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Cyclotides as templates for peptide GPCR ligand design—discovery of the target receptors of the oxytocin plant peptide kalata B7

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Cyclotides are plant peptides comprising a circular backbone and three conserved disulfide bonds that confer them with exceptional stability. They were originally discovered in Oldenlandia affinis based on their use in traditional African medicine to accelerate labor. Recently cyclotides have been identified in numerous plant species of the coffee-, violet-, cucurbit-, pea-, potato- and grass-families. Due to their unique structural topology, high stability and tolerance to sequence variation they are considered as promising templates for the development of peptide-based pharmaceuticals. However, the mechanisms underlying their biological activities remain largely unknown; specifically, a receptor for a native cyclotide has not been reported hitherto. Using bioactivity-guided fractionation of a herbal peptide extract known to indigenous healers as ‘kalata-kalata’ the cyclotide kalata B7 was found to induce strong contractility on human uterine smooth muscle cells. Radioligand displacement and second messenger-based reporter assays confirm the kalata B7 peptide as an oxytocin and vasopressin V1a-receptor, members of the G protein-coupled receptor (GPCR) family, as molecular targets for this cyclotide. Furthermore we show that cyclotides can serve as templates for the design of selective GPCR peptide ligands by generating oxytocin-like ligands with nanomolar affinities that induce dose-dependent contractions on human myometrium tissue. This provides a proof-of-principle for the design and development of cyclotide-based peptide ligands.

Introduction

Cyclotides are head-to-tail cyclized plant peptides containing three conserved disulfide-bonds in a knotted arrangement known as a cyclic cystine-knot motif (1). This confers them high stability (2) and improves their oral bioactivity relative to their linear counterparts (3). They were first discovered in a decoction of Oldenlandia affinis DC. (Rubiacaeae) leaves, a herbal remedy used in traditional African medicine during childbirth (4). The observed induction of labor and shortened delivery time were later studied on isolated rat and rabbit uteri and human uterine strips (4, 5). The peptides responsible for the contractility effects (5) raised interest as they survived boiling, presumably as a result of their unique three-dimensional structure, which was elucidated in 1995 (6). Since then several plant species of the coffee- (Rubiacaeae) (7), violet- (Violaceae) (8), legume- (Fabaceae) (9), potato- (Solanaceae) (10) and grass- (Poaceae) families (11) have been identified to produce cyclotides. Currently ~300 sequences have been reported (12) and the predicted number of >50,000 cyclotides in Rubiacaeae alone (7) suggests them to be one of the largest peptide classes within the plant kingdom. Their high intercysteine sequence variability and structural plasticity (13), together with intrinsic bioactivities make them interesting templates for the development of novel pharmaceuticals (14).

However, five decades after the discovery of cyclotides, there still isn’t any information about specific molecular targets and/or mechanisms underlying their biological activities. It is known that cyclotides can – at higher concentrations – disrupt phospholipid bilayers (15, 16), because they expose hydrophobic residues on their surface. This endows them with physico-chemical properties allowing for insertion into membranes and pore formation (17, 18). Although no cyclotide target receptor has been identified hitherto, the observed biological activities, (e.g., their uterotonic effects) may be explained by specific receptor-mediated mechanisms. In mammals – including humans – uterine muscle contractility can be elicited by activation of various signaling pathways. One physiological regulator of uterine contraction is the neuropeptide oxytocin. In uterine tissue this peptide activates oxytocin and vasopressin V1a-receptors (19-21), two members of the G protein-coupled receptor (GPCR) family. GPCRs are receptors for a large fraction of the currently marketed drugs elicit their actions by binding to these transmembrane receptors.

Significance

G protein-coupled receptors are considered as promising drug targets and a large fraction of the currently marketed drugs elicit their actions by binding to these transmembrane receptors. Low receptor subtype selectivity often hampers drug development efforts due to unwanted off-target effects. The ethnopharmacological use of herbal medicines containing cyclic peptides with uterotonic properties has been known for decades. Here we investigated the molecular mechanisms underlying this activity and report the first oxytocin plant peptide that modulates the human oxytocin receptor. This naturally-occurring peptide served as template for the design of an oxytocin-like nonapeptide with enhanced receptor selectivity, which highlights the potential of cyclotides for the discovery of peptide-based GPCR ligands.

