cINSLb in Figure 3.

In yet another aspect, the monomeric analogue is a relaxin analogue modified from a relaxin-1, relaxin-2, or relaxin-3 B-chain sequence set forth in SEQ ID NOs: 1, 2 and 3, respectively.

In a preferred form, the monomeric relaxin analogue is the relaxin analogue designated cRlx in Figure 3.

10

In a further aspect, the monomeric relaxin analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequences set forth in SEQ ID NO:2.

15 In yet a further form, one or more amino acids within the INSL3 or relaxin peptide analogue sequence, other than the cross-linked first and second amino acids, is optionally substituted to modify one or more biological activities of the monomeric analogue.

In a further preferred form, the monomeric analogue is an agonist, partial agonist, antagonist, partial antagonist or reverse antagonist at the relaxin superfamily receptor.

20

In another aspect, the analogues of the invention are conjugated to an A-chain of the relaxin superfamily protein.

25 In a further aspect, the monomeric analogues of the invention are conjugated to carrier molecules, such as labelling compounds, radioligands, radionucleotides, immunotherapeutic agents or other desirable chemical moiety.

In a further aspect, the invention seeks to provide a method of making a monomeric peptide analogue of a relaxin superfamily protein, the method comprising:

30

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- providing a B-chain, receptor-binding subunit of a relaxin superfamily protein; and
- identifying a first and a second amino acid on respective, opposing strands of a turn and/or loop moiety in the receptor-binding subunit of the protein, with an
5 alpha-helix or beta-strand carbon separation distance of less than six angstroms; and
- introducing a cross-link between the first and second amino acids;

wherein the cross-link constrains a three dimensional structure of at the B-chain, receptor-binding subunit.

10

In a particularly preferred form, the first and/or second amino acids are substituted with amino acids suitable for cross linking.

15

In one aspect, the method produces an INSL3 peptide analogue based on the sequence set forth in SEQ ID NO:7.

In a preferred form, the method produces the INSL3 analogue designated cINSLa in Figure 3.

20 In another preferred form, the method produces the INSL3 analogue designated cINSLb in Figure 3.

In a further aspect, the method produces a monomeric relaxin-1, relaxin-2 or relaxin-3 peptide analogue based on any of the sequences set forth in SEQ ID NOs:1, 2 and 3,
25 respectively.

Preferably, the method produces a monomeric relaxin analogue designated cRLx in Figure 3.

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In yet a further form, the method produces an INSL3 and/or relaxin analogue wherein one or more amino acids other than the cross-linked first and second amino acids, are optionally substituted to modify one or more biological activities of the peptide analogue.

- 5 In yet another aspect, there is provided a pharmaceutical composition including one or more of the monomeric analogues of the present invention, and pharmaceutically acceptable salts thereof.

10 Preferably, the pharmaceutical composition includes an INSL3 analogue or a relaxin analogue.

A method of treating, preventing or ameliorating the symptoms of a disorder in a patient, the disorder involving dysregulation of activity of a relaxin superfamily protein member, the method comprising administering to the patient an effective amount of a peptide
15 analogue of the invention.

In one aspect, the disorder is characterised by dysregulation of relaxin and/or INSL3 expression or activity.

20 In another aspect, the invention provides a method of treating, preventing or ameliorating the symptoms of a relaxin or INSL3-mediated disorder in a patient, wherein the disorder is selected from the group consisting of hyperplastic disorders, neoplastic disorders, scleroderma, uncontrolled or abnormal collagen or fibronectin formation or breakdown, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive
25 disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, dysregulation of spermatogenesis and reproductive development including descent of the gonads, the method comprising administering to the patient an effective amount of a peptide analogue of the invention.

30

In a further aspect, the invention contemplates the use of the analogues and/or

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pharmaceutical compositions of the present invention in the manufacture of a medicament for the treatment of a relaxin or INSL3-mediated disorder in a patient, the disorder selected from the group consisting of: hyperplastic disorders, neoplastic disorders, scleroderma, uncontrolled or abnormal collagen or fibronectin formation or breakdown, neurological
5 disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, dysregulation of spermatogenesis and reproductive development including descent of the gonads.

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Description of the Figures

FIGURE 1: Schematic representation of binding of INSL3, cINSLa and cINSLb analogues
5 to the LGR8 receptor.

FIGURE 2: Schematic representation of binding of the relaxin-like or INSL3 analogues to
the LGR7 receptor.

10 FIGURE 3: Sequences and constraints of the native relaxin and INSL3-B chains with
exemplary analogues designed.

FIGURE 4: Schematic representation of antagonistic activity of cINSLa in the inhibition of
the response of INSL3 (measured by cAMP response) to the native INSL3 receptor
15 (LGR8).

FIGURE 5: Schematic representation illustrating CD spectra of cINSLa showing
significant alpha-helical content in water and phosphate buffered saline.

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Detailed Description

In its broadest form, the present invention relates to peptide analogues of members of the relaxin superfamily of proteins, the analogues having a binding activity at a relaxin protein superfamily receptor, methods of producing the analogues, and pharmaceutical
5 compositions, methods of treatment, and uses of the peptide analogues.

In a first aspect, the analogue is a conformationally constrained, cyclic analogue of a B-chain of a relaxin superfamily member protein which binds to a biological target of a relaxin superfamily protein, and modulates an activity of the biological target, wherein the
10 relaxin superfamily protein is selected from insulin, IGF-I, IGF-II, relaxin 1, relaxin 2, relaxin 3, INSL3, INSL4, INSL5 or INSL6.

The term "modulates" as used herein includes, but is not limited to, initiation of activity at a biological target of a relaxin superfamily protein, up- or down-regulation of activity at a
15 biological target of a relaxin superfamily protein, or blockage of an activity at a biological target of a relaxin superfamily protein.