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A herbal extract that has been used for many generations by

Bioactivity-guided fractionation of uterotonic plant cyclotides. Results of GPCR ligands, thus opening new avenues for cyclotide-based cyclotides can serve as scaffolds for the design of new classes and for the first time identified a molecular target for native the mechanism underlying the oxytocic activity of cyclotides combined with pharmacological and structural analysis to elucidate In particular loop 3 of the cyclotide kalata B7 (-CYTQGC-) play sequence homology to human oxytocin, whereas cyclotides by MS and MS/MS peptide sequencing and 17 cyclotides were
demonstrated from 6.8-18.7% increased contractility over unstimulated cells (Fig. S2) (22). Compared to unstimulated cells, incubation with uterine smooth muscle cells using a collagen gel contractility assay HPLC and tested for their ability to induce contractions of human kalata B7 was isolated by HPLC and the purified cyclotide (Fig. S3) was analyzed (i) for its ability to stimulate contractions of uterine smooth muscle cells and (ii) for its affinity to human oxytocin or V$_{1a}$ receptors. Kalata B7 displaced tritiated oxytocin or vasopressin in a dose-dependent manner from the binding-site of the oxytocin or V$_{1a}$-receptor with a K$_D$ of 50 µM and 12 µM, respectively (Fig. 2 A, Table 1). It also provoked significant contraction of uterine cells, i.e., 8.4% increased contraction compared to unstimulated control cells (Fig. 2 C). We verified that the cyclotide kalata B7 acted via oxytocin and/or V$_{1a}$-receptors on uterus cells by applying kalata B7 together with the receptor antagonist atosiban (23); this co-application of both compounds resulted in a significant loss of contractility.

If kalata B7 was an agonist at the oxytocin and/or the V$_{1a}$-receptor, the peptide ought to trigger signaling via a G$_{i/o}$-dependent pathway. We verified this prediction by measuring the generation of inositol-1-phosphate (IP1) in response to the cyclotide in HEK293 cells heterologously expressing either receptor. The analysis of the concentration-response curve showed that kalata B7 was a partial agonist at both, the oxytocin and the V$_{1a}$- receptor (Fig. 2 B) with EC$_{50}$ of 12 µM and 4 µM, respectively. Agonistic activity was more pronounced at the oxytocin receptor (about 80% of the response elicited by oxytocin) than at the V$_{1a}$-receptor (about 40% of the response elicited by vasopressin).

**Structural characterization of kalata B7.** To understand the ligand-receptor interaction the structure of kalata B7 was determined by NMR (Fig. 3 A, Table S2). This revealed a well-defined backbone around the cyclic cystine knot motif, typical for Moebius cyclotides, a type I-$\beta$-turn between residues 9-12, a type II-$\beta$-turn between residues 16-19 and a type V$\alpha$I-$\beta$-turn between residues 22-25 as well as a $\beta$-hairpin between residues 26-28. As shown in Fig. 3, loop 3 of kalata B7 (-CYTQGC-) contains homology to the six-residue-ring sequence of human oxytocin (CYIQNC-). In particular, the tyrosine (Y15) and glutamine (Q17) residues of kalata B7 are in analogous position with those residues (Y2 and Q4) in oxytocin. NMR structural analysis of human oxytocin confirmed the presence of a type II-$\beta$-turn (Fig. 3 B, (24)). Therefore loop 3 of kalata B7 and human oxytocin share similarities in sequence and three-dimensional structure. Furthermore, the structure of kalata B7 indicated that the side chains of Tyr and Glu in loop 3 protruded from the backbone (Fig. 3 A) and hence they might be capable of interacting with the oxytocin receptor. The crucial role of the tyrosine and glutamine residues (loop 3) of the cyclotide was confirmed by generating mutated variants (Y replaced by A, S or F; Q replaced by A or E) and these were all inactive or did not bind to the receptor (Fig. S5).