The biological targets of relaxin superfamily proteins include receptors, such as but not limited to, LGR7, LGR8, GPCR135, GPCR142, the insulin receptor, the IGF-1 receptor,
20 and the IGF-II receptor.

Accordingly, the analogue can also be a ligand at a relaxin superfamily receptor, wherein the analogue ligand can have an activity including, but not limited to, that of an agonist, antagonist, mixed agonist, reverse agonist, partial agonist, partial antagonist or reverse
25 antagonist at the receptor.

The cyclic B-chain peptide analogues of the invention are produced by modification of a turn or loop moiety of the B-chain of the relaxin superfamily protein, the modification involving selection of at least a first and a second amino acid residue with an alpha-helix
30 or beta-strand carbon separation distance of less than six Angstroms and cross-linking the

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first and second amino acids, wherein the cross-link conformationally constrains the analogue.

In one aspect, the analogue is an INSL3 B-chain analogue modified from the sequence set
5 forth in SEQ ID NO:7.

In a further aspect, the INSL3 analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequences set forth in SEQ ID NO:7. Even more preferably, the
10 INSL3 analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8, including positions 2, 3, 4, 5, 6, 7, and 8, and a second amino acid within a range of positions 21 and 26, including 21, 22, 23, 24, 25 and 26, of the sequence set forth in SEQ ID NO:7.

15 In a particularly preferred aspect, the B-chain peptide analogue is the INSL3 analogue designated cINSLa in Figure 3.

In yet another preferred form, the B-chain peptide analogue is the INSL3 analogue designated cINSLb in Figure 3.

20

In yet another aspect, the analogue is a relaxin analogue modified from a relaxin-1, relaxin-2, or relaxin-3 B-chain sequence set forth in SEQ ID NOs: 1, 2 and 3, respectively.

In a further aspect, the relaxin analogue is constrained by a cross-link between a first
25 amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequences set forth in SEQ ID NO:2. Even more preferably, the relaxin analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8, including positions 2, 3, 4, 5, 6, 7, and 8, and a second amino acid within a range of positions 21 and 26, including 21, 22, 23, 24, 25 and 26, of the sequence set
30 forth in SEQ ID NO:2.

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In a preferred form, the B-chain analogue is the relaxin analogue designated CR1x in Figure 3.

In a particularly preferred form, the first and second amino acids are substituted with
5 amino acids suitable for cross linking.

Suitable chemical linking groups have 0 to 20 carbon atoms, 0 to 10 heteroatoms (N, O, S, P etc.), and can be straight chain or branched and contain saturated, unsaturated and/or aromatic rings, single and/or double bonds and chemical groups such as amide, ester,
10 disulfide, thioether, ether, phosphate, amine and the like.

The amino-acid substitution and formation of a cross-link, which is used to conformationally constrain the B-chain can be obtained by several methods, including but not limited to:

- 15 (i) cyclizing the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, for example by condensation with an M-amino carboxylic acid;
- (ii) cyclizing via the formation of a covalent bond between the side chains of two residues,
20 such as an amide bond between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulfide bond between two cysteine residues, or a thioether bond between a cysteine residue and an α -halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the B-chain sequence itself, or may be incorporated into or added on to the B-chain
25 sequence for this purpose; and,
- (iii) cyclizing via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or using a spacer group as described in (i) above. The residues contributing the side chains may be derived from the B-chain sequence itself, or may be incorporated into
30 or added on to the B-chain sequence for this purpose.

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Furthermore, the B-chain peptide analogue can be made in which one or more amino acid residues is replaced by its corresponding D-amino acid, substitutions or modifications are made to one or more amino acids in the sequence, peptide bonds can be replaced by a structure more resistant to metabolic degradation and different cyclizing constraints and dimerization groups can be incorporated.

With respect to compounds in which one or more amino acids is replaced by its corresponding D-amino acid, the skilled person is aware that retro-inverso amino acid sequences can be synthesized by standard methods; see, for example, Chorev and Goodman, *Acc. Chem. Res.*, 26: 266-273, 1993.

Olson *et al.*, *J. Med. Chem.*, 36: 3039-3049 1993 provides an example of replacing a peptide bond with a structure more resistant to metabolic degradation.

Analogues of the invention can also be made where individual amino acids are replaced by analogous structures, for example gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge. The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogs are well known in the art (see, for example, Gallop *et al.*, *J. Med. Chem.*, 37: 1233-1251, 1994). It is particularly contemplated that the analogues of the invention are useful as templates for design and synthesis of compounds of improved activity, stability and bioavailability.

Preferably where amino acid substitution is used, the substitution is conservative, *i.e.* an amino acid is replaced by one of similar size and with similar charge properties.

As used herein, the term "conservative substitution" denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative

- 15 -

substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids which can be substituted for one another include asparagine, glutamine, serine and threonine. The term "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid.

As such, it should be understood that in the context of the present invention, a conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in the following Table A from WO 97/09433.

15

TABLE A**CONSERVATIVE SUBSTITUTIONS I**

Side Chain Characteristic	Amino Acid
Aliphatic non-polar	G A P I L V
Polar – uncharged	C S T M N Q
Polar – charged	D E K R
Aromatic	H F W Y
Other	N Q D E

20

Alternatively, conservative amino acids can be grouped as described in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY: NY(1975), pp. 71-77] as set out in the following Table B.

25

TABLE B**CONSERVATIVE SUBSTITUTIONS II**

30

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Side Chain Characteristic	Amino Acid
Non-polar (hydrophobic)	
A. Aliphatic:	A L I V P
B. Aromatic:	F W
C. Sulphur-containing:	M
D. Borderline:	G
Uncharged-polar	
A. Hydroxyl:	S T Y
B. Amides	N Q
C. Sulfhydryl:	C
D. Borderline:	G
Positively Charged (Basic):	K R H
Negatively Charged (Acidic):	D E

Exemplary conservative substitutions are set out in the following Table C.