Cyclotides as peptide templates for oxytocin and vasopressin GPCR ligand design. Cyclotides typically comprise 28-37 amino acids. Therefore, they are larger and more bulky than the nonapeptide ligands oxytocin and vasopressin. Thus we used the sequence of kalata B7 as a scaffold for the synthesis of oxytocin-like nonapeptides. Based on the sequence of loop 3 of kalata B7 four peptides were synthesized (Table 1). NMR analysis revealed negligible differences in structure relative to oxytocin, as determined by $H$ chemical shifts (Fig. S5) and structural calculations (Fig. 3 and Table S3). A comparison of the structural ensembles of the solution structure of oxytocin (Fig. 3 B) with the kalata B7-OT$_1$ (Fig. 3 C) reveals a similarly dynamic exocyclic tail and a defined region comprising residues 1-6 that overlap well (RMSD: 0.65 Å, Fig. 3 D). The synthetic oxytocin-like peptides were tested for binding and receptor activation (Fig. 4, Fig. S4). [G5, T7, S9]-oxytocin (kalata B7-OT$_1$) had improved binding affinity (K$_D$ = 218 nM; Fig. 4 A). The increased affinity was also evident, when assessing its ability to raise luciferase transcription on the human oxytocin receptor cells (EC$_{50}$ IP$_1$ formation = 145 nM, EC$_{50}$ luciferase-NFAT induction = 356 nM; Table 1), where it acted as a full agonist (cF$\_E_{\text{max}}$ of oxytocin and kalata B7-OT$_1$: in Fig. 4 B).
Peptide Sequence Binding affinity IP1 formation

<table>
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<th>Peptide</th>
<th>Sequence</th>
<th>Binding affinity (Ki (M))</th>
<th>IP1 formation (nM)</th>
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<tr>
<td>kalata B7</td>
<td>cyclo-GPVCGETGCGTCYQGCWPIKCRN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[G5,T7,S9]-OT</td>
<td>CYIQGCLS-NH₂</td>
<td>5.0 ± 1.1 x10⁻⁵</td>
<td>1.2 ± 0.1 x10⁻⁵</td>
</tr>
<tr>
<td>B7-OT</td>
<td>CYIQGCLS-NH₂</td>
<td>2.2 ± 0.2 x10⁻⁷</td>
<td>1.0 ± 0.1 x10⁻⁵</td>
</tr>
<tr>
<td>[T3,G5,T7,S8,5]-OT</td>
<td>CYTPGCSST-NH₂</td>
<td>&gt;1.0 x10⁻⁵</td>
<td>&gt;1.0 x10⁻⁵</td>
</tr>
<tr>
<td>[P4,G5,S7,9]-OT</td>
<td>CYTPGCSST-NH₂</td>
<td>&gt;1.0 x10⁻⁵</td>
<td>&gt;1.0 x10⁻⁵</td>
</tr>
<tr>
<td>kalata B7-OT</td>
<td>CAIQGCLS-NH₂</td>
<td>&gt;1.0 x10⁻⁵</td>
<td>n.d.</td>
</tr>
<tr>
<td>kalata B7-OT</td>
<td>CYIQGCLS-NH₂</td>
<td>1.0 ± 0.1 x10⁻⁹</td>
<td>n.d.</td>
</tr>
<tr>
<td>oxytocin</td>
<td>CYIQNPCGL-NH₂</td>
<td>1.8 ± 0.1 x10⁻⁹</td>
<td>n.d.</td>
</tr>
<tr>
<td>vasopressin</td>
<td>CYFQNCPRG-NH₂</td>
<td>n.d.</td>
<td>8.0 ± 0.2 x10⁻¹⁰</td>
</tr>
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* Binding affinity (Ki) and functional receptor activation (EC₅₀) data are mean ± SEM of two to four independent experiments; Ki values were calculated using IC₅₀ values according to Cheng and Prusoff (44) with a Kᵣ value of 1.5 nM for oxytocin (OT) on the oxytocin receptor and 0.6 nM for vasopressin on the V₁₄-receptor. If no IC₅₀ value has been determined, the given values represent the highest concentration tested.
† Measurement of luciferase-coupled NFAT induction
‡ Not determined

Similarly to the mutated cyclotide, the kB7-OT; mutants [Y2A] and [Q4A] had lost their affinity and were not capable to activate the oxytocin receptor (Table 1, Fig. S4). Interestingly kalata B7-OT proved to be specific for the oxytocin receptor, because it did not compete for binding of radiolabeled vasopressin on any of the three vasopressin (V₁₄-, V₁₃- and V₁₂-) receptors at concentrations up to 10 nM (Fig. C) in contrast to native oxytocin (25).

**Uterostimulant effects of O. affinis extract and kalata B7-OT on human myometrium.** The crude ‘kalata-kalata’ extract and the nonapeptide kalata B7-OT were applied directly to small bath containing strips of human myometrium superfused with physiological saline solution. Application of 1 mg mL⁻¹ crude extract resulted in stimulation of contraction amplitude by +8.1 ± 3.5% whilst the area-under-curve increased by 313.5 ± 96% (mean ± SEM, n=6) (Fig. 5.A). Pre-treatment with atosiban significantly reduced the stimulatory effect of the extract (Fig. S6). Application of the selective nonapeptide kB7-OT resulted in a dose-dependent increase in both contraction amplitude (1 nM: +6.0 ± 3.2%; 10 nM: +41.4 ± 6.4%; 100 nM: +73.2 ± 5.4%) and area-under-curve (1 nM: +2.7 ± 2.3%; 10 nM: +48.8 ± 20.3%; 100 nM: +218.3 ± 143.6%; n= x) which was inhibited by pretreatment with atosiban (amplitude 1 nM: -0.8 ± 2.7%; 10 nM: -13.6 ± 7%; 100 nM: -2.8 ± 5.8%; area-under-curve 1 nM: -14.5 ± 6.9%; 10nM, -25.5 ± 4.8%; 100 nM: -11.1 ± 13.7%; n=3) (Fig. 5.B and C).

**Discussion**
A decoction of Oldenlandia affinis induces strong uterine contractions after both, oral administration as a tea or intravaginal instillation as aqueous solution (4, 5). In line with this activity, we identified the active principle in peptide-containing extracts of O. affinis and HPLC purified fractions containing various cyclotides based on the following criteria: (i) the peptide mixture and HPLC purified fractions containing various cyclotides were antagonized by the oxytocin receptor blocker atosiban; (ii) they displaced radiolabeled oxytocin from its heterologously expressed cognate receptor and vasopressin from the V₁₄-receptor, the closest relative of the oxytocin receptor; (iii) consistent with...
The endogenous ligand oxytocin is used clinically to induce labor and to prevent life-threatening post-partum bleeding (20). The kalata B7 cyclotide is a partial agonist on uterine smooth muscle cells and cells expressing the human oxytocin and V1a-receptors (Fig. 2 B). The extracellular face of both receptors are highly conserved and it is therefore not surprising that many drugs engage both receptors (26, 27). In fact, the oxytocin receptor antagonist atosiban, which is used clinically to delay pre-term birth, is a potent antagonist at the V1a-receptor (23). Adverse events reported after administration of oxytocin and the original remedy kalata-kalata include a decrease in blood pressure and cardiotoxic effects (4, 28); these may be related to the observed cross-reactivity, in particular if the partial agonistic action of effect of kalata B7 on V1a-receptor is taken into account (Fig. 2 B).

Based on the observed pharmacological properties of kalata B7 we performed a structural analysis to define candidate interaction sites on the oxytocin receptor. Cyclotides are three times larger than oxytocin and presumably cannot enter deep into the binding pocket of receptors. However, the NMR structure showed that the side chains of the Tyr and Gln residues in loop 3 protruded from the backbone. Hence they were capable of interacting with the oxytocin receptor. To our knowledge, kalata B7 is the only cyclotide containing a tyrosine and a glutamine residue in this loop. Both residues are also present in native oxytocin.

In fact, the tyrosine at position 2 in oxytocin has been shown to be important for receptor-ligand interaction with residues Y209 and F284 of the oxytocin receptor (29-31). In addition, loop 3 of kalata B7 contains a type II β-turn, which is also important for the activity of oxytocin (24).