TABLE C

5

CONSERVATIVE SUBSTITUTIONS III

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr, Phe
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

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The cyclic peptide analogues of the invention can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the peptides of the invention.

The peptide analogues also can be modified to create peptide derivatives by forming covalent or noncovalent complexes with other moieties. Covalently-bound complexes can be prepared by cross-linking the chemical moieties to functional groups on the side chains of amino acids comprising the peptides, or at the N-or C terminus.

The B-chain peptide analogues can be conjugated to an A-chain of a relaxin superfamily protein, and preferably, the A-chain of the relaxin superfamily protein to which they share B-chain analogy.

The B-chain peptide analogues can be conjugated to a reporter group, including, but not limited to a radiolabel, a fluorescent label, an enzyme (e.g., that catalyzes a colorimetric or fluorometric reaction), a substrate, a solid matrix, or a carrier (e. g., biotin or avidin).

In yet a further form, one or more amino acids within the peptide analogue sequence, other than the first and second amino acids, is optionally substituted to modify one or more biological activities of the analogue.

The term "biological activities" as used herein includes, but is not limited to, binding affinity for a biological target, effector activity at the biological target and *in vivo* stability.

In a further aspect, the invention seeks to provide a method of making a peptide analogue of a relaxin superfamily protein, the method comprising:

- providing a B-chain, receptor-binding subunit of a relaxin superfamily protein;
- and

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- identifying a first and a second amino acid on respective, opposing strands of a turn and/or loop moiety in the receptor-binding subunit of the protein, with an alpha-helix or beta-strand carbon separation distance of less than six angstroms; and
 - 5 - introducing a cross-link between the first and second amino acids;
- wherein the cross-link constrains a three dimensional structure of at the B-chain, receptor-binding subunit.

10 In a particularly preferred form, the first and second amino acids are substituted with amino acids suitable for cross linking.

In one aspect, the method produces an INSL3 peptide analogue based on the sequence set forth in SEQ ID NO:7.

15

In a preferred form, the method produces an INSL3 analogue designated cRlx, cINSL3a or cINSL3b in Figure 3.

In a further aspect, the method produces a relaxin-1, relaxin-2 or relaxin-3 peptide
20 analogue based on any of the sequences set forth in SEQ ID NOs:1, 2 and 3, respectively.

Preferably, the method produces a relaxin analogue designated cRlx in Figure 3.

In yet a further form, the method produces an INSL3 and/or relaxin analogue wherein one
25 or more amino acids other than the cross-linked first and second amino acids, are optionally substituted to modify one or more biological activities of the peptide analogue.

One aspect of the invention utilises the ability of the B-chain peptide analogues of the invention to modulate an activity of a biological target of at least one relaxin superfamily
30 protein member, that is, induce or modify an activity of at least one relaxin superfamily protein member, and in particular, relaxin or INSL3, which are mediated through the

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actions of the LGR7 or LGR8 receptors. The analogues can thus induce an activity of a relaxin superfamily member, in the absence of endogenous expression of the protein, or suppress or antagonise the activity of a protein endogenously expressed. Alternatively, the analogues of the invention can up-regulate the level of an existing biological activity of a relaxin superfamily member. Such biological activities include, but are not limited to, collagen degradation and remodelling, vascular dilation, growth and differentiation of the mammary gland, increased blood flow and organ perfusion, increasing heart rate, increasing coronary blood flow, increasing glomerular filtration rate and renal plasma flow, maturation of the gonads, promotion of cellular division, survival of sperm cells, development of ovarian follicles and oocyte maturation. As LGR8 is also expressed in the kidney, bone, peripheral blood leukocytes, muscle, brain, thyroid, and uterus. Hence INSL3 analogues have potential actions on kidney and thyroid function, central actions on the brain, modulation of muscle function, effects on bone and blood cell function and reproductive actions on the uterus.

15

Accordingly, modification of any one or more of the above-mentioned biological activities of the relaxin superfamily proteins requires administration of an effective, biological-activity interfering amount of a B-chain analogue of the invention.

20 Clinical applications of the invention include, but are not limited to, hyperplastic disorders, neoplastic disorders, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, dysregulation of spermatogenesis, male and female infertility and reproductive development including descent of the gonads.

25

In yet another aspect, there is provided a pharmaceutical composition including one or more of the analogues of the present invention, and pharmaceutically acceptable salts thereof.

30

Another aspect of the invention concerns the provision of a pharmaceutical composition

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comprising a B-chain analogue of the invention, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. A preferred pharmaceutical composition will inhibit or interfere with a biological activity induced by at least one member of a relaxin superfamily protein. A particularly preferred
5 pharmaceutical composition will inhibit the biological activity induced by relaxin and/or INSL3.

Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose,
10 microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms.

15

The formulation is carried out to suit the mode of administration. Compositions comprising a peptide of the invention will contain from about 0.1% to 90% by weight of the active compound(s), and most generally from about 10% to 30%.

20 The dose(s) and route of administration will depend upon the nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian.

Suitable routes include oral, subcutaneous, intramuscular, intraperitoneal or intravenous
25 injection, parenteral, topical application, implants etc. For example, an effective amount of a monomeric monocyclic peptide of the invention or a dimeric bicyclic peptide of the invention is administered to an organism in need thereof in a dose between about 0.1 and 1000 μ g/kg body weight.

30 The B-chain analogues of the invention may be used as therapeutic compositions either alone or in combination with other therapeutic agents.

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Preferably, the pharmaceutical composition includes an INSL3 analogue or a relaxin analogue.

- 5 In one aspect, the disorder is characterised by dysregulation of relaxin and/or INSL3 expression or activity.

As previously mentioned, it is understood that relaxin binds to at least two biological targets: LGR7 and LGR8, while INSL3 appears to mediate its biological effects via LGR8
10 only. Without being bound to any particular theory or mode of action, it is believed that an analogue with relaxin-like binding activity which exhibits affinity for LGR7 and LGR8 will modulate the effects of both relaxin and INSL3, given that INSL3 shares a common receptor with relaxin. Given the fact that LGR8 binds both relaxin and INSL3, it is possible that many of relaxin's effects are modulated by INSL3 levels, and vice versa.

15

In another aspect, the invention provides a method of treating, preventing or ameliorating the symptoms of a relaxin or INSL3-mediated disorder in a patient, the disorder selected from the group consisting of hyperplastic disorders, neoplastic disorders, scleroderma, uncontrolled or abnormal collagen or fibronectin formation or breakdown, neurological
20 disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, male and female infertility, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, dysregulation of spermatogenesis and reproductive development including descent of the gonads, the method comprising administering to the patient an effective amount of a
25 peptide of the invention.

As used herein, the term "conditions associated with pregnancy" includes, but is not limited to, conditions of fertilisation, pregnancy, parturition and lactation.

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The term "relaxin or INSL3-mediated" as used herein refers to conditions where relaxin or INSL3 are involved or implicated in the development, maintenance or progression of the condition.

- 5 In a further aspect, the invention contemplates the use of the analogues and/or pharmaceutical compositions of the present invention in the manufacture of a medicament for the treatment of a relaxin or INSL3-mediated disorder in a patient, the disorder selected from the group consisting of: hyperplastic disorders, neoplastic disorders, scleroderma, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive
10 disorders, conditions associated with pregnancy, renal disease, male and female infertility, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, disregulation of spermatogenesis and reproductive development including descent of the gonads.
- 15 This invention will now be described with reference to the following, non-limiting examples.

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Example 1Methods and Materials*Molecular design of INSL3 analogues*

- 5 Using SYBYL molecular modelling software (Tripos) on a Silicon Graphics O2 workstation, we designed a model of INSL3 B-chain from the X-ray crystal structure of human Gene 2 relaxin B-chain as a template (Eigenbrot *et al.*, 1991, *J Mol Biol* 221: 15-21). After modifying the sequence to resemble that of INSL3, an energy minimisation
- 10 using a Tripos forcefield with Gasteiger-Marsili charges was carried out. From the resulting energy minimised model, two residues were identified (Glu⁴ and Arg²⁶) to have C β atoms within 4Å of each other. These residues were then replaced with cysteine and a disulphide bond formed to give the cyclic peptide cINSLa.
- 15 A second cyclic analogue cINSLb was formed by changing His¹² to Arg, to mimic a relaxin-like binding motif of Arg-X-X-Arg-X-X-Val.

Solid-phase peptide synthesis:

- 20 The synthesis of the INSL3 analogues were achieved using the continuous flow Fmoc-methodology as previously described (Dawson *et al.*, *J Peptide Res* 53:542-547, 1999). The solid support was Fmoc-PAL PEG-PS (PerSeptive Biosystems, USA), and HBTU--activated Fmoc-amino acids were used throughout.. N ^{α} -Fmoc deprotection was with 20% piperidine in DMF. All derivatives were purchased from Auspep (Melbourne, Australia).
- 25 Cleavage of the peptides from the solid support and side chain deprotection was achieved by a 3 hour treatment with trifluoroacetic acid (TFA) in the presence of phenol, thioanisole, ethanedithiol and water (82.5/5/5/2.5/5, v/v). The crude peptides were subjected to reversed-phase high performance liquid chromatography (RP-HPLC) on a
- 30 TFA. Peptides were then oxidised in a buffer containing 10% dimethylsulfoxide (DMSO) for 24 hours and the reaction monitored on HPLC and by mass spectrometry.

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Characterization:

The purity of the synthetic peptide was assessed by analytical RP-HPLC, and matrix-assisted laser desorption time of flight (MALDITOF) mass spectrometry using a Bruker
5 BiflexTM instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptide quantitation was by amino acid analysis of a 24 hour acid hydrolyzate using a GBC instrument (Melbourne, Australia).

Circular dichroism (CD) spectroscopy:

10

CD spectra were taken on a Jasco J-720 instrument at room temperature in a 1 mm path-length cell, Doubly distilled water, 10 mM sodium phosphate buffer containing 120 mM NaCl pH 7.4, and spectroscopy grade trifluoroethanol (TFE) were used as solvents. The peptide concentrations were made to 1 μ M, determined by quantitative RP-HPLC and
15 amino acid analysis (ref). Curves were smoothed by the algorithm provided by Jasco, Mean residue ellipticity ($[\theta]_{MR}$) is expressed in degrees^xcm²/dmole by using the molecular mass. CD spectra evaluations were based on comparison with known peptide conformations (Otvos, L., Jr. 1996. from Neuropeptide protocols. Irvine, G. & Williams, C., Eds. pp 153-161. Humana Press, Totowa, NJ).

20

Functional cAMP assays:

Human 293T cells stably transfected with human LGR7, or LGR8 were preincubated in the presence of 0.25mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma) before various
25 treatments for 30 minutes. At the end of the incubation cells were lysed for measurement of cAMP using a well-characterized cAMP ELISA (Satoko *et al.*, *J Biol Chem*, 278:7855-7862). All experiments were repeated at least three times using cells from independent transfections, Results are plotted as pmol cAMP/10⁴ cells.

30 *Ligand binding assays:*

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The method for binding of ³³P-labelled relaxin to 293T cells stably transfected with either LGR7 or LGR8 has been described previously (Satoko *et al.*, *J Biol Chem*, 278:7855-7862). Data are expressed as mean $\pm$ SEM of % specific binding of triplicate determinations performed on a least three independent plates of transfected cells. Data
5 were plotted using the one-site competition functions of the PRISM program (Graphpad Inc., San Diego, USA).