Nonapeptide-analogs of oxytocin are flexible. Hence they can adopt several conformations that may possibly allow for accommodating differences in the ligand binding pocket of their target receptors. This conjecture predicts that a rigid scaffold may increase receptor selectivity. In fact, of the several peptides that were designed using the kalata B7 intercysteine loop 3 as a template, kalata B7-OT1 was found to be a selective agonist at the oxytocin receptor, since it stimulated the receptor in the sub-micromolar range, but did not bind to any of the other related receptors -- i.e., V1a-, V1b-, and V2-receptor -- up to concentrations of 10 μM (Fig. 4). The NMR data suggest that kalata B7-OT1 and authentic oxytocin are highly similar in their overall structure (cf. 1H chemical shifts in Fig. S5). A comparison of the structural ensembles of the first solution structure of oxytocin with the selective oxytocin receptor agonist kalata B7-OT1 reveals a similarly dynamic exocyclic tail whereas the more defined regions of the structure overlay well (Fig. 3). The sequence of kalata B7-OT1 (CYIQGCTLS) is a sequence combination of the kalata B7 cyclotide (-CYTQGCTCS-) and that of oxytocin (CYIQNCPLG). Tyr2, Ile3 and Leu8 are known to be important for receptor recognition of oxytocin-like peptides (30). This is in line with our data (Table 1). A change of Asn to Gly in position 5 of oxytocin does not impede the ability to bind and activate the receptor, but contributes to receptor selectivity. This feature has been previously appreciated for oxytocin- and V1a-receptor antagonists (26). Based on our observations, we also consider this of relevance in the future development of selective agonists for the human oxytocin and vasopressin receptors. In the last two decades, there was only a modest progress in identifying selective agonists for individual receptor subtypes (23). Hence, it is per se of interest that a selective agonist for the oxytocin receptor was discovered by extracting sequence information derived from loop 3 of kalata B7 that was found to be effective at stimulating intact human myometrium (Fig. 5).

At the more general level, our work provides a proof-of-principle for the concept that naturally-occurring peptides serve...
molecules that have been created by various synthetic strategies. Thus, at the very least, they can be anticipated to complement the existing collections of compounds that are used in drug discovery by high throughput screening and related approaches. In fact, cyclotides have been recently used as scaffolds to improve the stability of peptides that have interesting biological activities. This grafting introduced peptide sequences into cyclotide loops and resulted in chimeric molecules, which bound to G protein-coupled receptors (33-35), inactivated VEGF (35-36), stimulated angiogenesis (36), blocked entry of HIV via CXCR4 (37) and inhibited serine proteases (38). Here we used the reverse approach, i.e., we extracted the active segment of the cyclotide to create a selective ligand. To the best of our knowledge, this is the first report, which documents that a plant peptide shares similarity in sequence and activity with oxytocin. The discovery of the active ergot ingredients produced by the fungus Claviceps purpurea was instrumental to the development of modern pharmacology. Accordingly, C. purpurea has been referred to as the treasure trove of pharmacology (39). Incidentally, ergot also contains (methyl)ergometrine/ (methyl)ergonovine, i.e., the first selective uterotonic compounds introduced into clinical medicine. We are aware of the limitations of historical comparisons. Nevertheless, we believe that the rich diversity of cyclotides justifies that they also be considered as a potential treasure trove for drug discovery.

Methods
(Detailed materials and methods are given in Supplementary Information Text S1.)

Plant extraction, RP-HPLC fractionation and peptide isolation. Aerial parts of Oldenlandia affinis DC, were extracted and purified as described previously (40) yielding a starting extract of peptides. Fractionation and isolation of cyclotides were carried out using RP-HPLC on a Dionex Ultimate 3000 unit (Thermo-Scientific Dionex).

Mass spectrometry and peptide identification. Analysis of peptides was performed on a MALDI-TOF/TOF 4800 Analyzer (AB Sciex). MS and MS/MS experiments were carried out as described previously (8). Prior to MALDI analysis, samples were desalted using C18 ZipTips™. Spectra were processed using DataExplorer Software and cyclotides were characterized by manual peptide sequencing.

Cloning, cell culture, transfection and membrane preparation. Oxytocin, V1a, V1b, and V2 receptor DNA sequences were inserted into pEGFP-N1 plasmids to yield C-terminal GFP fusion proteins. Preparation of stably transfected HEK293 cell lines, propagation and membrane isolation were performed on a MALDI-TOF/TOF 4800 Analyzer (AB Sciex).

Radioligand displacement assays. Isolated membranes were incubated with radioactive agonists ([3H]oxytocin (2 nM) or [3H]vasopressin (0.75 nM) and various concentrations of competing peptide. The reaction was stopped by filtration over glass fiber filters using a cell harvester. Non-specific binding was determined in the presence of 1 µM oxytocin or vasopressin, respectively.