Example 2

Sites for the incorporation cyclising constraints to create B chain analogues were
10 determined by molecular modelling studies. In the case of relaxin, distances between β -carbon atoms in the B chain were measured in the X-ray crystal structure of relaxin [6RLX], using the molecular modelling program SybylTM (Tripos Inc St Louis MO). Residue 25 (Met) within the C chain helix and residue 3 (Trp) in the strand were found to have a β -carbon to β -carbon distance of 3.855 angstroms. These residues were thus deemed suitable
15 for replacement by Cys and subsequent formation of a disulphide bond. This constrained two ends of the molecule in a similar fashion to the native protein, resulting in the cyclic relaxin B chain mimetic cRlx (Figure 3). Methods are as described in International Patent Applications Nos. WO 00/75176 and WO 01/52875.

20 In the case of INSL3 we constructed a model of the 3D structure of INSL3 by substituting the appropriate amino acids into the relaxin structure, and carrying out an energy minimisation of the resultant INSL3 B chain. We then identified pairs of residues as potential sites for the incorporation of a Cys to Cys constraint, including the pair Glu4 and Arg26, residues adjacent to those replaced in the relaxin analogue, giving the cyclic INSL3
25 mimetic cINSLA (Figure 3).

Following the biological characterisation of peptides cRlx and cINSLa (Figure 3), an additional compound was prepared incorporating the Cys to Cys constraint of peptide cINSLa. This compound, designated cINSLb, was a INSL3-based sequence, in which
30 His12 from the INSL3 sequence was replaced by Arg in an attempt to obtain an analogue of the putative relaxin receptor binding cassette which is absent in INSL3.

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The same approach is used to prepare insulin B chain analogues and other relaxin superfamily analogues.

Although several preferred embodiments have been described in detail, it should be understood that various changes, substitutions, and alterations can be
5 made herein by one ordinarily skilled in the art without departing from the scope of the present invention as defined in the claims.

SEQUENCE LISTING

<110> Howard Florey Institute of Experimental Physiology and Medicine
The University of Melbourne

<120> Relaxin Superfamily Peptide Analogues

<130> 12262441/PAS/SET

<150> AU 2003903124

<151> 2003-06-20

<160> 25

<170> PatentIn version 3.1

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Ala	Gln	Ile	Ala	Ile	Cys	Gly	Met	Ser	Thr	Trp	Ser
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Arg	Ala	Gln	Ile	Ala	Ile	Cys	Gly	Met	Ser	Thr	Trp	Ser
			20				25					

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Ala	Val	Ile	Phe	Thr	Cys	Gly	Gly	Ser	Arg	Trp
			20				25			

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 1 5 10 15

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Pro

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 1 5 10 15

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 20 25 30

Gly

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<400> 10
 Ser Asp Ile Ser Ser Ala Arg Lys Leu Cys Gly Arg Tyr Leu Val Lys
 1 5 10 15

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 20 25 30

Glu

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1				5					10					15	

Leu	Val	Arg	Val	Ser	Gly	Gly	Pro	Cys	Trp	Ser
			20					25		

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Thr	Pro	Cys	Met	Arg	Glu	Lys	Leu	Ser	Gly	Arg	His	Phe	Val	Arg	Ala
1				5					10					15	

Leu	Val	Arg	Val	Ser	Gly	Gly	Pro	Cys	Trp	Ser
			20					25		

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1				5					10					15	

Gln	Val	Ile	Ala	Ile	Gly	Gly	Pro	Cys	Trp	Ser
			20					25		

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Leu	Val	Arg	Val	Ser	Gly	Gly	Cys	Arg	Trp	Ser
			20					25		

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5

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1				5					10					15	

Lys	Arg	Ser	Leu	Ala	Lys	Tyr	Cys
			20				

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<213> Homo sapiens

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1				5					10					15	

Lys	Arg	Ser	Leu	Ala	Arg	Phe	Cys
			20				

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<213> Homo sapiens

<400> 18

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1				5					10					15	

Lys	Ser	Glu	Ile	Ser	Ser	Leu	Cys
			20				

<210> 19

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<400> 19

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1				5					10					15	

Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn
			20					25	

<210> 20

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<400> 20

Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu	Cys	Cys	Phe	Arg	Ser	Cys	Asp
1				5					10					15	

Leu	Arg	Arg	Leu	Glu	Met	Tyr	Cys	Ala
			20					25

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 Leu Ala Leu Leu Glu Thr Leu Cys Ala
 20 25

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 1 5 10 15
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 20 25

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 1 5 10 15
 Met Thr Asp Leu Ser Ala Leu Cys
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 1 5 10 15

Lys Glu Glu Leu Ser Ile Ala Cys
20

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CLAIMS:

1. A monomeric, cyclic peptide analogue of a B-chain of a relaxin superfamily member protein which binds to a biological target of the relaxin superfamily protein, and modulates an activity of the biological target, wherein the analogue is produced by
5 modification of a turn or loop moiety of the B-chain of the relaxin superfamily protein, the modification involving selection of at least a first and a second amino acid residue with an alpha-helix or beta-strand carbon separation distance of less than six angstroms and cross-linking the first and second amino acids, wherein the cross-link conformationally constrains the analogue wherein the relaxin superfamily protein is selected from IGF-I, IGF-II, relaxin 1,
10 relaxin 2, relaxin 3, INSL3, INSL4, INSL5 and INSL6.
2. The analogue according to claim 1, wherein the biological target is selected from insulin receptors, IGFR-I, IGFR-II, LGR7 and LGR8.
3. The analogue according to any one of claims 1 to 2, wherein the analogue is an INSL3 B-chain analogue modified from a sequence set forth in SEQ ID NO:7.
- 15 4. The analogue according to claim 3, wherein the INSL3 analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequences set forth in SEQ ID NO:7.
5. The analogue according to any one of claims 1 to 2, which is a relaxin analogue modified from a relaxin-1, relaxin-2, or relaxin-3 B-chain sequence set forth in SEQ
20 ID NOs: 1, 2 and 3, respectively.
6. The analogue according to claim 5, wherein the relaxin analogue is constrained by a cross-link between a first amino acid within a range of positions 2 and 8 and a second amino acid within a range of positions 21 and 26 of the sequence set forth in SEQ ID NO:2.
7. The analogue according to any one of claims 1 to 6, wherein the first and/or
25 second amino acids are substituted with alternative amino acids suitable for cross-linking.

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8. The analogue according to claim 7 wherein at least one of the alternative amino acids is a cysteine residue.

9. The analogue according to claim 8 wherein both of the alternative amino acid residues are cysteine residues.

5 10. The analogue according to claim 9 wherein the analogue is cross-linked by oxidizing the cysteine residues to form a disulfide bond between the cysteine residues.

11. The analogue according to any one of claims 1 to 4 or 7 to 10, wherein the analogue is an INSL3 analogue with the sequence and structure:



10 12. The analogue according to any one of claims 1 to 4 or 7 to 10, wherein the analogue is an INSL3 analogue with the sequence and structure:



13. The analogue according to any one of claims 1, 2, 5, 6, or 7 to 10, wherein the analogue is a relaxin analogue with the sequence and structure:



14. The analogue according to any one of claims 1 to 13 wherein the biological target of the analogue is LGR7 and/or LOR8.

15. The analogue according to claim 14, wherein activity of the biological target is initiated, up-regulated, down-regulated or otherwise blocked.

20 16. The analogue of any one of claims 1 to 15, wherein the analogue is conjugated to an A-chain of a relaxin superfamily protein.

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17. The analogue according to claim 16, wherein the A-chain of the relaxin superfamily protein is derived from the relaxin superfamily protein from which the B chain analogue is derived.

18. The analogue according to any one of claims 1 to 17, wherein the analogue is
5 conjugated to a reporter group.

19. The analogue according to claim 18, wherein the reporter group is a radiolabel.

20. The analogue according to claim 18, wherein the reporter group is a fluorescent label.

21. The analogue according to claim 18, wherein the reporter group is an enzyme.

10 22. The analogue according to claim 18, wherein the reporter group is a carrier.

23. A method of making a peptide analogue of a relaxin superfamily protein, the method comprising:

- providing a B-chain, receptor-binding subunit of a relaxin superfamily protein; and

15 - identifying a first and a second amino acid on respective, opposing strands of a turn and/or loop moiety in the receptor-binding subunit of the protein, with an alpha-helix or beta-strand carbon separation distance of less than six angstroms; and

- introducing a cross-link between the first and second amino acids;

20 wherein the cross-link conformationally constrains a three dimensional structure of at the B-chain, receptor-binding subunit.

24. The method according to claim 23, wherein the first and/or second amino acids are substituted with amino acids suitable for cross-linking.

25. The method according to claim 23 or 24, wherein the method produces an INSL3 peptide analogue derived from the sequence set forth in SEQ ID NO:7, with the first

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amino acid within a range of residues 2 and 8 and the second amino acid within a range of residues 21 and 26.

26. The method according to claim 23 or 24, wherein the method produces a relaxin peptide analogue derived from the sequence set forth in any one of SEQ ID NOS:1 to 3, with the first amino acid within a range of residues 2 and 8 and the second amino acid within a range of residues 21 and 26.

27. A pharmaceutical composition including one or more analogues as claimed in any one of claims 1 to 22, together with a pharmaceutically acceptable carrier.

28. Use of a peptide analogue according to any one of claims 1 to 22 for the manufacture of a medicament for treating, preventing or ameliorating the symptoms of a disorder associated with deregulation of activity of a relaxin superfamily protein member, wherein the disorder is selected from the group consisting of hyperplastic disorders, scleroderma, uncontrolled or abnormal collagen formation, uncontrolled or abnormal collagen breakdown, uncontrolled or abnormal fibronectin formation, uncontrolled or abnormal fibronectin breakdown, neoplastic disorders, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Raynaud's disease, Raynaud's phenomenon, cryptorchidism, male or female infertility, dysregulation of spermatogenesis and reproductive development.