Fig. 4. Pharmacological selectivity of synthetic kalata B7-OT, on oxytocin/vasopressin receptors. (A) The binding of [3H]oxytocin (2 nM) to membranes from HEK293 cells (30-100 µg/assay) expressing the human oxytocin receptor was measured in the an excess of oxytocin (OT) peptide (0.1 nM to 1 µM) and kalata B7-OT (0.3 nM to 3 µM). (B) The ability of the peptides (0.03 nM to 10 µM OT; 3 nM to 30 µM kalata B7-OT) to signal through Gq and activate downstream DNA binding elements of GPCR activation in HEK293 cells stably transfected with the human oxytocin receptor was measured with a luciferase reporter gene assay. Data were fitted with non-linear regression (sigmoidal, variable slope) and are shown as mean ± SEM of three independent experiments. Binding data are normalized to percentage (%) of specific binding; the 100% value refers to an average of 1.57 pmoles of ligand bound per mg of membrane. Activation data are normalized to the number of cells and fold induction above baseline. K and EC50 values are listed in Table 1. (C) The selectivity of kalata B7-OT was tested on all four human receptors, i.e., the oxytocin-, vasopressin V1a-, V1b- and V2-receptors. 100% specific binding refers to values of 1.57 pmoles/mg for the oxytocin-, 0.97 pmoles/mg for the V1a-, 0.78 pmoles/mg for the V1b- and 0.30 pmoles/mg for the V2-receptor, respectively. Statistical differences were analyzed using an unpaired t-test with *p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant.

Fig. 5. Uterostimulant effects of Oldenlandia affinis extract and kalata B7-OT on human myometrium. Spontaneous contractions of term and non-laboring human myometrium superfused with physiological saline solution at 37°C. (A) Application of 1 mg mL⁻¹ extract of O. affinis followed by 0.5 nM oxytocin. (B) Dose-responses from 1 nM to 100 nM of kalata B7-OT. (C) The effects of kalata B7-OT, in the presence of the oxytocin- and vasopressin V1a-receptor antagonist, atosiban (1 µM).

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Functional receptor activation assays. Luciferase-based reporter assays were performed as described previously (41). Briefly, HEK cells were transfected with firefly luciferase containing plasmid pGL4.30 luc2P. After transfection cells were seeded into 96-well plates and incubated with logarithmically spaced concentrations of peptides. Following incubation, medium was removed and cells frozen at -80°C. Following cell lysis, luciferase activity was measured using a Promega luciferase reagent kit. Inositol-1-phosphate (IP1) accumulation measurements were carried out using the Ciskin IP1 HTRF® assay. Cells were incubated with peptides for 1 h prior to fluorescence measurements on a Synergy4 microplate reader according to the manufacturer’s recommendation.

Collagen gel contractility assays. Human uterine myometrial smooth muscle cells in miniprep were isolated and collagen gel contractility studies prepared as described previously (42). Gel images were taken using a Fluorochrome™ 8900 imager and the gel area measured using AlphaEaseFC software. Collagen contraction, correlating to decrease in gel area, was determined in quadruplicate. Data were statistically analyzed using one way ANOVA.

Cyclotide and peptide synthesis. Peptides were synthesized using Boc or Fmoc solid phase peptide synthesis. After cleavage from resin, peptides were oxidized in 0.1 M ammonium bicarbonate at pH 8.2 for 24 h and purified on RP-HPLC to yield >95% purity.

NMR spectroscopy. Samples were dissolved in 90% H2O/10% D2O or 100% D2O (for ECOSY) and spectra recorded on a Bruker 600 MHz spectrometer at temperatures of 290K and 298K with mixing times of 100 and 200 ms for 1D, TOCSY, NOE, DQF-COSY and ECOSY experiments. Spectra were analyzed and integrated with SPARKY® software. Structures were calculated with DYANA, CYANA and CNS packages. The Protein Data Bank (www.pdb.org) ID code for kalata B7 is 2MSO.

Organ bath myometrial contractility assays. Isometric force recordings were made on strips of human myometrium obtained from the lower uterine incision site at Caesarean Section. Crush extract (1 mg mL−1) or the selective nonapeptide ketB-OT-(1-100 nM) was added directly to the organ bath. In some experiments, strips were pre-treated with atosiban (10−6 M). Contraction amplitude and area under the contraction curve was measured and compared to control activity (100%) using OriginPro 8.5 software as previously described (43).

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