29. The use of claim 28 wherein the disorder is in descent of the gonads.

30. The use according to claim 28, wherein the disorder is a female reproductive disorder.

31. The use according to claim 28, wherein the disorder is a pregnancy-related disorder.

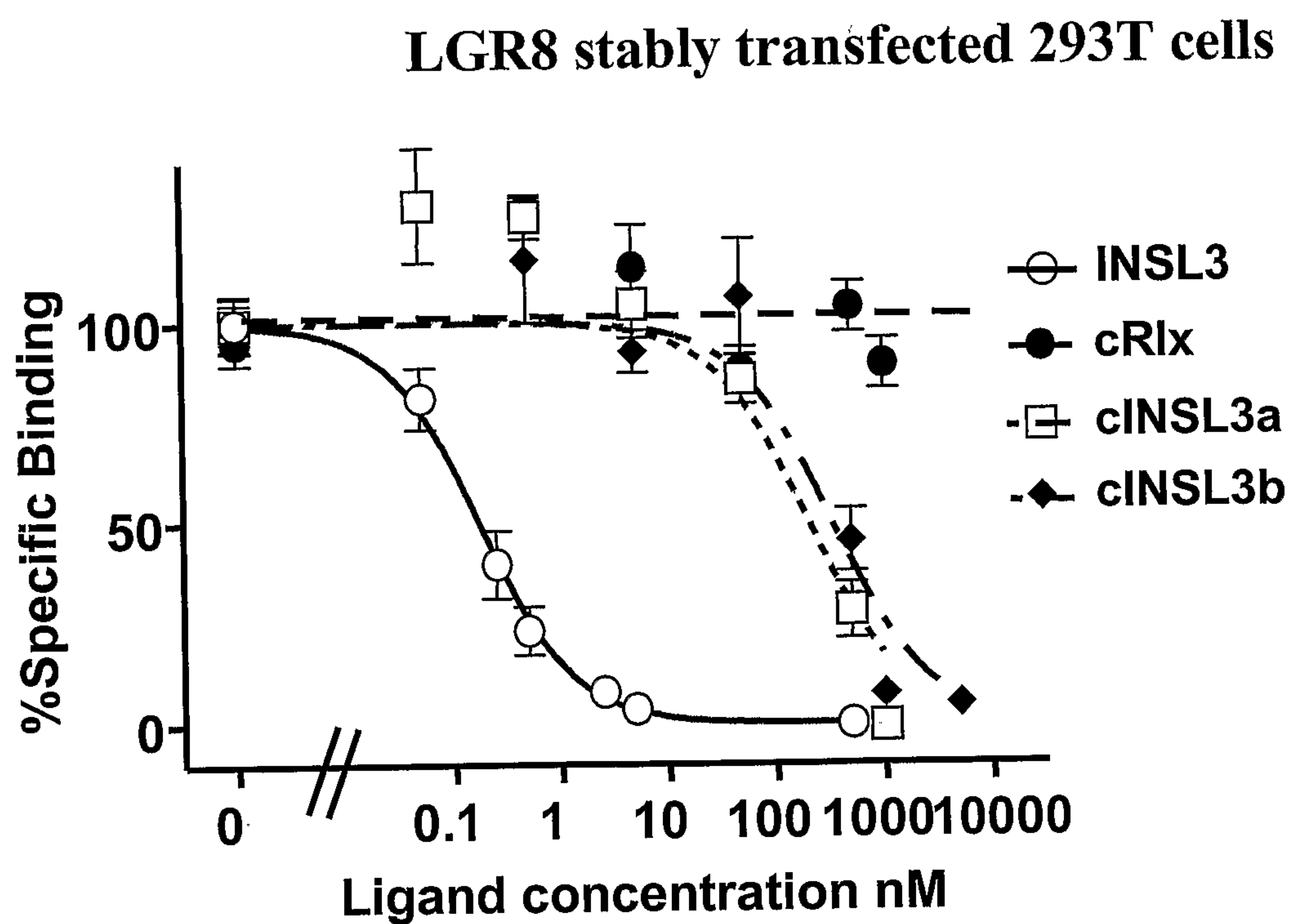
32. The use according to claim 28, wherein the disorder involves abnormal reproductive developments.

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33. The use according to claim 28, wherein the disorder is a cardiovascular disorder.
34. The use according to claim 28, wherein the disorder is an angiogenic disorder.
35. The use according to claim 28, wherein the disorder is a neoplastic disorder or
5 a hyperplastic disorder.
36. The use according to claim 35, wherein the neoplastic or hyperplastic disorder involves the thyroid gland.
37. The use according to claim 28, wherein the disorder is scleroderma.
38. The use according to claim 28, wherein the disorder is renal disease.
- 10 39. The use according to claim 28, wherein the disorder involves uncontrolled or abnormal collagen or fibronectin formation or breakdown.
40. The use according to claim 28, wherein the disorder is male or female infertility.

Figure 1



LGR8 pEC₅₀(n=3 to 4)

GB- 0

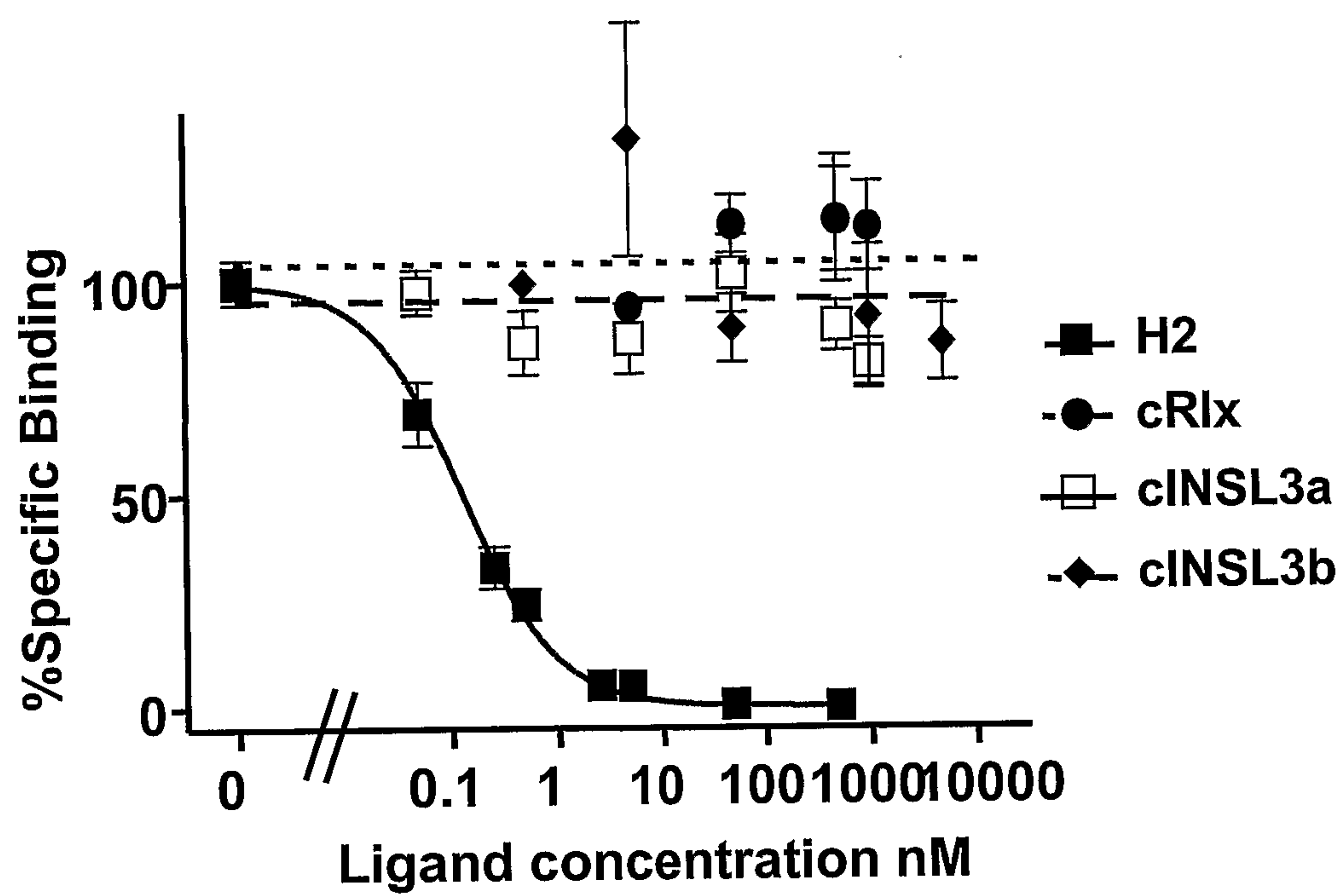
GBII - -6.79 ± 0.27

GBIII - -6.26 ± 0.23

} ns

Figure 2

LGR7 stably transfected 293T cells

LGR7 pEC₅₀ (n=3 to 4)

H2 -10.04±0.37

cRlx -0

cINSLaII - 0

cISNLb - 0

Sequences of relaxin superfamily B-chain peptide analogues

relaxin

DSWMEEVIKLCGRELVRAQIAICGMSTWS

INSL3 B-chain

PTPEMREKLCGHHFVRALVRVCGGPRWSTEA

cR1x

SCMEEVIKLCGRELVRAQIAISGCS

cINSL3a

TPCMREKLCGHHFVRALVRVSGGPPCWS

cINSL3b

TPCMREKLSGRHFVRALVRVSGGPPCWS

Figure 3

Figure 4

Antagonism of INSL3 at LGR8 by cINSL3a

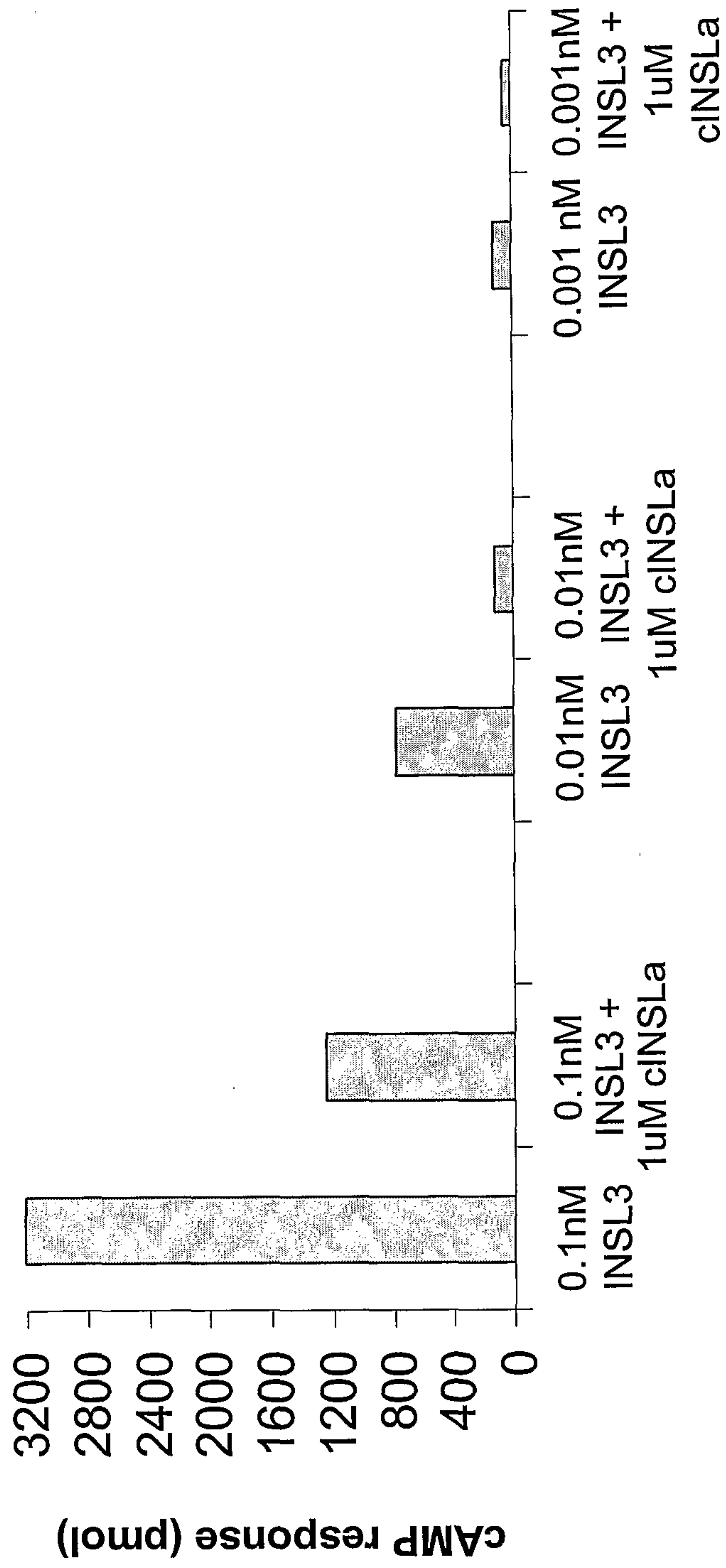


Figure 5

CD spectra of cINSLa in various solvents

